Mineral Resource Reviews

Antony van der Ent · Alan J. M. Baker ·
Guillaume Echevarria · Marie-Odile Simonnot ·
Jean Louis Morel *Editors*

Agromining: Farming for Metals

Extracting Unconventional Resources Using Plants

Second Edition

Springer

Mineral Resource Reviews

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SOCIETY for GEOLOGY APPLIED to MINERAL DEPOSITS

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Editors
Antony van der Ent
Centre for Mined Land Rehabilitation,
Sustainable Minerals Institute
The University of Queensland
Brisbane, QLD, Australia

Guillaume Echevarria
Laboratoire Sols et Environnement
Université de Lorraine, INRAE
Nancy, France

Jean Louis Morel
Laboratoire Sols et Environnement
Université de Lorraine, INRAE
Nancy, France

Alan J. M. Baker
School of BioSciences
The University of Melbourne
Parkville, VIC, Australia

Marie-Odile Simonnot
Laboratoire Réactions et Génie
des Procédés
Université de Lorraine, CNRS
Nancy, France

ISSN 2365-0559 ISSN 2365-0567 (electronic)
Mineral Resource Reviews
ISBN 978-3-030-58903-5 ISBN 978-3-030-58904-2 (eBook)
https://doi.org/10.1007/978-3-030-58904-2

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Preface

Metallophytes (metal-tolerant plants) have been used for centuries to locate valuable metallic ore deposits (as early as 1556 in Georgius Agricola's *De Re Metallica*). A subset of metallophytes, those plants that accumulate extraordinary amounts of metals or metalloids within their living tissues, is called hyperaccumulators. Although first described in 1865 in the zinc hyperaccumulating *Noccaea caerulescens*, reporting of the exceptional blue-green latex containing 25 wt% Ni, in the endemic New Caledonian tree *Pycnandra acuminata* by Jaffré et al. 40 years ago, has greatly spawned scientific interest in these unusual plants. We have come a long way since to find innovative uses for these plants. One such emerging technology is phytomining or agromining, which involves the cultivation of hyperaccumulator plants and harvesting their biomass to obtain particular metals or metalloids. Whereas phytomining describes the process of exploiting plants for valuable elements, agromining refers to the full agronomic chain in using accumulator/hyperaccumulator plants as 'metal crops'. The process involves the farming of 'metal crops' on sub-economic deposits or industrial or mineral wastes in order to obtain valuable element(s) from their harvest biomass via the production of a 'bio-ore'. The demand for critical metals, including rare earth elements (REEs), platinum group elements (PGEs), nickel and cobalt, even gold, is more under pressure in the twenty-first century as a result of resource depletion and geopolitical factors. Agromining is expected to be transformative in future extraction of unconventional resources of these elements not accessible by traditional mining techniques.

This seminal book presents the complete chain of metal farming—'agromining'. It brings together for the first-time individual contributions by active research scientists and practitioners currently engaged in studying fundamental and applied aspects of hyperaccumulator plants. The emergence of the great opportunities they present has taken several decades for acceptance, a fact that is discussed in detail in the introductory chapter. Other chapters address the agronomy of 'metal crops', the latest developments in the processing of bio-ores and associated products, the current state of knowledge on the global distribution and ecology of hyperaccumulator plants, biogeochemical pathways involved in the basic processes, the influence of rhizosphere microbes, as well as aspects of propagation and conservation of these unusual plants. This book then summarizes the state of the art in using new tools for hyperaccumulator discovery and in the understanding of their physiology and molecular biology. The opportunities for incorporating agromining into rehabilitation and mine closure strategies are presented and ecosystem service provision and life cycle analysis discussed. The possibilities for agromining nickel, cobalt, manganese, selenium, arsenic, thallium, REEs, and PGEs are discussed in separate chapters devoted to case studies. Finally, an overview concludes the book, looking ahead to prospects for the development of agromining in the future.

This book is edited and authored by pioneers in the field who have been at the forefront of the development of agromining over the past three decades. The book is timely, as agromining is now on a pivotal point in its development, with the rapid expansion of activities occurring in the field worldwide. As such, the book will be of significant interest to environmental professionals in the minerals industry, government regulators, and academia. This second edition takes into account the most recent development in the agromining chain, fundamental advances as well as technological developments.

Brisbane, Australia	Antony van der Ent
Parkville, Australia	Alan J. M. Baker
Nancy, France	Guillaume Echevarria
June 2020	Marie-Odile Simonnot
	Jean Louis Morel

Contents

The Long Road to Developing Agromining/Phytomining

Rufus L. Chaney, Alan J. M. Baker, and
Jean Louis Morel

Abstract

The concept of phytomining is a natural extension of botanical prospecting and the study of metal biochemistry and biogeography of metal hyperaccumulator plants. Some elements may be phytoextracted to remediate soils, but the recovered biomass would have little economic value (e.g. Cd, As, etc.) and disposal of the biomass would be a cost. A few elements may have sufficient economic value in phytomining biomass to support commercial practice (Ni, Co, Tl and Au). The development of agro/phytomining requires (i) selection of high-biomass hyperaccumulator plant species; (ii) evaluation of genetic diversity and breeding of improved strains with higher yields of the phytoextracted element; (iii) development of agronomic practices to maximize economic return; and (iv) development of methods to recover the agromined element(s) from the plant biomass. Plant species and methods for agromining of soil Ni have been demonstrated for several species and locations (temperate and tropical climates). Production of Ni metal in an electric-arc furnace smelter, and of Ni $(NH_4)_2SO_4$ using a hydrometallurgical method, and other metals (e.g. Zn, REEs) have been demonstrated. Full commercial agromining of Ni is underway in Europe (e.g. the Balkans, France) using Brassicaceae (notably *Odontarrhena chalcidica*—formerly *Alyssum murale*), and major trials in Malaysia are underway using *Phyllanthus rufuschaneyi*. Variable prices of commodity metals add constraints on the development of commercial agromining.

1 Background

Phytoremediation is the use of plants to solve environmental problems. Phytoremediation of soil metals to prevent adverse effects in the environment includes: (i) phytostabilization (converting the metals to forms that are not phyto- or bio-available); (ii) phytodegradation (using plants and soil microbes to biodegrade xenobiotics); (iii) phytovolatilization (using plants and soil microbes to cause release of volatile forms of elements into the surrounding

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R. L. Chaney (✉)
Chaney Environmental, Beltsville, MD 20705, USA
e-mail: rufuschaney@verizon.net

R. L. Chaney · A. J. M. Baker
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD 4072, Australia

A. J. M. Baker
School of BioSciences, The University of Melbourne, Parkville, VIC 3010, Australia

A. J. M. Baker · J. L. Morel
Laboratoire Sols et Environnement, Université de Lorraine, INRAE, 54000 Nancy, France

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_1

atmosphere (Hg, As); (iv) phytoextraction (using plants to remove metals from the soil); (v) and agromining/phytomining (growing plants to 'mine' soil metals as an alternative agricultural technology). After research on metals in the environment began in the 1970s, research on each of these technologies has been intensive and productive. These technologies are only useful if they are economic compared to engineering alternatives such as removal and replacement of the tillage depth of soil, which cost, for example, US \$2.5 million ha^{-1} in Japan where more than 1000 ha of rice paddy soils (which produced rice that caused *itai-itai* Cd disease in farm families who consumed the home-grown rice) were remediated using engineering methods (Iwamoto 1999).

Chaney (1983a) and Chaney et al. (1981a, b) introduced the concepts of phytoextraction and phytomining in papers dealing with land treatment of hazardous wastes. Recently, Morel (2013) proposed using the name "agromining" to consider the entire chain of agronomic and metallurgical processes and their combinations, to produce commercial elements from mineralized or contaminated matrices using plants. Phytomining and agromining have the same basic meaning, that is, using plants to recover elements of commercial interest; they can be used alternately. While phytomining focuses more on the plant and its potential to extract elements of interest, agromining emphasizes technological processes and their combination (agronomy and metallurgy) to produce commercial compounds. Here, in the text, we refer to phytomining for previous research and development activities, and to agromining for more recent ones that consider the entire production of bio-based elements.

Chaney (1983a, b) developed the concept of the 'soil-plant barrier' to characterize the food-chain transfer of elements in contaminated or mineralized soils. Food chains are protected from nearly all trace elements in contaminated soils because animals tolerate, in lifetime diets, the element concentrations that accumulate in shoots of nearly all plant species when the plants suffer phytotoxicity for that element (exceptions include Se, Cd and Tl). For example, nearly all crop species suffer Zn phytotoxicity when shoot Zn reaches 500 $\mu g\ g^{-1}$ dry weight (DW), and the most sensitive livestock tolerate this level of diet Zn (see Chaney 1993). Crop plants suffer Ni phytotoxicity at ≤ 100 mg kg^{-1} DW, but even sensitive ruminant livestock species tolerate >100 $\mu g\ g^{-1}$ Ni in foliar biomass. Metal hyperaccumulator biomass would be expected to cause toxicity to wildlife and livestock if only that biomass were consumed; however, livestock avoid most known hyperaccumulators (e.g. Cannon 1960; Chaney and Baklanov 2017). At the same time, hyperaccumulator plants (Ernst 1974, 1975; Jaffré and Schmid 1974; Brooks et al. 1977) of several potentially toxic elements had been reported in the literature. Results of the first paper that reported a Ni hyperaccumulator, Minguzzi and Vergnano (1948), have been repeatedly confirmed, but because analysis of trace levels of Ni was difficult in 1948, little attention was paid to this original evidence, until Jaffré, Brooks and colleagues began studying Ni hyperaccumulators.

2 The Need for Cleaning Up Contaminated Land in the USA

The risk-assessment research and papers that Chaney prepared focused on land application of municipal sewage sludge (now biosolids) and treated municipal wastewater effluent applied to agricultural land. In the 1970s, many biosolids were highly enriched in specific metals discharged by local industry, such that metal phytotoxicity (Zn, Cu, Ni, Co) could occur if amended soils became acidic. For example, in even 1980, a single N-fertilizer application of 5 dry t ha^{-1} of a typical biosolids containing 2500 $\mu g\ g^{-1}$ Zn and 25 $\mu g\ g^{-1}$ Cd would apply 12.5 kg Zn and 125 g Cd ha^{-1}, much more than that removed in a 20 t ha^{-1} crop of maize forage (0.5 kg Zn and 4 g Cd) (Table 1). Growth of hyperaccumulator crops could remove much higher amounts of Zn and Cd than crop species, which might be an alternative to reverse soil contamination.

Table 1 Estimated removal of Zn and Cd in crop biomass versus hyperaccumulator biomass, or improved phytoextraction crop (Remediation)

Soil	Species	Yield (t ha^{-1})	Zn in Crop			Zn in Ash
			mg kg^{-1}	kg ha^{-1}	% of soil	%
Control	Maize	20	25	0.5	1	0.025
Contam	Maize	10	500	5	0.0125	0.50
Contam	*Noccaea*	5	25,000	125	3.12	25
Contam	Remed	10	25,000	250	6.25	25
Crop		Yield (t ha^{-1})	Cd in Crop			Cd in Ash
			mg kg^{-1}	kg ha^{-1}	% of soil	%
Control	Maize	20	0.2	0.004	0.01	–
Contam	Maize	10	5	0.05	0.125	0.005
Contam	*Noccaea* (Prayon)	5	250	1.75	4.4	0.25
Contam	*Noccaea* (Ganges)	5	2000	10	25	2
Contam	Remed	10	2000	20	50	2

Presume soil is highly contaminated by smelter emissions, and pH is managed to achieve moderate Zn phytotoxicity (50% yield reduction) due to Zn phytotoxicity of crop species. The contaminated soil is assumed to contain 2000 μg g^{-1} Zn = 4000 kg Zn (ha 15 cm)$^{-1}$; and 20 kg Cd kg^{-1} = 40 kg Cd (ha 15 cm)$^{-1}$, whereas the control soil is assumed to contain 50 μg g^{-1} Zn [100 kg (ha 15 cm^{-1})] and 0.20 μg g^{-1} Cd [0.4 kg (ha 15 cm^{-1})]

Because of recognition in the early 1970s that soil Cd could cause adverse health effects in rice farmers (Kobayashi 1978), many questions were raised about the accumulation of Cd and how to remove Cd from contaminated soils. The mining and smelting industries had caused Zn and Cd contamination of large areas of soils in many countries, whilst historic application of biosolids had caused excessive Cd accumulation in some cities. Public concern about hazardous wastes followed several internationally recognized cases, and regulatory agencies were starting to identify methods to remediate the risks of contaminated soils.

In 1980, a US-EPA scientist, Carlton Wiles, who worked on problems of hazardous waste-contaminated soils, contacted Chaney about extending the USDA research on metals from biosolids to include remediation of hazardous soils. An Inter-Agency Agreement between US-EPA and USDA-ARS was initiated to support a review of the literature, and then fund US$1 million/year for four years of research and demonstrations of hazardous soil remediation technologies dealing with both metals and xenobiotics. Scientists in the Biological Waste Management Laboratory, led by Dr James F. Parr, worked on the literature review, preparing both a formal report for US-EPA (Chaney et al. 1981b), and a book (Parr et al. 1983). In the 1980s, with a new president caring less about the environment, the US-EPA received reduced funding and redirected many programs. The Inter-Agency Agreement was cancelled after the literature review was completed. Lacking the US-EPA funding, USDA-ARS terminated that research program and redirected research of the team.

As part of that review process, Chaney reviewed the important findings of Ernst, Baker, Brooks, Jaffré, Rascio, Reeves, Wild, and others about the hyperaccumulation of metals by rare plants. It was conceivable that growing high yields of hyperaccumulator plants on contaminated soils could remove enough metal to alleviate the hazardous nature of the soils at considerably lower cost than removal and replacement of the contaminated soil. The potential to remove Cd from contaminated soils (phytoextraction) seemed promising, and a need had been clearly identified for the technology to remove Cd from contaminated soils in order to protect food chains. The phytoextraction/phytomining concept was summarized with regard to Cd, Zn and Ni hyperaccumulation (Chaney 1983a). Table 1 presents the potential removal of Zn and Cd by crop plant maize (*Zea mays*) grown as a forage crop, compared to removal by the hyperaccumulator *Noccaea* (formerly *Thlaspi*) *caerulescens*. It is clear that crop plants remove so little Cd or Zn that growth for centuries would not deplete soil levels from contaminated soils, but that some Cd hyperaccumulators can accumulate 1000 mg kg^{-1} Cd in their dry above-ground plant tissue when growing in soils in which they normally occur (van der Ent et al. 2013), and hence other hyperaccumulators might remove significant amounts of Cd. Table 2 summarizes similar information about Ni phytomining by hypernickelophore species that accumulate $\geq 1\%$ Ni, such as *Odontarrhena chalcidica*. Although phytoextraction of Zn and Ni would not be rapid, by using the southern France or so-called ‘Ganges’ populations of *N. caerulescens*, Cd removal might be fast enough to achieve soil remediation goals that protect food safety by phytoextraction of soil Cd at low cost (see Simmons et al. 2015).

Rice genotypes with high accumulation of Cd under aerobic acidic soil conditions (null HMA3 gene) grow much better in tropical rice paddy soils (Murakami et al. 2009) than does the temperate species *N. caerulescens*. Because these rice genotypes can give high yields of shoots with 100 µg g^{-1} Cd in dry biomass, harvestable Cd yield is much greater than *N. caerulescens* on those soils, and the crop is adapted to the tropical regions where rice that caused human Cd disease is grown. Because *N. caerulescens* is relatively sensitive to excessive Cu in soils, those with Cu co-contamination with Cd and Zn may be addressed using maize inbred lines having relatively high Cd accumulation (Broadhurst et al. 2015). For soil with high levels of Zn, Ni, Cu, Pb and some other metals, phytostabilization is quite effective in reducing plant uptake and even bioavailability of the metal in soils ingested by children, wildlife, and livestock (see Ryan et al. 2004; Chaney et al. 2014; Chaney and Baklanov 2017).

In 1979, Chaney attended a Trace Element Symposium in Los Angeles, CA, which was also attended by Alan Baker. Their discussion included metal tolerance and accumulation and the unusual hyperaccumulator species, and Chaney’s concept of phytoextraction (see Moskvitch 2014). Until that time, Baker, Brooks, Reeves, and others (Cannon 1960) had conceived of botanical prospecting for metal ores by analysis of herbarium specimens for elements (Brooks et al. 1977), and were studying the metal tolerance and biogeography of such species as *N. caerulescens*, *Odontarrhena*, etc. In the Proceedings of that Symposium, a classic paper by Baker reviewed metal tolerance by exclusion or accumulation (Baker 1981). Baker and Brooks (1989), in a seminal review, summarized the concepts of hyperaccumulator species and closed with a discussion of the potential for phytoextraction/phytomining that spread the meme of phytoextraction widely. This concept was also promoted independently in an article in *New Scientist* by Baker et al. (1988), which received wide international interest and triggered fundamental research worldwide on hyperaccumulator plants.

3 The Situation and Response in Europe—Focus on France

In Europe, soil contamination by heavy metals, their absorption by roots, and subsequent transfer to the food chain were important issues in the 1970s (e.g. in France, research programs launched by the Ministry of Environment and the Anred, now Ademe). In the same way, as in the

Table 2 Potential of crop species maize (*Zea mays*) and Ni hypernickelophore *Odontarrhena chalcidica* to phytomine Ni from soils

Plant species	Biomass	Ni in shoot biomass			Ash-Ni (%)
	Yield (t ha^{-1})	mg kg^{-1}	kg ha^{-1}	% of soil	
Maize (Control)	20	1	0.02	0.0002	0.002
Maize (50% YD)	10	100	1	0.01	0.2
Alyssum in pasture	3	10,000	30	0.3	5–10
Wild *Alyssum* crop	10	15,000	150	1.5	20–30
Alyssum+Agric[a]	20	20,000	400	4	20–30
Alyssum cultivar[a,b]	20	30,000	600	6	25–30

Assume soil contains 2500 μg g^{-1} Ni which equals 10,000 kg Ni (ha 30 cm)$^{-1}$ deep. Presume soil pH is low enough to reduce maize yield by 50%, or high enough to achieve maximum Ni in *Odontarrhena* species

[a] Appropriate agronomic practices: N, P, K, S, Ca, and B fertilizers; herbicides; planted seeds

[b] Improved cultivar bred to maximize shoot Ni content (yield-×-concentration) at annual harvest

United States much research was conducted on the fate of metals in agroecosystems, because the use of sewage sludge and urban waste in agriculture raised great concerns about the risks of soil pollution and crop contamination. In fact, during this period, some urban sludge exhibited abnormally high concentrations of metals (i.e. >150 μg g^{-1} Cd; Morel 1977; Juste and Solda 1988). In addition, application rates were much higher than those currently permitted, and the probability of transfer of toxic metals to plants was very high at that time (Morel and Guckert 1984; Morel et al. 1988). Processes and mechanisms that control the uptake and transfer of metals to plants were being studied in the 1980s and 1990s, allowing for a better understanding of plant contamination, considering soil pH and metal adsorption, root activity, and plant metabolism. For example, at the soil-root interface, the potential role of exudates (root secretions), either soluble or mucilage exudates, in metal dynamics was demonstrated (Morel et al. 1986; Mench et al. 1986). Regulation for the use of biosolids in agriculture and resulting improvement of the quality of urban biosolids considerably reduced the risk of contamination of agroecosystems with heavy metals, whilst ensuring the recycling of essential elements such as phosphorus (Sommellier et al. 1996). Still, large surface areas had been contaminated by heavy metals not only by urban wastes, but also by industrial atmospheric deposits (e.g. smelters) (Sterckeman et al. 2000). In general, soils strongly affected by human activities in urban and industrial areas may contain elevated concentrations of metals (De Kimpe and Morel 2000). Some soils are mineralized from parent rocks that had higher Cd and Cd:Zn ratio (Birke et al. 2017), but the vast majority of contaminated soils are contaminated by urban and industrial wastes and atmospheric deposits, threatening human health by the direct ingestion of soil particles by children and the indirect consumption of contaminated water and vegetables produced in polluted gardens (e.g. Mielke et al. 1983; Schwartz et al. 1998; Paltseva et al. 2018).

Over time, the strong economic transformations of the 1980s that impacted mining and heavy industry, the closure of activities such as coal, iron and non-ferrous metal mines, steel and textile mills, led to the identification of numerous industrial wastelands. If these metal-contaminated areas were used for new economic activities; unless remediated, they might be a threat to human and environment health. Organic and inorganic contaminants were present and very commonly firmly attached to the soil matrix, thus making their elimination a difficult task. Soil remediation technologies were based on excavation and land disposal in landfills or ex situ treatment using biological, thermal, or physicochemical techniques. However, in spite of their efficiency, these techniques were not suitable for large-scale sites, for technical as well as economic reasons. This problem triggered the emergence of multidisciplinary structures during this period, in order to develop innovation in the treatment of large polluted sites and soils. The Gisfi (Groupement d'Intérêt Scientifique sur les Friches Industrielles) is an example of groups that combined a wide range of disciplines to cope with complex situations; launched in France in 2000, this agency (www.gisfi.univ-lorraine.fr) is still active in basic research and development of new technologies and strategies to deal with contaminated areas. Research activity was directed to in situ treatments, including chemical and biological, to treat soils having persistent pollutants on very large areas such as brownfields and agricultural land contaminated with metals from atmospheric deposition. Phytoremediation was one of the options.

In the early 1990s, the phytoremediation technology proposed by Chaney (1983a) was considered a potential alternative. However, at that time in Europe, remediation professionals rather designated phytoremediation a "sweet dream of plant lovers" and did not take it seriously. It is true that knowledge then about the effects of plants and their possible contribution to the cleaning of polluted soils was rather scarce. But this did not prevent some unwise industrialists from promoting too fast the technology and presenting it as a reliable solution for dealing with contaminated environments. The consequence of the lack of solid scientific foundations inevitably led to a series of failures in the 1990s that marginalized the technology for several years. In the late 2010s, as site owners were looking for other ways to manage polluted sites, phytoremediation sparked new interest among companies involved in site restoration, with new companies focusing on phytoremediation or large existing companies creating new branches dedicated to the phytoremediation approach. Phytoremediation combined with soil construction is now a new alternative for site restoration, with the production of biomass for industrial use, such as energy, fibre, and agromining as demonstrated by the LORVER project (see below).

4 The Saga of Chelator-Enhanced Pb Phytoextraction

Much has been learned about the potential for phytoextraction to remove enough metal to achieve phytoremediation. The concept laid in the hands of basic researchers until Ilya Raskin and colleagues at Rutgers University invited a lecture by Chaney on metal accumulation. Raskin et al. recognized that Pb-contaminated soils were a significant industrial and urban problem, and that if plants could remove soil Pb efficiently, it would create a significant market for a phytoextraction technology. Unfortunately, Raskin et al. tested Pb uptake under conditions that kept Pb highly soluble. It was well known at that time if a nutrient solution was deficient in phosphate, that Pb was readily absorbed and translocated to plant shoots thus causing Pb phytotoxicity (Miller and Koeppe 1971). Kumar et al. (1995) chose to test *Brassica juncea,* and grew the crop with low levels of P and S, then washed the sand and added a P- and S-free nutrient solution having a high level of soluble Pb, and when the plants suffered Pb phytotoxicity, harvested and analyzed the plant shoots. Some accessions of this species accumulated >1% Pb (10 000 μg g^{-1}) in shoots (Kumar et al. 1995). Raskin et al. acquired a U.S. Patent for phytoextraction of essentially all elements (Raskin et al. 1994) and obtained investment to start commercialization. Of course, *B. juncea* grown on Pb-contaminated field soils did not accumulate much Pb, typically <100 mg kg^{-1} DW. Their team looked at alternatives to increase Pb accumulation (P-deficiency in soil, foliar P fertilization), then tested application of chelating agents such as ethylenediaminetetraacetate (EDTA) to the soil surface when the plants had grown significant biomass but before flowering changed growth patterns. With the addition of EDTA and other chelators, they achieved high shoot Pb concentrations (Blaylock et al. 1997) but actually more metals were leached as EDTA chelates than were taken up by the plants. The mathematics of the Pb budget just did not add up.

Testing of reported Pb-accumulating plant species such as *Noccaea rotundifolia*, containing very high Pb levels where it grew naturally on highly Pb-contaminated mine wastes (Reeves and Brooks 1983), found <300 μg g^{-1} DW Pb when grown in field-Pb-contaminated soils using methods to prevent soil splash contamination of the plants. Even a locally adapted strain of *Ambrosia artemisiifolia* (ragweed), which was found to accumulate more Pb than other plant species occurring on Pb-contaminated land controlled by the DuPont Corp., only accumulated about 300 mg kg^{-1} DW (Huang and Cunningham 1996). It is now recognized that the very high Pb levels in the field-collected *N. rotundifolia* was likely due to soil particle contamination of this very small plant (e.g. Faucon et al. 2007). In the field testing by Phytotech, in addition to the effect of EDTA addition and growth of *B. juncea*, tillage caused dispersal and dilution of Pb concentration in the surface soil 'hot spots' that most required remediation. It should be noted that their process achieved the legal environmental standard for the site (400 mg kg^{-1} Pb soil in surface inch of soil). Deep tillage and soil movement provided most of the reduction of surface soil Pb concentration to meet the clean-up goal.

As Chaney et al. (2002) reported, the cost of adding EDTA also made this an unacceptable technology; leaching of Pb-EDTA and other metal chelates to groundwater was also unacceptable in the environment. Reviews of chelator-induced phytoextraction discuss many reasons why this use is environmentally unacceptable (e.g. Nowack et al. 2006). As a result, US and EU governments prohibited the application of EDTA and other chelating agents to induce phytoextraction. In an estimate (Chaney et al. 2014), the cost was lower if one used the lower cost of bulk-purchased EDTA (≥ 20 t truckload lots); the addition of 5 mmol of EDTA kg^{-1} contaminated soil would cost more than US\$23,500 ha^{-1} yr^{-1}. Thus, induced phytoextraction was neither economic nor acceptable in the environment. Field tests showed the extent of metal chelate leaching. These problems were reviewed in detail by Chaney et al. (2014). Unfortunately, the reports and patent of Raskin et al. and the Phytotech program led many people to believe that growing many plant species (especially sunflower) in garden soils could remove Pb to protect children. The web has many posts about growing sunflower to reduce Pb soil risks. Today, Pb uptake is much better understood. Kopittke et al. (2008) showed that soluble Pb can be taken up by roots but is immediately precipitated as chloropyromorphite [$Pb_5(PO_4)_4Cl$], a highly insoluble form of Pb, nearly stopping Pb translocation to shoots. Only the application of excess EDTA to cause the root membranes to become leaky, allows high uptake of PbEDTA (Vassil et al. 1998). Fortunately, phytostabilization is now available to greatly reduce bioavailable soil Pb to protect children, livestock and wildlife (Ryan et al. 2004).

Raskin et al. (1994) had obtained a patent for Phytoremediation of Metals that they described as covering all metals and all plant species. Because the Chaney-Angle-Baker-Li team was working to develop a patentable technology for Cd or Ni phytoextraction, they contacted their patent advisers (University of Maryland and USDA) and immediately submitted a patent application for Ni phytomining. Fortunately, Raskin et al. had stressed in their patent that non-crop metal accumulator plant species were so small and difficult to grow that they were not useful for phytoextraction, which left us the possibility of patenting Ni phytoextraction/phytomining. The team was also advised that obtaining economic support to develop the full commercial technology would likely be impossible without a patent. Hence, we conducted the research needed to support a patent application for Ni phytomining and worked with the USDA and Universities of Maryland and Sheffield patent advisers, and eventually obtained patents for a method of phytomining Ni, Co and other metals from soil (e.g. Chaney et al. 1998, 2004). Phytotech Inc. licensed the Raskin et al. patent.

Phytotech ultimately went bankrupt when it became evident that chelator-induced Pb phytoextraction would not be permitted in the USA.

5 Developing the First Phytomining Trials in the USA

In about 1990, the Chaney-Angle-Baker-Li team began collaborating to test potential phytoextraction technologies starting with Cd and Zn, but quietly also starting a study of potential Ni phytomining. Although much research has been conducted to understand how plants hyperaccumulate Cd, and large land areas have become contaminated with Cd and cause excessive food-chain transfer of Cd, governments have not ordered remediation of the large land areas where Cd phytoextraction would be applicable. Without legislation to mandate the remediation of soil Cd from polluted soils, there was no commercial market for such a technology and hence no commercialization of Cd phytoextraction has occurred. Both Cd phytoextraction using hyperaccumulator plant species and the use of high biomass energy species (Ruttens et al. 2017) or rice genotypes (Murakami et al. 2009) have been demonstrated in the field, but the lack of any government impetus to remediate Cd-polluted areas prevents progress in use of these technologies. The production of energy crops such as willow or poplar trees, which may also accumulate appreciable amounts of Cd and Zn, might be cost effective if the energy value alone would provide profitable agricultural use of the contaminated soils, as phytoextraction is achieved very slowly with these species. Cadmium and Zn would need to be effectively recovered from incinerator stack emissions in order to make products of the combustion safe in the environment.

The Chaney et al. team worked with artist Mel Chin to establish a field test of Cd, Zn and Pb phytoextraction by *N. caerulescens* and several crop species from a landfill in St. Paul, Minnesota. In the process of seeking a grant to pay for the art piece (entitled 'Revival Field'), Chin's proposal was initially rejected by the U.S. National Endowment for the Arts (NEA). This caused a large response from the art community and eventually a half-page article on Revival Field was published in *Science* (Anonymous 1990). The grant rejection led to many national newspaper stories and the subsequent note in *Science* spread the word about the potential value of phytoextraction to the scientific community, and jump-started phytomining research in the USA. The 'Revival Field' art piece was then reconsidered and funded by the NEA.

Nickel phytomining clearly offered greater economic opportunity than did the phytoextraction of cadmium. Chaney had obtained seed of *Dicoma niccolifera* Wild from the renowned botanist Hiram Wild in Zimbabwe (Wild 1970) in 1980 and began research, but it was not a hypernickelophore by testing at Beltsville. Baker and colleagues in the UK had begun basic research and field phytoextraction trials with *N. caerulescens* (e.g. Baker et al. 1994) and provided seed of the Cd-accumulating Prayon population of *N. caerulescens* from Belgium to many researchers to promote studies.

Ernst (1996, 2000) raised several questions about the practicality of phytoextraction. He had published many papers on native metal hyperaccumulators and reported the small size of harvestable shoots of *N. caerulescens* and the low density of plants in the field sites he had examined. He reasoned that the harvest of standing biomass at contaminated sites that included hyperaccumulator plants would remove so little metal mass that no benefit could result. We looked at this information and argued that agronomic methods to maximize metal amounts in annual harvests (herbicides, fertilizers and pH management), and breeding improved phytoextraction cultivars of hyperaccumulators, are required for maximally successful phytoextraction and especially for the agricultural technology of phytomining.

In spite of all the ensuing "hype" (Ernst 2000), it was soon recognized that nickel phytomining could be profitable if developed. So, the Chaney et al. team quietly began research with seeds of several *Odontarrhena* species supplied by Baker from accessions collected in

Mediterranean Europe and Eurasia. Independently, they obtained seeds of cold-tolerant *O. chalcidica* collected from ultramafic soils in the southern Bulgarian mountains. As the Chaney et al. team conducted research on Ni phytomining, they contacted potential sources of funding to develop Ni phytomining technology. Separate work with INCO Ltd., Ontario, Canada, had addressed risk assessment for soil Ni phytotoxicity and methods to alleviate the toxicity (reported in Kukier and Chaney 2000, 2001, 2004; Siebielec et al. 2007; Chaney 2019). Angle and Chaney visited several Ni industrial companies to present our developing Ni phytomining technology and seek funding.

Separately, U.S. Bureau of Mines scientists undertook testing of *Streptanthus polygaloides* (Nicks and Chambers 1995, 1998; Brooks et al. 1998) on an ultramafic soil in California. This species was reported to be able to accumulate 1% Ni in dried leaves (Reeves et al. 1981). However, the trials with *Streptanthus* were of limited success because of the growth pattern of this species. Although its leaves can accumulate greater than 1% Ni, leaves represent only a small portion of the shoot biomass at harvest, which has to occur at flowering before the leaves fall to the soil. Nicks and Chambers' field studies were the subject of a news note in *Discover Magazine* (Anonymous 1994), a paragraph which was read by Jay Nelkin (of Viridian LLC) who contacted Nicks and Chambers, who referred Nelkin to Chaney and Angle about the commercialization of Ni phytomining. We described concepts and data on Ni phytomining in the USA and solicited funding for a Cooperative Research and Development Agreement (CRADA) with the US Department of Agriculture, which would allow the CRADA commercial co-operator to obtain licenses to any patents of the technology and germplasm developed during the CRADA. After prolonged discussion of what might be possible, a meeting was held in Beltsville (attended by the Nelkin family, Chaney, Angle, Li, our managers and Alan Baker) at which the extraordinary Ni accumulation in leaves of hyperaccumulators was demonstrated by showing the reaction of hyperaccumulator leaves to dimethylglyoxime-impregnated filter paper (resulting in an instant purple colour-response). Negotiations occurred over several years during which time a US patent for Ni phytomining using Ni hyperaccumulators was granted (Chaney et al. 1998). With the patent issued, commercial interest greatly increased. The CRADA started in June 1998 and led to our collaborative development of commercial Ni phytomining technology (see Angle et al. 2001; Li et al. 2003b; Chaney et al. 2007).

6 Advancing the Development of Agromining/Phytomining

Brooks extended the evaluation of potential phytomining by estimating the value of hyperaccumulator biomass for elements with known hyperaccumulator plants (Brooks et al. 1998, 1999). Table 3 is from his paper with some additional adjustments shown for current metal prices. The price of metals varies somewhat widely with global economic conditions, among others, thus making it difficult to decide on developments in phytomining. Several approaches to modelling or improving phytoextraction and phytomining have been reported by researchers. Robinson et al. (2003, 2009) developed a computer program to estimate annual metal removals and reviewed the phytomanagement of trace elements in soils.

One greatly unexpected outcome from the basic research by the CRADA team was the recognition that higher Ni accumulation by *Odontarrhena* species occurred when soil pH was raised (Li et al. 2003a; Kukier et al. 2004), which was opposite the effect of soil pH on the solubility, extractability, and phytotoxicity of soil Ni (e.g. Kukier and Chaney 2004). Several estimates of potential phytomining of Ni from different soils were premised on DTPA-extractable or ammonium acetate-extractable Ni, both of which increase as pH declines. But this is opposite the response of *Odontarrhena* species Ni accumulation to soil pH (Kukier et al. 2010). Furthermore, added EDTA actually reduced Ni accumulation by several studied Ni hyperaccumulator species (Nkrumah et al. 2018a). In the

Table 3 Example first reported hyperaccumulator plant species, their estimated biomass and element concentrations together with the value of the biomass metals

Element	Species	Concentration (μg g^{-1})	Biomass (t ha^{-1})	Reference
Au	*Brassica juncea*	57	5	Anderson et al. (2005)
Cd	*Noccaea caerulescens* 'Ganges'	3000	4	Reeves et al. (2001)
Co	*Haumaniastrum robertii*	10,200	4	Brooks (1977)
Cu	*Haumaniastrum katangense*	8356	5	Brooks (1977)
Pb	*Thlaspi rotundifolium*	8200	4	Reeves and Brooks (1983)
Mn	*Macadamia neurophylla*	55,000	30	Jaffré (1979)
Ni	*Alyssum bertolonii*	13,400	9	Minguzzi and Vergnano (1948)
	Berkheya coddii	17,000	18	Morrey et al. (1992)
Se	*Astragalus pattersoni*	6000	5	Cannon (1960)
Tl	*Iberis intermedia*	3070	8	Leblanc et al. (1999)
U	*Atriplex confertifolia*	100	10	Brooks et al. (1998)
Zn	*Noccaea caerulescens*	10,000	4	Brooks et al. (1998)

Re-interpretation of some species changes the potential value of Cu and Co hyperaccumulators

case of phytoextraction of Cd and Zn using *N. caerulescens*, this species accumulates more Cd and Zn at lower pH for which these metals have much higher solubility (Wang et al. 2006).

Chaney et al. (2020) evaluated the role of convection of a soil solution containing Ni to *Odontarrhena* roots versus diffusion of Ni from soil solid phases. For a Brockman gravelly loam soil from Oregon having 4700 μg g^{-1} Ni, the Ni in soil saturation extracts was only 0.047 mg L^{-1} or 0.8 μM. Assuming the plants used 250 mL of soil solution to produce 1 g of shoot dry matter, and the shoot dry matter contained 15,000 μg g^{-1} Ni, the soil solution would have needed to contain 60 mg L^{-1} or 1010 μM Ni to provide the shoot Ni by convection in soil solution to roots. Hence, convection could account for only 0.8/1010 or 0.079% of *O. corsica* absorbed soil Ni. Many reviews have since been published, and valuable up-to-date reviews are included in the present book. In particular, readers are referred to Chapter "Agronomy of 'Metal Crops' Used in Agromining" by Nkrumah et al. on the agronomic management of Ni agromining/phytomining to attain commercial agromining of Ni.

During the 1990s, the Morel et al. team studied two directions for phytoremediation in Europe: phytodegradation of organic pollutants with the accelerated degradation of petroleum hydrocarbons in the rhizosphere (Chaîneau et al. 1995, 2000), and phytoextraction to remove heavy metals and metalloids from contaminated or mineralized soils. Their encounter with Alan Baker in 1993 initiated joint research with the hyperaccumulator *N. caerulescens* (Prayon, Belgium; Baker and Brooks, 1989), and helped to demonstrate the potential of the species to extract Cd and Zn from the soil and from different matrices, including wastes (Schwartz 1997). It is with this species that they demonstrated the proliferation of roots in contact with hotspots of Cd and Zn pollution (Schwartz et al. 1999). Beyond the Prayon population, known to hyperaccumulate both Zn and Cd but only accumulate useful amounts of Zn, they conducted a survey in France that led to the discovery of a number of *N. caerulescens* populations (generically, but mistakenly, called 'Ganges ecotypes'). These populations were able to accumulate 10-fold higher Cd levels than Prayon, giving this species the ability to alleviate Cd risk from contaminated soils in temperate zones (Reeves et al. 2001). The availability of the 'Ganges' *N. caerulescens* led to the initiation of extensive research work on Cd phytoextraction (Schwartz et al. 2001a, b, 2003, 2006; Perronnet et al. 2003; Sterckeman et al. 2004; Saison et al. 2004; Sirguey et al. 2006). In parallel, thanks to collaborations established in the late 1980s with colleagues in Albania, Morel et al. conducted in 1996 a survey of the flora of ultramafic environments in the Balkans (Shallari 1997; Shallari et al. 1998). The survey made it possible to establish a list of species growing on these ultramafic environments, which represent >10% of the Albanian territory, and to identify Ni hyperaccumulators, in particular *O. chalcidica*, widespread in this geographic region. Later, other plants originating from the Balkans were also added to the list of potential agromining crops (e.g. *Bornmuellera* (formerly *Leptoplax*) *emarginata* and *B. tymphaea*) (Chardot et al. 2005; Bani et al. 2009, 2010, 2013).

The success of phytoextraction, hence agromining/phytomining, depends closely on soil properties, in particular metal phytoavailability (Gérard et al. 2001; Morel 2012), i.e. the capacity of the solid phase to supply the soil solution with metal ions where they can be absorbed by roots (Morel 1997). This property has generated a lot of work since the eighteenth Century among agronomists wishing to measure the size of the available nutrient reserve, e.g. phosphate. Much progress has been made with the use of isotopic techniques, with the measurement of L- and E-values (Larsen 1952) that give a quantification of the pools of phytoavailable nutrients (e.g. Fardeau 1981). The availability of trace elements such as Cd, Hg, Ni and Zn was later assessed using these techniques (Fardeau et al. 1979; Morel 1985; Echevarria et al. 1998; Sinaj et al. 1999; Gérard et al. 2001; Kukier et al. 2010).

The extraordinary ability of hyperaccumulators to absorb soil metals had led to the belief that these plants would be capable of extracting metal ions from the soils that were not able to be extracted by other 'normal' plants. However, with isotopic techniques it was possible to demonstrate that, in fact, hyperaccumulators and 'normal' plants absorb Cd and Ni from the same labile pool of soil metals (Echevarria et al. 1998; Shallari et al. 2001; Gérard et al. 2001). Hyperaccumulator plants exhibit an amazing ability to deplete this pool. For example, a single crop of *N. caerulescens* was shown to take up more than 20% of the available Cd in a soil contaminated by a Zn smelter (Gérard et al. 2001). In the soil pH adjustment greenhouse pot study of Wang et al. (2006), *N. caerulescens* from Viviez, France, was grown in a farm soil contaminated by a Zn smelter. At the optimum pH, 40% of soil Cd was removed. These finding proved that hyperaccumulators are excellent agents to reduce the risks associated with soil-to-plant transfer of Cd. Field trials run in South China showed that co-cropping hyperaccumulators (e.g. *Sedum alfredii*) and maize (*Zea mays*) significantly reduced the intake of Cd by maize, in comparison with a control lacking hyperaccumulators (Wu et al. 2007, 2006, 2010). In a pot test to see if co-cropping with a grass which would secrete phytosiderophores to obtain soil Fe might affect Ni hyperaccumulation by *O. corsica* or *Alyssum montanum*, Broadhurst and Chaney (2016) found no interactions among these species.

Cutting and harvesting hyperaccumulators breaks the natural cycle of metal in the soil-plant-litter system. The harvest is aimed either at removing the metal contained in the above-ground biomass or recycling the metal if it has a sufficient economic value. Data accumulated over the years have shown that agromining is a relevant option. However, there was a need for tests to be run at large scale in order to demonstrate the commercial feasibility of this approach. As a continuation of the work carried out on *O. chalcidica* (Shallari 1997), the French-Albanian team initiated a series of multi-year field trials to establish the practical potential of the species to agromine Ni from ultramafic soils. The thesis conducted by Bani (2009) showed that under field plot conditions, it was possible to harvest up to 120 kg of Ni ha^{-1} yr^{-1}, making it feasible to implement the technology on a wider scale (Bani et al. 2007, 2010). Hence, complementary to the Oregon studies (Li et al. 2003a), the Morel et al. team demonstrated the economic feasibility of the production of biomass containing metals of industrial interest. A subsequent phase of optimization of the agronomic part of the chain allowed the refining of conditions necessary for fertilization and pesticide treatment (Bani et al. 2007, 2009, 2015a, b), the selection of the best individuals, the improvement of substrate fertility, the decrease of toxicity, and the increase of availability of metals of interest (e.g. Rees et al. 2015, 2016), taking into account the whole cycle of processes (Rodrigues et al. 2016, Rodríguez-Garrido et al. 2018). Similarly, testing of *P. rufuschaneyi* in Sabah (Malaysia) estimated the net value of the annual crop reached \$250 ha^{-1} yr^{-1} (Nkrumah et al. 2018b, 2019). Agronomic and soil fertility practices for most economic Ni agromining are reviewed in Chapter "Agronomy of 'Metal Crops' Used in Agromining" by Nkrumah et al. (2020).

In natural environments, hyperaccumulating plants contribute to a change in the chemical status of soil metals. Metal ions are extracted from the soil available compartment in the rooting depth, transferred to the aerial parts, and then deposited on the soil surface as litter during senescence. The Morel group demonstrated that metals are much more phytoavailable when present in litter than in the soil (e.g. Cd—Perronnet et al. 2000; Ni—Zhang et al. 2007), but still less phytoavailable than added soluble Ni salts (Zhang et al. 2007). Consequently, the plant contributes to increase size of the available metal pool of the surface of the soil, a pool where it preferentially picks up metal ions during its life. Therefore, in natural environments, hyperaccumulators thrive on a restricted metal pool that is permanently renewed by litter and root deposits. The role of rhizosphere microorganisms was also investigated (e.g. Aboudrar et al. 2007, 2013; Abou-Shanab et al. 2003; Chardot-Jacques et al. 2013) with applications in the inoculation of PGPR to enhance phytoextraction of metals (Durand et al. 2016). More recent work showed that, using high-throughput sequencing techniques, the structure and diversity of the rhizosphere bacterial communities of Ni hyperaccumulator plants is strongly affected by the presence of available metal concentration in the rhizosphere, as demonstrated for *Rinorea bengalensis* in Borneo and *Odontarrhena chalcidica* in the Balkans (Lopez et al. 2020a, b).

Other work using stable isotopes in the soil-plant-system has shown that isotopic fractionation during the various processes taking place in hyperaccumulators yields new insights into the mechanisms of uptake, translocation, sequestration, and secondary redistribution into the plant of Cd and Ni (Montargès-Pelletier et al. 2008; Tang et al. 2012a, 2016; Deng et al. 2014; Estrade et al. 2015). The collaboration of the Morel group with groups in Guangzhou (PR China) contributed to increasing our knowledge about the physiology of various hyperaccumulators, including *Picris divaricata* and *Sedum alfredii* (Ying et al. 2010; Du et al. 2011; Tang et al. 2013; Wu et al. 2007), and to revealing strategies to design cropping systems suitable to obtain value from mineralized or contaminated environments (Tang et al. 2012b). This dynamic gave birth in 2015 to an international joint laboratory, named ECOLAND (Ecosystem Services Provided by Contaminated Land), with a strong focus on the use of phytoremediation technologies to increase value from metal-rich lands. During the last years, intensive work was conducted on former rare earth elements (REEs) mine sites to develop technologies, including phytoremediation coupled with amendments, in order to restore the ecosystems. An option is the agromining of the residual REEs present in the mine tailings using plants such as the fern *Dicranopteris linearis* (syn. *D. dichotoma*) (Liu et al. 2019, 2020) and *Phytolacca americana* (Yuan et al. 2017). From natural ultramafic soils, agromining was applied to industrial sites contaminated by metals, and now is mining industrial wastes such as industrial sludge containing high amounts of metals together with other elements of interest (e.g. P). These wastes can be particularly toxic, exhibiting rather low pH and hence need soil construction to be able to support crops. This issue was studied and developed by Rue et al. (2019) and Tognacchini et al. (Tognacchini et al. 2020) at BOKU, Vienna (see Chapter "Agromining From Various Industrial Wastes").

The agromining chain would have been incomplete without the second phase, i.e. metal recovery and subsequent production of commercial products, which is crucial for an economic technology. Dealing with biomass is a challenging task for the metallurgical sector that scientists in chemical engineering and separation engineering have tried to solve. In 2004, the group of Simonnot et al. started research on the recovery of Ni from *O. chalcidica* biomass. They established a cooperation with INRS of Québec, which led to an original patented procedure to produce Ni salt (Mercier et al. 2011; Barbaroux et al. 2012). The recovery of the metal contained in the biomass had gained interest since with a focal point on Ni leading to several Ni upgrading routes, metals, salts, or catalysts (Li et al. 2003a; Barbaroux et al. 2009, 2011, 2012; Chaney 2019; Harris et al. 2009; Losfeld et al. 2012; Zhang et al. 2014; Vaughan et al. 2017). Research to develop unique Ni catalysts from hyperaccumulator plant biomass has been reported (Kidd et al. 2018). In general, combustion is commonly used to eliminate the organic matter, then hydrometallurgical processes are applied to the ash that contains high concentrations of Ni, 10–25% according to the burnt biomass. Other means, which consist of a direct leaching of metals, have proven effective in recovering Ni directly from hyperaccumulator biomass (Guilpain et al. 2018). Besides Ni, the main target for two decades, other metals have been considered and technologies are being developed. For example, a procedure was developed for Cd and Zn, and more recently for REEs, using the *D. linearis* accumulator (Chour et al. 2018), and the separation of REEs from other elements such as Al. Overall, agromining involves not only the recycling of the target metal, but also other by-products such as potassium that can be recycled in agriculture. Recovery of heat produced during the combustion processes has also been studied and implemented with the use of dedicated boilers.

The 'ups and downs' of phytomining in the USA, described above, probably had a negative effect in the development of the whole chain. However, in France, two start-ups were created by Stratoz (2013) and Econick (2016), to market metal compounds derived from harvested hyperaccumulator biomass. Econick has established a partnership with the industrial sector, which uses Ni and other metals and wishes to develop the use of bio-based elements in their applications. An example is the use of Ni oxide produced by agromining in the colouring of works of art, such as the leatherback turtle manufactured by the Daum crystal-maker with nickel produced by Econick (see Chapter "Processing of Bio-Ore to Products: Nickel"; Fig. 3.5).

7 Outlook

The term 'agromining' was introduced as it reflects the entire chain of agronomic processes for the production of metals of economic value from the cultivation of plants on metalliferous environments (Morel 2013; van der Ent et al. 2015). Agromining is similar in concept and complexity to the chain of processes that are required to produce cash crops. The word also stresses the need for multi-disciplinary studies. Indeed, agromining would not have emerged without a dynamic that favoured the gathering of a large set of disciplines. Multi-disciplinarity can be illustrated by projects such as the LORVER project, funded by both the Region Grand Est in France and the EU (ERDF), aimed at the production of biomass for industrial use (e.g. energy, fibre or agromining) on polluted sites and even on polluted matrices (e.g. industrial wastes), using a series of plant species and strategies to restore soil functions with only secondary materials, avoiding clean soil material. It is also one of the reasons for the success of the Laboratory of Excellence 'Ressources21' aimed at developing the green mining of strategic metals, and for supporting research on agromining. Hence, agromining is no longer an idea, it is now a chain of processes that is being implemented at field scale thanks to research and demonstration projects funded by the National Agency for Research (e.g. Agromine, ANR, France; Simonnot 2014a, b) and the EU (e.g. LIFE; Echevarria 2015), wherein international teams combine efforts to make agromining feasible for different substrates and climatic conditions (Bani et al. 2015a, b; Pardo et al. 2018). As well as Ni, phytoextraction of Cd and Zn is already implemented at field and commercial scale with plants such as *N. caerulescens*. The selection of improved cultivars for Cd removal from contaminated soils is underway and has been advanced to produce efficient lines (Sterckeman et al. 2019) in order to improve phytoextraction and subsequent recycling of metals of interest.

Tropical regions have substantial unrealized opportunities for Ni agromining operations. Recent advances by the team of van der Ent et al. in Australia, Malaysia, and elsewhere in SE Asia have revealed that the extensive ultramafic outcrops in these regions have suitable characteristics that include high Ni phytoavailability for phytomining species and good soil physical properties, required for profitable agromining. Furthermore, Ni hypernickelophores adapted to tropical rainforest environments are being developed commercially (e.g. Nkrumah et al. 2018b, 2019). Currently, some ultramafic areas in these regions are not readily accessible, whereas others have challenging topography, and the rocky nature of other substrates limit usage. Nonetheless, large expanses of ultramafic substrates are available for consideration. Attempts by Viridian LLC to capitalize on this huge expanse of ultramafic substrates by employing the widely used Mediterranean-climate hyperaccumulator *Odontarrhena* species, did not yield useful outcomes (van der Ent et al. 2013). In view of this, van der Ent and colleagues (2015) have embarked on extensive field surveys and systematic herbarium screening that have led to the discovery of more than 50 new hyperaccumulator plant species in Sabah (Malaysia) and Halmahera (Indonesia). Potential ‘metal crops’ were selected from these hyperaccumulator species for agronomic trials, which have begun in order to ascertain growth performance, nutrient requirements, and Ni yield (see Nkrumah et al. 2018a, b, 2019). Pioneering studies in Sabah are currently underway and consist of a detailed, large, randomized block growth trial using *Phyllanthus rufuschaneyi* and *Rinorea bengalensis* undertaken over 12 months, and a 1.5 ha field using *P. rufuschaneyi* (Nkrumah et al. 2019). The pot trial was aimed at testing, under controlled conditions, the effects of N, P, K, Ca and S fertilization, pH adjustment, and organic matter amendments, which will ultimately be critical for successful field-scale agronomic systems. The field trial is aimed at demonstrating the agronomic management needed for best commercial-scale Ni agromining in tropical regions, including spacing in the field and coppicing schedules that are most profitable. Early results from the pot trial suggest that a Ni yield of 200–300 kg ha^{-1} can be achieved under appropriate agronomic systems—the highest so far achieved with agromining, which is indicative of hitherto untapped metal resources in tropical regions. It is envisaged that economic tropical Ni agromining could replace marginal agriculture on poor ultramafic soils not useful for commercial farming, thus serving as an income source for local communities in Malaysia, Indonesia, and the Philippines to farm for metals.

Other benefits likely to emerge include improvement of the fertility of these soils and mine wastes for future usage such as in productive agriculture and agroforestry. The agromining technology could also be an integral part of Ni ore strip-mining operations, during the initial project phase, by cropping the surface soil and then as part of the rehabilitation strategy for the mine waste disturbed areas. Efforts are underway to explore and secure more potential sites for implementation of tropical Ni agromining; the success of the first field trial will be critical in providing baseline information. Unknown is whether improved agronomic management, breeding improved cultivars of hypernickelophores, or development/bioengineering of transgenic, high-yielding crop plant species with hyperaccumulator ability will be the most effective direction for progress in phytomining. Transgenic *Brassica juncea* accumulated more Se than the wild type, but still far lower than the amount accumulated by *Astragalus* and *Stanleya* species (Pilon-Smits and Pilon 2002; Parker et al. 2003). Consideration of the public acceptance of crop plants made into trace element hyperaccumulators suggests, however, that public acceptance would be difficult (Angle and Linacre 2005). Application of the concepts discussed in this paper regarding agromining of Ni are

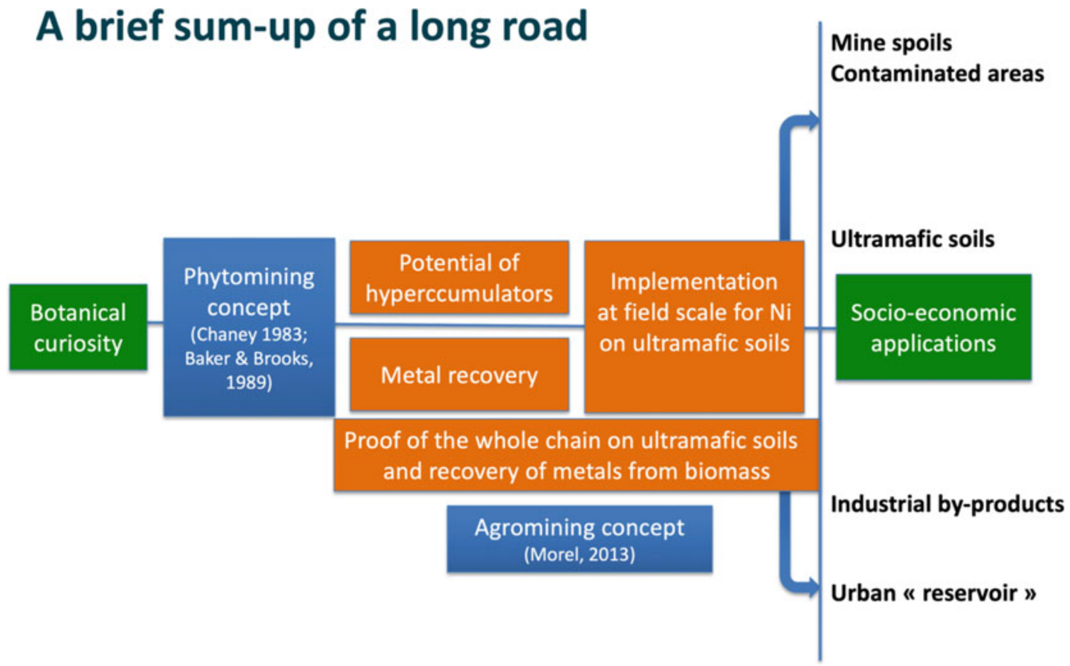

Fig. 1 The main steps towards phytomining then agromining (Morel et al. 2017)

extended to other elements, which may be mined if similar research is conducted to demonstrate efficacy, develop agronomic management practices, breed improved agromining cultivars, and demonstrate methods for recovery of valuable ‘ores’ from the biomass (Remigio et al. 2020).

Finally, as developed in the Conclusion and Outlook section of this book, agromining is not only a chain of processes aimed at producing bio-based metals, it is also a way to mine with the minimum impact on ecosystems, even with a goal of improving their state. Therefore, when run in field conditions, agromining should fulfil a wide range of functions that are able to augment a large series of ecosystem services that can be rendered by contaminated lands and matrices, such as pollution attenuation, recycling of wastes and land, mitigation of climate change through carbon storage, improvement of soil fertility, improvement and preservation of ecosystems and biodiversity, and cultural services such as income for famers, hence a better life for people, etc (Fig. 1).

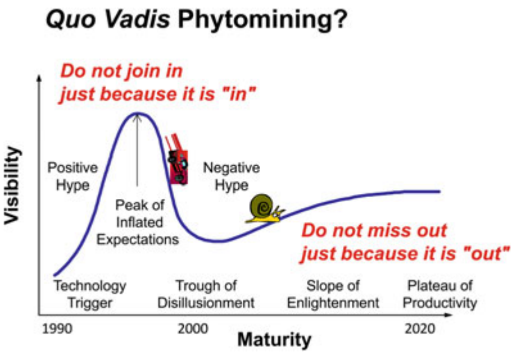

Fig. 2 The time course of research and development of phytomining and agromining

The saga of the ‘long and winding road’ for the development of phytomining and now agromining continues even in the face of the current low world price for base metals. The story has been well told in a *New Scientist* article by Moskvitch (2014) and is also summarized in the stylized time-line illustrated in Fig. 2. We are now at a point where, at least for nickel, the prospect for commercialization of Ni products through agromining represents a real economic and socially desirable prospect. The first tropical Ni agromining farm has begun operations in Sabah (Nkrumah et al. 2019), complementing the Ni agromining in Albania by Bani et al. (2015a, b). The future also clearly holds great opportunities for similar approaches for other metals and metalloids of industrial and commercial interest. The following chapters document our present state of knowledge on agromining/phytomining and their applications.

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Agronomy of ‘Metal Crops’ Used in Agromining

Philip Nti Nkrumah, Rufus L. Chaney,
and Jean Louis Morel

Abstract

Agromining involves growing selected hyperaccumulator plant species (‘metal crops’) on low-grade ore bodies or mineralized (e.g. ultramafic) soils, or anthropogenic metal-rich materials (e.g. contaminated soils, mine spoils, industrial sludge), followed by harvesting and incineration of the biomass to produce a ‘bio-ore’ from which target metals or salts may be recovered. This chapter begins with an introduction section that clarifies the concepts of phytomining and agromining. We then acknowledge the role of agronomy in enhancing the metal yield of ‘metal crops’, with emphasis on Ni. Highlighted are the selection of sites section, potential agromining substrates and discussion of the role of metal phytoavailability in economic agromining. We present the criteria for selecting potential ‘metal crops’ and possible regions where these species are most suited for successful agromining operations. We then discuss thoroughly the experimentally demonstrated soil and plant management practices that have been proposed to increase biomass and the metal yield of ‘metal crops’. We report also on progress of the pioneering tropical agronomic trials. Finally, we provide a conclusion and present an outlook on the agronomy of ‘metal crops’ that may be used in agromining.

1 Introduction

Chaney (1983) was the first to propose the use of metal hyperaccumulator plant species for soil remediation, and introduced the concept of phytomining (a technology that then used such plants to accumulate soil Ni into the plant shoots where they could be harvested and used as an alternative ore for Ni). Nearly three decades later, Morel (2013) proposed the adoption of the term ‘agromining’ to broaden the scope of the technology to include the entire soil-plant-ore agrosystem (Fig. 1). Agromining involves the growing of selected hyperaccumulator plant species (‘metal crops’) on low-grade ore bodies or mineralized (ultramafic) soils, or on anthropogenic metal-rich materials (e.g. contaminated soils, mine spoils, industrial sludge), followed by harvesting and incineration of the biomass to produce a ‘bio-ore’ from which target metals or salts may be recovered (Barbaroux et al. 2012; van der Ent et al. 2015a). The target elements may include As, Se,

P. N. Nkrumah (✉)
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, Australia
e-mail: p.nkrumah@uq.edu.au

R. L. Chaney
Chaney Environmental, Beltsville, MD 20705, USA

J. L. Morel
Laboratoire Sols et Environnement, UMR 1120, Université de Lorraine, INRAE, 54518 Vandoeuvre-lès-Nancy, France

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
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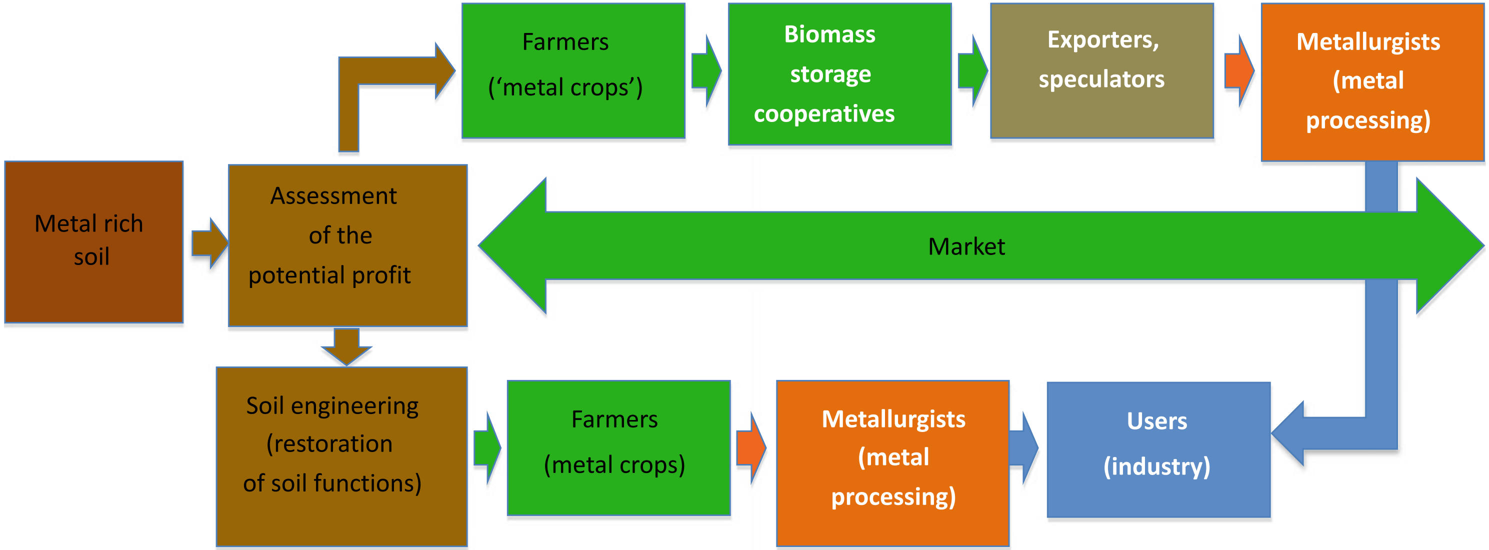

Fig. 1 The concept of agromining (Morel et al. 2017)

Cd, Mn, Ni, Tl and Zn, as well as rare earth elements, but most research has focussed on the development of Ni agromining. Large-scale demonstration of agromining of Ni with *Odontarrhena chalcidica* has been undertaken in the USA (Fig. 2) (Li et al. 2003b) and Albania (Fig. 3) (Bani et al. 2015a, b), with *Berkheya coddii* in South Africa (Howes et al. 1998) and with *Phyllanthus rufuschaneyi* in Sabah, Malaysia (Fig. 4) (Nkrumah et al. 2019a).

Appropriate agronomic practices, based on insights from laboratory (Fig. 5) and field tests,

Fig. 2 Field of flowering *Odontarrhena* species near Cave Junction, Oregon, USA. Soil was derived from ultramafic parent materials and is rich in Ni

Fig. 3 Large-scale demonstration of agromining of Ni with *Odontarrhena chalcidica* growing on ultramafic substrate in the Balkans (Albania)

Fig. 4 Large-scale demonstration of agromining of Ni with *Phyllanthus rufuschaneyi* cultivated on ultramafic substrate in Sabah (Malaysia)

Fig. 5 Large pot experiment with *Odontarrhena* species conducted at Beltsville, USA

have been proposed to maximize yields of the selected 'metal crops' (Li et al. 2003a; Bani et al. 2007; Kidd et al. 2018; Nkrumah et al. 2016, 2019a, b, c, d). Here we discuss the agronomy of 'metal crops' that are more important for developing efficient agromining with a special emphasis on Ni as a realistic example. In the past, most of these agronomic systems have been tested in temperate regions mainly using *O. chalcidica* and *O. corsica* (Bani et al. 2015a, b; Li et al. 2003a, b). More recently, a large growth trial has been undertaken in Sabah, Malaysia, using two tropical species (*P. rufuschaneyi* and *Rinorea* cf. *bengalensis*) to develop an agronomic system for tropical Ni agromining (Nkrumah et al. 2018a, b, 2019a, b, c, d).

2 Selection of Sites for Cultivation of 'Metal Crops' Used in Agromining

The cultivation of 'metal crops' could be undertaken on large metal-rich surface areas. For Ni, cultivation is feasible on ultramafic areas with suitable topography, where soils are otherwise unsuitable for food production; or degraded Ni-rich land that includes Ni laterite mine sites, smelter contaminated areas, and ore beneficiation tailings (van der Ent et al. 2015a). Figure 6 shows a typical agromining site in a large ultramafic area in Sabah that has been prepared for cultivation. Ultramafic soils develop from the weathering of ultramafic bedrock (Brooks 1987), and are characterized by relatively high concentrations of Mg, Fe, Mn, Cr, Ni and Co, whereas the concentrations of Ca, N, P, K, Mo and B typically range from low to deficient levels (Baker and Brooks 1989; Echevarria 2018; Proctor and Woodell 1975).

High Ni phytoavailability is essential for successful Ni agromining (Massoura et al. 2004; Nkrumah et al. 2018a, b), because Ni hyperaccumulator plants take up Ni from the same soil labile Ni pools as 'normal' plants (Echevarria et al. 1998, 2006; Shallari et al. 2001). Nickel hyperaccumulator plants have efficient root absorption mechanisms that deplete the phytoavailable Ni pools to the extent that chemical equilibrium in the soil Ni is changed (Centofanti et al. 2012; Deng et al. 2014). As a result, Ni

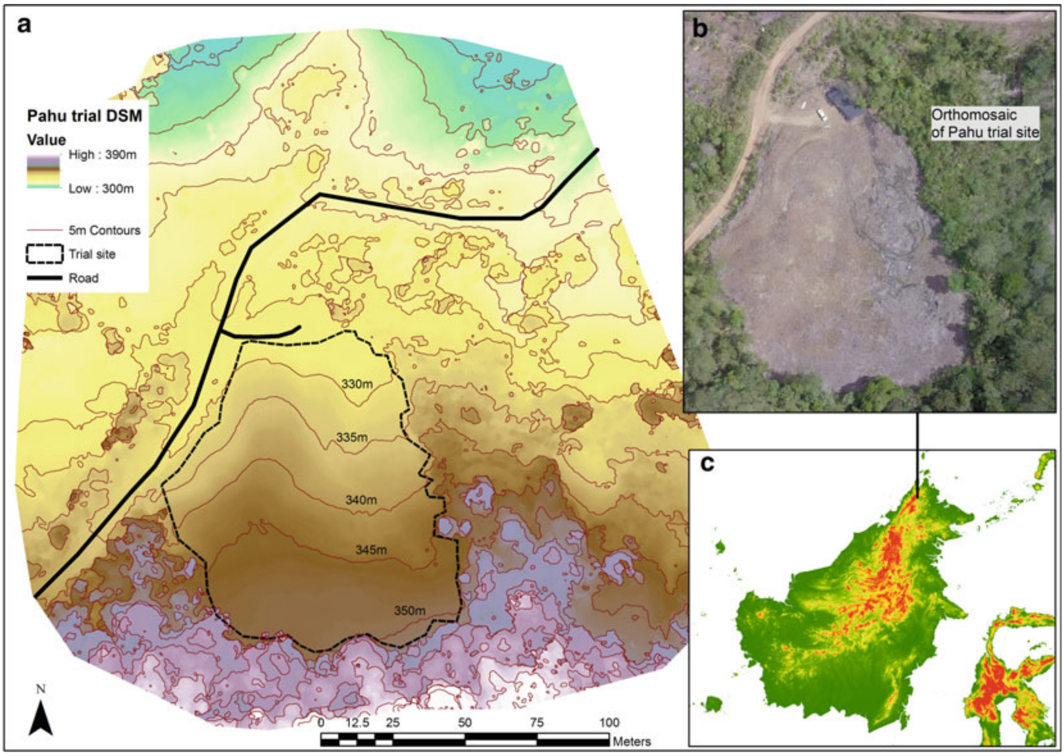

Fig. 6 Large-scale field site for tropical Ni agromining demonstration in Sabah, Malaysia

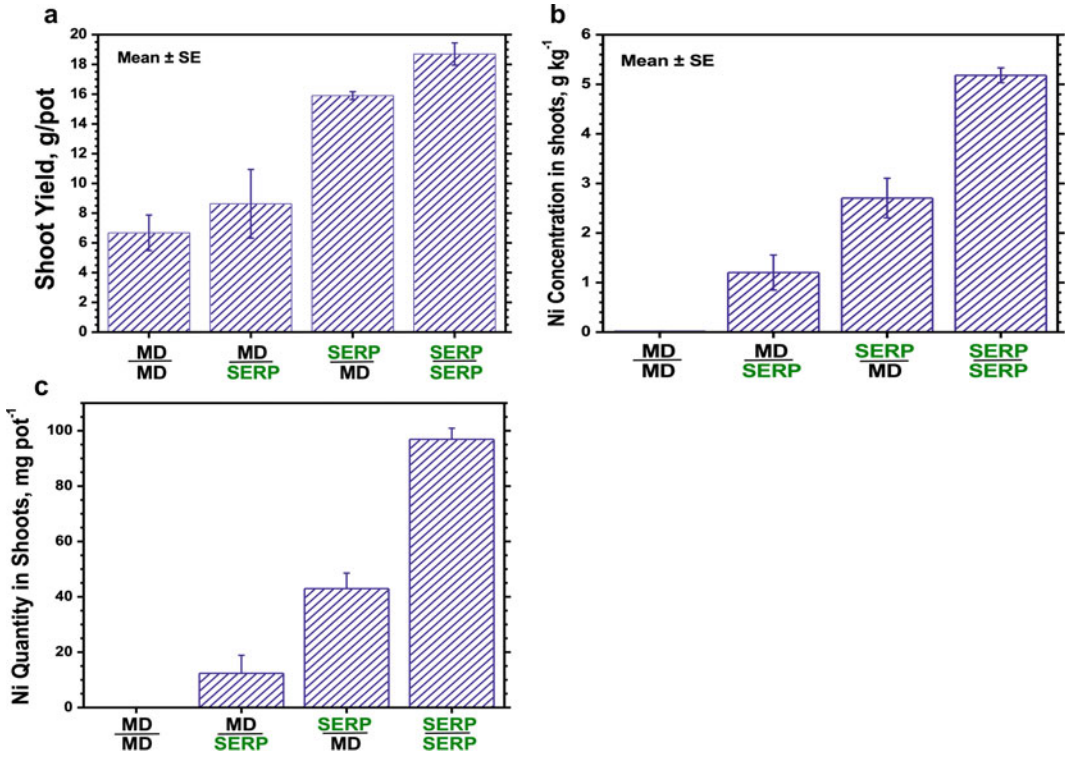

Fig. 7 Effect of normal soil from Maryland, USA (Christiana fine sandy loam) or ultramafic soil from Oregon, USA (Brockman variant cobbly loam) used at topsoil or subsoil on: **A** growth, **B** Ni concentration, and **c** Ni content in shoots of *Odontarrhena corsica* (Chaney et al. unpublished)

from non-labile pools replenishes the labile pool over time to maintain equilibration (Centofanti et al. 2012), but this is a slow process and depends on the local buffering system (Massoura et al. 2004). Metals in the hyperaccumulator biomass exhibit a higher phytoavailability than in the soil from which they derive, as reported for Cd in the hyperaccumulator *Noccaea caerulescens* (Perronnet et al. 2000) and Ni in *O. chalcidica* (Zhang et al. 2007). Recent stable isotope fractionation studies have revealed the contribution of leaf litter from hyperaccumulator plants to phytoavailable Ni in local ultramafic substrates (Estrade et al. 2015), but the Ni is rapidly adsorbed on the Fe and Mn oxides in the soil (Zhang et al. 2007). We stress that although the Ni-rich shoot biomass will be removed by harvest during agromining operations, the next crop will obtain Ni from the remaining pools of Ni enough for economic recovery. Recently, Chaney et al. (unpublished) have demonstrated the role of subsoil metal on the overall metal yield of a ‘metal crop’ (Fig. 7). Although there have been no long-term repeated hyperaccumulator cropping experiments to ascertain the number of crop years that are possible for profitable Ni phytomining in the same area, our previous study suggests that Ni agromining could be sustainable for 15–50 years (Nkrumah et al. 2016). As there is currently no chemical extraction method that can accurately predict Ni uptake by hyperaccumulator plants (van der Ent et al. 2019) (as shown in Fig. 8), future studies need to develop Ni phytoavailability assays in order to predict Ni yield in ‘metal crops’ as already established for ‘normal’ plants using DTPA extraction (e.g. Echevarria et al. 1998) or with 0.01 M $Sr(NO_3)_2$ (Siebielec et al. 2007). The suggested cycle of Ni evolution in a hyperaccumulator cropping system is shown in Fig. 9.

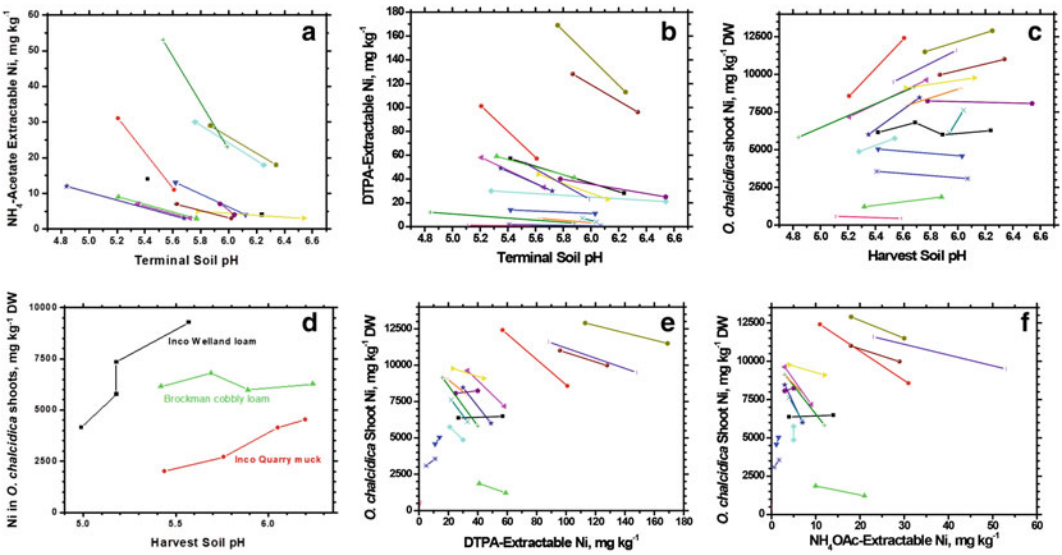

Fig. 8 The effect of adjusted pH on **a** 1.0 M NH_4-acetate-extractable; **b** DTPA-extractable Ni; and **c** Ni accumulation in shoots of *Odontarrhena* species grown for 120 days in 17 ultramafic soils from Oregon and Maryland, USA. **d** Effect of pH on Ni accumulation in shoots of *Odontarrhena* species grown for 120 days on 2 soils collected near a Ni refinery at Port Colborne, Ontario (Quarry muck; Welland loam; organic and mineral soils respectively), and the Brockman cobbly loam. **e** The effect of adjusted pH (control treatment 2, and acidified treatment 6, which have been described in Table 3) on 1.0 M NH_4-acetate-extractable and; **f** DTPA-extractable Ni in relation to Ni phytoextraction from 17 ultramafic soils from Oregon and Maryland, USA. Soil pH was adjusted by addition of HNO_3, followed by leaching of soluble ions, and fertilization for the growth of *Odontarrhena* species to test the effect of soil pH on Ni accumulation

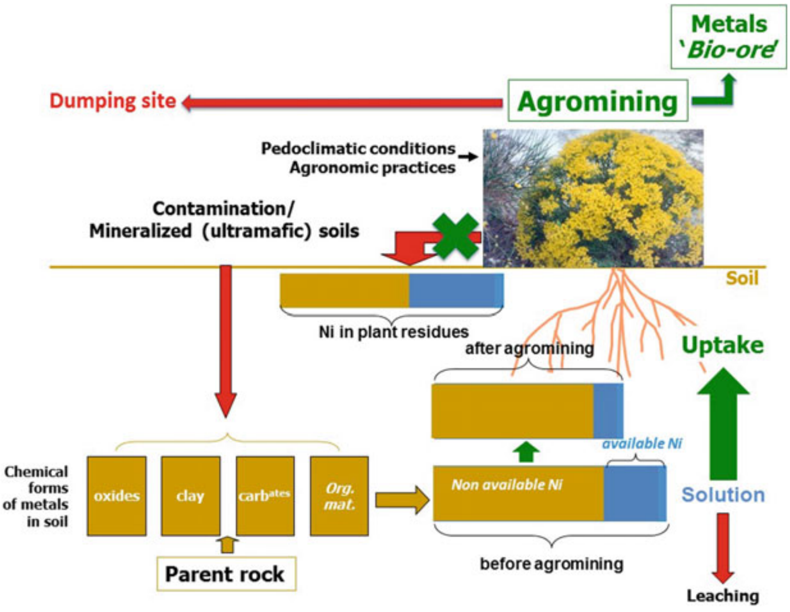

Fig. 9 Influence of hyperaccumulators and agromining on metal cycle. After Morel (2013)

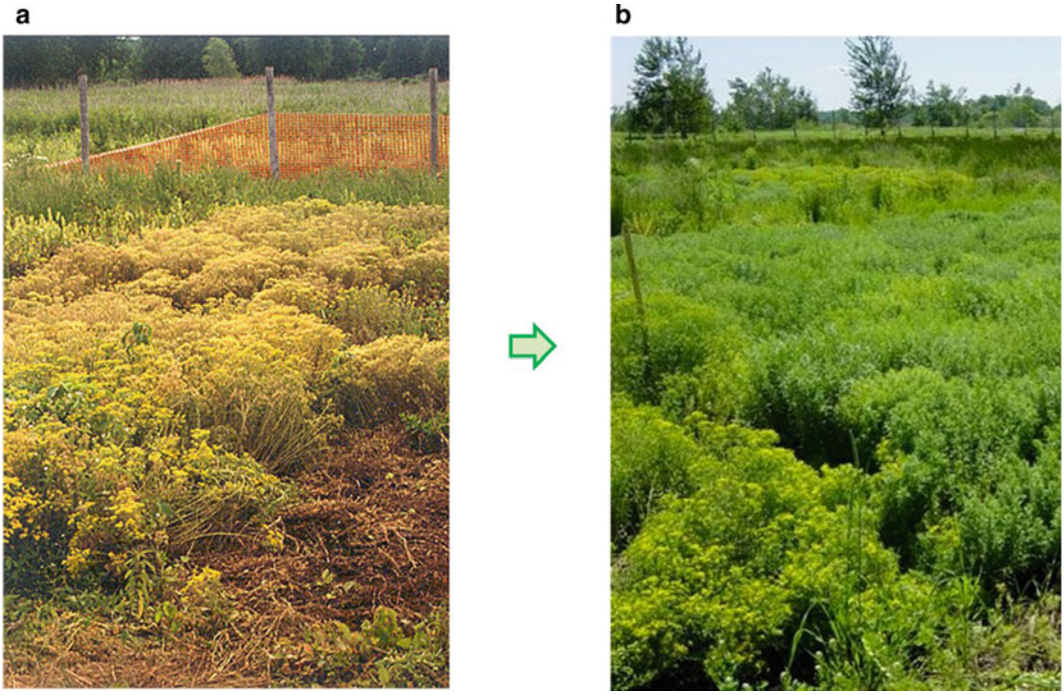

Fig. 10 *Odontarrhena chalcidica* phytomining Ni from: **a** poorly drained, and **b** better drained muck field at Port Colborne, Ontario, Ni-refinery contaminated soils

Soil physical properties influence the Ni yield of hyperaccumulator plants. For example, adequate soil drainage is an important factor in the agronomy of Ni phytomining. In a wet climate where soils are poorly drained, the growth of hyperaccumulator plants (e.g. *P. rufuschaneyi*) is adversely affected and plants may die before normal harvest time. Chaney et al. (2007a) have demonstrated in field trials that such conditions may be corrected by establishing plants with ridge tilling and surface drainage (Fig. 10); tilling ensures maximum root penetration. Good soil water-holding capacity is also important for economic Ni phytomining. Soil moisture affects soil Ni extractability, Ni uptake by hyperaccumulator species, plant growth, and ultimately Ni yield (Angle et al. 2003). *Odontarrhena chalcidica* and *Berkheya coddii* grow well at high moisture content; Ni foliar concentration increases with increasing soil moisture content despite a decreasing trend in extractability of the soil Ni (Angle et al. 2003).

3 Selection and Breeding of ‘Metal Crops’ for Nickel

The criteria for selection of ‘metal crops’ for Ni agromining include high biomass yield combined with high Ni concentrations in the above-ground biomass (Chaney et al. 2007a). Most Ni hyperaccumulator plants accumulate 0.1–0.5% Ni in their biomass, but for economic agromining only so-called ‘hypernickelophores’ (>1% Ni) are potentially suitable (Chaney et al. 2007a, b; van der Ent et al. 2015a). Table 1 lists the Ni ‘hypernickelophore’ species that have been identified as having especially high Ni agromining potential for use as ‘metal crops’. Local plant species are recommended because of their adaptation to local climatic and edaphic conditions (Baker 1999; Bani et al. 2007). Suitable species must be relatively easy to collect as bulk seed accessions and have high success rates of germination, establishment and growth (Angle et al. 2001).

Table 1 Nickel hyperaccumulator species with over 1% Ni ('hypernickelophores') which have high potential for application as 'metal crops' in Ni phytomining operations

Species	Potential application area	Native distribution	Height (m)	Cropping system	Shoot Ni (%)	References
Odontarrhena spp.	Mediterranean and Eurasian Region	S & SE Europe, Turkey, Armenia, Iraq, Syria	0.5–1	Perennial herb	1–2.5	Brooks (1998)
Bornmuellera (*Leptoplax*) spp.	Mediterranean and Eurasian Region	Greece	1–1.5	Perennial herb	1–3.5	Reeves et al. (1980)
Bornmuellera spp.	Mediterranean and Eurasian Region	Greece, Albania, Turkey	0.3–0.5	Perennial herb	1–3	Reeves et al. (1983)
Buxus spp.	Tropical Central America	Cuba	0.3–12	Ligneous shrub	1–2.5	(Reeves et al. 1996)
Leucocroton spp.	Tropical Central America	Cuba	1–3.3	Ligneous shrub	1–2.7	Reeves et al. (1996)
Phyllanthus spp.	Tropical Asia-Pacific Region	Southeast Asia and Central America	1–6	Ligneous shrub	2–6	van der Ent et al. (2015b)
Rinorea cf. *bengalensis*	Tropical Asia-Pacific Region	Southeast Asia	5–20	Ligneous shrub	1–2.7	Brooks and Wither (1977)
Berkheya coddii	Southern Africa	South Africa, Zimbabwe	1–2	Perennial herb	1.1	Morrey et al. (1989)
Pearsonia metallifera	Southern Africa	Zimbabwe	0.35–1.5	Perennial herb	1.4	Wild (1974)

Adapted from Nkrumah et al. (2016)

The selected species may be propagated via direct seeding, transplantation, or by using cuttings (Brooks et al. 1998). Hyperaccumulators show large natural variation for trace metal accumulation as shown for *Noccaea caerulescens* (Reeves et al. 2001; Schwartz et al. 2006; Gonneau et al. 2014).

In the only study to date that has used plant breeding techniques to produce improved 'metal crop' cultivars, Li et al. (2003b) showed a wide range of shoot Ni concentrations and yield of diverse *O. chalcidica* and *O. corsica* germplasm (Fig. 11). Using recurrent selection (required for self-incompatible species), the authors significantly increased shoot Ni concentration and yield of Ni during three cycles of selection. In their study, plant lines were also selected for retention of leaves during flowering so that the high Ni foliar biomass was not lost before harvest of the flowering crop. Collection of diverse germplasm followed by normal plant breeding techniques to improve the 'metal crop' is clearly a key step in developing agromining.

4 Inorganic Fertilization to Increase Biomass Production

4.1 NPK Fertilizers

Inorganic fertilization plays a major role in maximizing the growth and metal yield of 'metal crops' (Álvarez-López et al. 2016; Bani et al. 2007, 2015a; Li et al. 2003a; Nkrumah et al. 2019a, b). The hyperaccumulator species tested on ultramafic soils have proven to react strongly to increased levels of soil N, P and K, due to the very low fertility of their native habitats (e.g. Bani et al. 2015a). Trials using ultramafic soils have shown that fertilizer application enhances shoot biomass

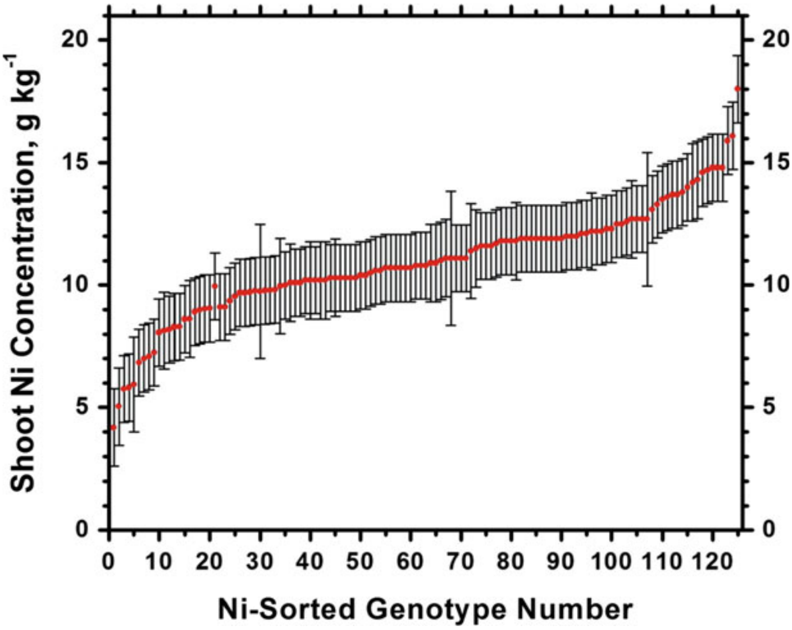

Fig. 11 Variation in shoot Ni concentration among *Odontarrhena chalcidica* genotypes grown to (mid-flowering) harvest stage on an Oregon Brockman variant ultramafic soil with 5500 mg Ni kg^{-1}. Adapted with permission from Li et al. (2003b)

production of Ni hyperaccumulator plants and also increases their overall Ni yield (Table 2). There was a significant increase in the biomass of *O. chalcidica* from 3.2 t ha^{-1} in unfertilized plots to 6.3 t ha^{-1} in the fertilized treatment (Bani et al. 2015a). Table 3 shows the effect of P and Ca fertilizers and pH adjustment on Ni and other element accumulation by *O. chalcidica* from an Oregon ultramafic soil that had received little fertilizer from previous land use.

A significant effect on biomass production has been observed for N application, and split N application could be employed to minimize excessive N leaching (Li et al. 2003a). Phosphorus has a particularly strong effect on the biomass yield and Ni uptake by hyperaccumulator species growing on soil not previously fertilized (Table 3), whereas previously fertilized soils show less of a response to P fertilization (Bennett et al. 1998; Robinson et al. 1997). In ultramafic Vertisols in Albania, Bani et al. (2015b) found that *O. chalcidica* yield increased 10-fold with application of 120 kg NPK and 77 kg Ca ha^{-1} plus monocot herbicide to control Gramineae (Poaceae), as opposed to encouraging co-cropping (Fig. 12). These agronomic practices increased Ni phytoextraction yield from 1.7–105 kg ha^{-1}. Thus, in a long-term field agromining situation, standard inorganic fertilization may be both adequate and preferable.

4.2 Calcium Application

Odontarrhena hyperaccumulator species native to typically low-Ca and low Ca/Mg quotient ultramafic soils accumulate Ca in the absence of fertilization. However, Ca supply to high-Mg ultramafic soils will be required to maintain the annual Ni uptake in *Odontarrhena* species, because biomass harvest and removal reduces the pool of phytoavailable Ca in these soils, for example, the annual removal of 10 t of biomass removes 200 kg of Ca (Chaney et al. 2007a, 2008). Calcium supply in the form of $CaCO_3$ increases Ni accumulation in hyperaccumulator plants growing on some soils, as a result of the combined effect of Ca addition and increased soil pH (Li et al. 2003a; Kukier et al. 2004). In addition, Ni tolerance in hyperaccumulator plants may be improved by $CaSO_4$ addition independent of soil pH (Chaney et al. 2008). The sequestration mechanisms for Ni are distinct from Ca handling or storage in Ni hyperaccumulator plants

Table 2 Outcomes of Ni phytomining agronomic trials showing that N+P+K fertilization and organic matter additions significantly increase the biomass of Ni hyperaccumulator plants, with the former causing negligible dilution in shoot Ni content whereas the latter may reduce it

Agronomic practices	Species	Field and/or pot trials	Locations	Substrates	Effects on Ni yield	References
N+P+K fertilization	*Odontarrhena bertolonii* *Berkheya coddii* *Streptanthus polygaloides* *Odontarrhena chalcidica* *Odontarrhena serpyllifolium s.l.* *Noccaea goesingense* *Phyllanthus rufuschaneyi* *Rinorea* cf. *bengalensis*	Field trial Pot trials	Tuscany (Italy) New Zealand Pogradec (East of Albania) Spain Sabah, Malaysia	Ultramafic soil 3:1 mixture of bark: crushed serpentine rock	Increases Ni yield by increasing biomass production while causing negligible dilution in shoot Ni	Álvarez-López et al. (2016), Bani et al. (2015a), Bennett et al. (1998), Nkrumah et al. (2019b)
Sulfur addition	*Berkheya coddii* *Phyllanthus rufuschaneyi* *Rinorea* cf. *bengalensis*	Pot trials	Palmerston North, New Zealand Sabah, Malaysia	1:1 ultramafic soil: pumice soil mixture	Increases Ni yield by increasing Ni phytoavailability	Robinson et al. (1999), Nkrumah et al. (2019c)
Soil pH adjustment	*Odontarrhena chalcidica* *Odontarrhena corsica* *Phyllanthus rufuschaneyi* *Rinorea* cf. *bengalensis*	Pot trials	Ontario, Canada Sabah, Malaysia	Ni refinery contaminated soil (Welland loam and Quarry muck) Ultramafic soil	Increases Ni yield while decreasing Ni extractability	Kukier et al. (2004), Li et al. (2003b), Nkrumah et al. (2019d)
Different substrates on the basis of soil Ni phytoavailability	*Bornmuellera emarginata* *Bornmuellera tymphaea* *Noccaea caerulescens* *Odontarrhena chalcidica* *Phyllanthus rufuschaneyi* *Rinorea* cf. *bengalensis*	Pot trials	France Sabah, Malaysia	Ultramafic soil agricultural Calcaric Cambisol acid agricultural soil (Haplic Luvisol)	High Ni phytoavailability increases Ni yield	Chardot et al. (2005), Nkrumah et al. (2019d)
Bacterial inoculation	*Odontarrhena chalcidica* *Noccaea tymphaea* *Bornmuellera tymphaea*	Pot trials	Oregon, USA France	Ultramafic soil	Increases Ni yield by increasing Ni uptake and biomass production	Abou-Shanab et al. (2006), Durand et al. (2016)

(continued)

Table 2 (continued)

Agronomic practices	Species	Field and/or pot trials	Locations	Substrates	Effects on Ni yield	References
Plant growth regulators	*Odontarrhena corsica* *Odontarrhena serpyllifolia s. l.* *Odontarrhena chalcidica* *Noccaea goesingense*	Pot trials	Spain	Ultramafic soil	Positive effects on biomass, but the effects on Ni yield is not clear	Cabello-Conejo et al. (2014)
Weed control	*Odontarrhena chalcidica*	Field trial	Oregon, USA, Pogradec, East of Albania	Ultramafic soil	Enhances Ni yield by reducing competition for essential nutrients and water between the 'metal crop' and weeds	Li et al. (2003a, b), Bani et al. (2015a)
Plant density	*Odontarrhena chalcidica*	Field trial	Oregon, USA, Pojskë and Domosdovë, Albania	Ultramafic soil	Optimum plant density increases Ni yield	Angle et al. (2001), Bani et al. (2015b)
Organic matter additions	*Odontarrhena serpyllifolia s. l.* *Odontarrhena chalcidica* *Odontarrhena bertolonii* *Noccaea goesingense* *Phyllanthus rufuschaneyi* *Rinorea* cf. *bengalensis*	Pot trials	Spain USA Sabah, Malaysia	Ultramafic soil	No significant effect on Ni yield	Álvarez-López et al. (2016), Broadhurst and Chaney (2016), Nkrumah et al. (2019c)
Biochar amendment	*Odontarrhena chalcidica*	Pot trials	France	Ultramafic soil Constructed soil	Increase plant growth and Ni yield	Rue et al. (2019)

Plant growth regulators increase the biomass of Ni hyperaccumulator plants, but the effect on Ni yield is not clear. In addition, soil pH adjustment, S addition, N fertilization and bacterial inoculation increase uptake and accumulation of Ni shoots in Ni hyperaccumulator species
Adapted from Nkrumah et al. (2016)

(Broadhurst et al. 2004a), but a negative (Bani et al. 2013) or positive correlation (van der Ent and Mulligan 2015) exists in the foliar concentrations of Ca and Ni in some 'metal crop' species. Nickel hyperaccumulator species absorb more Ca relative to Mg, which leads to a high Ca/Mg quotient in the leaf tissues (Bani et al. 2014). This selective Ca accumulation from soils having very low Ca/Mg quotients may reduce Mg and Ni phytotoxicity (Kruckeberg 1991). Unless Ca is actually deficient, Ca addition has little effect on Ni concentration in the above-ground biomass and

Table 3 Effect of amending Brockman cobbly loam ultramafic soil (fine, magnesic, mesic Vertic Haploxerepts) from a unmanaged pasture field in Josephine County, Oregon, USA with phosphate (kg ha^{-1} P), pH adjusting, or Ca fertilizer ($CaSO_4{\cdot}H_2O$, t ha^{-1}) treatments on terminal soil pH, mean yield and macronutrient composition of shoots of two *Odontarrhena* species (*O. chalcidica* and *O. corsica*) grown for 120 days (GM designates geometric mean)

Treatment	Treatment		Final pH	GM-Yield (g pot^{-1})	GM-P (g kg^{-1})	Mg (g kg^{-1})	Ca (g kg^{-1})	K (g kg^{-1})
	1	None	6.56 a[a]	4.1 c	1.04 e	4.06 d	17.5 ab	9.1 d
Phosphate treatments								
	3	0 P	5.82 e	1.6 d	0.61 f	6.47 a	17.5 ab	10 cd
	2	100 P	6.24 b	24.5 a	2.16 cd	6.20 bc	17.1 ab	16.5 b
	4	250 P	6.14 bcd	23.2 ab	3 b	6.46 bc	19.8 a	19.9 a
	5	500 P	6.16 bc	26.5 a	3.59 a	6.40 bc	18.2 ab	19.8 a
pH treatments								
	6	Lo pH	5.42 g	27.4 a	2.03 d	4.92 cd	16.7 ab	18.4 ab
	7	MLo pH	5.69 f	26.2 a	2.12 d	6.42 bc	18.5 ab	17 b
	8	MHi pH	5.89 e	27 a	2.07 d	5.31 bcd	16.2 ab	18.4 ab
	2	As is pH	6.24 b	24.5 a	2.16 cd	6.20 bc	17.1 ab	16.5 b
Ca:Mg treatments								
	9	0 Ca	6.10 cd	19.3 b	2.43 c	5.66 bc	14.8 b	12.4 c
	2	1 Ca	6.24 b	24.5 a	2.16 cd	6.20 bc	17.1 ab	16.5 b
	10	2.5 Ca	6.04 cd	25.2 a	2.10 d	6.74 b	18.4 ab	17.7 ab
	11	5 Ca	6.03 d	24.2 a	1.94 d	6.26 bc	16.2 ab	17.2 ab

For single variable treatments, all other nutrients were applied as in treatment 2 (100 kg ha^{-1} P; 1.0 t $CaSO_4{\cdot}2H_2O$ ha^{-1}). Bray-1 extractable P was 0.49, 11.1, 49.9 and 100 mg kg^{-1} soil for the 0, 100, 250 and 500 kg ha^{-1} P treatments (applied as $Ca(H_2PO_4)_2{\cdot}2H_2O$); all except treatment 1 received 200 kg ha^{-1} N as NH_4NO_3. The experimental design, set-up and conditions have been described by Li et al. (2003b) in which the data from the Port Colborne soils were reported similar to the serpentine soil treatments

[a] Means followed by the same letter are not significantly different ($P < 0.05$ level) according to the Duncan-Waller K-ratio t-test

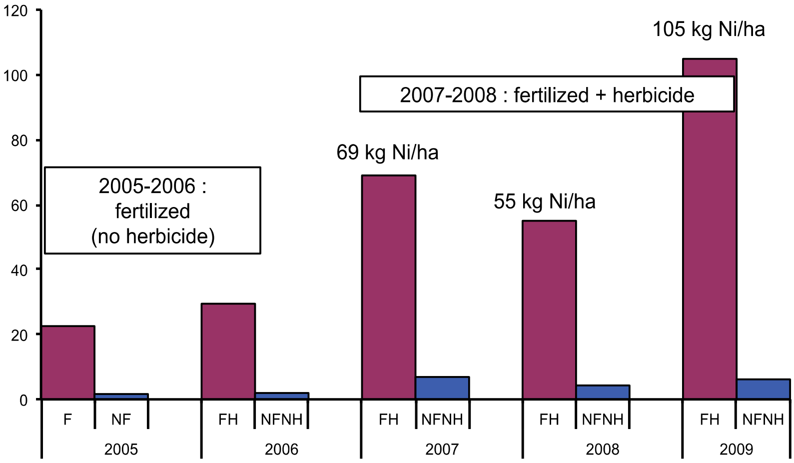

Fig. 12 Agromining of Ni in ultramafic areas of Albania using *O. chalcidica*—plant response to fertilization and weed control. Adapted from Bani et al. (2015a, b)

shoot yield as well as root-to-shoot translocation of Ni, but may increase Ni tolerance (Chaney et al. 2008). Broadhurst and Chaney (2016) reported that *O. chalcidica* accumulated about 13 g Ca kg^{-1}, which was consistent with all previous observations in which $CaCO_3$ nodules cover the surface of the trichomes (Krämer et al. 1997; Psaras et al. 2000; Küpper et al. 2001; Kerkeb and Krämer 2003; Broadhurst et al. 2004a, b, 2009; Tappero et al. 2007). Most Ni hyperaccumulators are very specific to Ni and to a lesser extent Mn and Co and even Zn (e.g. *N. caerulescens*), and do not non-selectively accumulate/hyperaccumulate other transition metals such as Fe, Cr or Cu. Using the isotopic fractionation of Ni, Deng et al. (2014) demonstrated that Ni in the hyperaccumulator *N. caerulescens* is taken up mainly via a low-affinity transport system across root cell membranes, which seems to be a Zn transport system. This pattern of Zn-Ni competition is not found in the serpentinophyte Ni hypernickelophores. In the case of Cu, despite 3600 mg kg^{-1} Ni accumulation, *O. chalcidica* Cu concentrations were only 2 mg kg^{-1} (Broadhurst and Chaney 2016). These observations support a specific relationship between Mn accumulation and Ni hyperaccumulation (Broadhurst et al. 2009; Ghaderian et al. 2015), rather than a general situation for *Odontarrhena* species wherein Mn uptake and storage may be related to enhanced Ca uptake to synthesize the unique trichome tissues (McNear and Küpper 2013). Moreover, Ca is present in ultramafic soils at low concentrations, hence Ca depletion should be avoided in Ni agromining operations (Bani et al. 2015a, this volume; Chaney et al. 2007b, 2008).

4.3 Sulfur Application

Sulfur is taken up extensively by all Brassicaceae (notably *Odontarrhena* spp.) owing to their specific metabolic requirements in, for example, glucosinolates (Booth et al. 1995) apart from any hyperaccumulation traits. Basic studies on Ni tolerance by *Noccaea* Ni hyperaccumulators (*N. goesingense*, *N. oxyceras* and *N. rosularis*) indicate that glutathione plays a role in Ni tolerance in these species (Freeman et al. 2004), and that a S-rich precursor of glutathione (L-cysteine) may be involved in Ni hyperaccumulation in *Noccaea* (Na and Salt 2011). Broadhurst et al. (2004b, 2009) reported co-localization of Ni and S in vacuoles of *O. chalcidica* and *O. corsica* grown on ultramafic soils and in nutrient solutions, and suggested that SO_4^{2-} could be a counter-ion to maintain the charge balance in vacuoles storing high concentrations of Ni. Concentrations of Ni and anions in vacuole contents have not been reported for Ni hyperaccumulators, only in total leaf extractions that have constitutive high levels of organic acids. In *N. caerulescens*, vacuolar contents were reported for moderately high Zn plants (Küpper et al. 1999).

Additions of micronutrients have also been considered during fertilization trials because ultramafic soils are locally deficient in B and Mo (Li et al. 2003b). Some ultramafic soils rich in Fe have proven deficient in Mo (Walker 1948, 2001), whilst low levels of B fertilizers may be beneficial for many previously unfertilized soils.

4.4 Soil pH Adjustments to Optimize Ni Uptake

Soil pH strongly influences the uptake of Ni from ultramafic soils by Ni hyperaccumulator plants (Chaney et al. 2000, 2007b; Kukier et al. 2004). In both strongly acidic and alkaline soil conditions, Ni uptake is low in ultramafic soils (Table 4; Fig. 8c), but not in smelter-contaminated soils (Fig. 8d) (Chaney et al. 2007a). Smelter-contaminated soils low in Fe-oxides show an increase in *Odontarrhena* species (*O. chalcidica* and *O. corsica*) shoot Ni concentration across the range of pH from about 5–7 or higher, whereas ultramafic soils reveal a maximum shoot Ni concentration near pH 6.5 (Table 4; Fig. 8c). Within the pH range of 5–6.5 in ultramafic soils, Ni accumulation by Ni hyperaccumulator plants increases (Chaney et al. 2007b). Because of the different effects of soil pH on extractability of soil Ni and uptake by *Odontarrhena* species, it is not possible to accurately predict shoot Ni from traditional

Table 4 Effect on terminal soil pH, mean yield and microelement composition of shoots of *Odontarrhena* species grown for 120 days (GM designates geometric mean) under conditions noted in Table 3

Treatment	Treatment		Final pH	GM-Yield (g pot^{-1})	GM-Ni (mg kg^{-1})	GM-Co (mg kg^{-1})	GM-Mn (mg kg^{-1})	GM-Zn (mg kg^{-1})	GM-Fe (mg kg^{-1})	Cu (mg kg^{-1})
	1	None	6.56 a[a]	4.1 c	14740. a	34.3 c	56.5 e	63.4 bc	154. b	3 cd
Phosphate treatments										
	3	0 P	5.82 e	1.6 d	6250. cd	19.4 ef	62.3 cde	118. a	273. a	2.8 d
	2	100 P	6.24 b	24.5 a	6270. cd	19.9 ef	60.9 cde	59.9 bc	112. cd	3.6 bc
	4	250 P	6.14 bcd	23.2 ab	6810. bc	22.6 def	65.2 cde	60.2 bc	104. d	4.2 ab
	5	500 P	6.16 bc	26.5 a	5690. d	18.1 f	67.2 cde	55.1 cd	92. d	4 ab
pH treatments										
	6	Lo pH	5.42 g	27.4 a	6150. cd	224. a	462. a	63.1 bc	144. bc	4.4 ab
	7	MLo pH	5.69 f	26.2 a	6800. bc	50.4 b	132. b	68.7 b	117. bcd	4.6 a
	8	MHi pH	5.89 e	27 a	5990. cd	28.8 cd	73.1 cd	58.2 bcd	96. d	3.6 bc
	2	As is pH	6.24 b	24.5 a	6270. cd	19.9 ef	60.9 cde	59.9 bc	112. cd	3.6 bc
Ca:Mg treatments										
	9	0 Ca	6.10 cd	19.3 b	7860. b	21.1 ef	55.6 e	49.4 d	87. d	3.1 cd
	2	1 Ca	6.24 b	24.5 a	6270. cd	19.9 ef	60.9 cde	59.9 bc	112. cd	3.6 bc
	10	2.5 Ca	6.04 cd	25.2 a	6050. cd	18.4 ef	58.2 de	59.6 bc	87. d	3.8 bc
	11	5 Ca	6.03 d	24.2 a	5630. d	24.4 de	78.5 c	63.3 bc	93. d	3.6 bc

[a] Means followed by the same letter are not significantly different ($P < 0.05$ level) according to the Duncan-Waller K-ratio t-test

extractable soil Ni data (Fig. 8e, f). On the other hand, when Ni accumulation from 17 ultramafic topsoils was regressed (linear or quadratic) onto the total soil Ni or DTPA-extractable Ni, the DTPA-extractable Ni had a R^2 of 0.47 for linear and 0.53 for quadratic regressions, but soil total Ni had R^2 of 0.65 for linear and 0.72 for quadratic regression of shoot Ni (Fig. 13). Higher values of soil Ni will always be a desired property of soils intended for commercial phytomining. More research is required in order to characterize the ways in which hyperaccumulator plants access Ni pools in the soil, and to develop predictive tools for Ni uptake in hyperaccumulator plants.

4.5 Soil Organic Matter Additions and Ni Accumulation

Broadhurst and Chaney (2016) utilized a standard, mature, dairy manure compost product from USDA Beltsville to investigate the effect of organic matter amendments on the growth and metal yield of *O. chalcidica*. The authors did not observe any significant positive effect on the biomass and yield of the ‘metal crop’. However, in the inorganic fertilized ultramafic soil, the *Odontarrhena* species grew very well with no evidence of disease or phytotoxicity. In a similar recent study, Álvarez-López et al. (2016) grew hyperaccumulator accessions of *O. serpyllifolium*

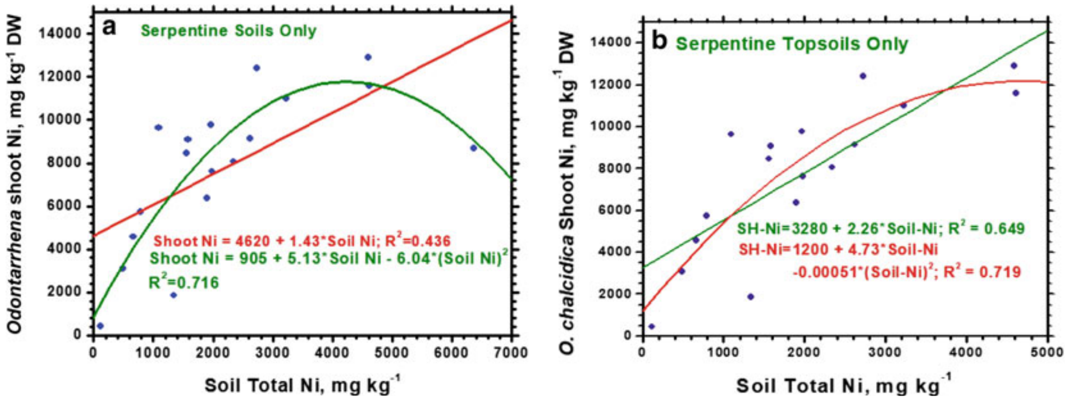

Fig. 13 **a** Prediction equations for shoot Ni concentration versus soil total Ni; **b** DTPA-extractable Ni for *Odontarrhena* species grown for 120 days on 17 ultramafic soils

s.l. and *O. bertolonii* in their native ultramafic soil with 2.5, 5 and 10 wt% commercial municipal solid waste compost added. These species grow slowly thus did not achieve the large shrub size that *O. chalcidica* can reach in one season. The lower levels of compost addition significantly increased yield but no further benefits were achieved with 10% additions. All levels of compost addition reduced extractable Ni; at the 10% level the reduction was 11-fold. Overall yield was lower without compost but both organic matter and inorganic NPK fertilization reduced shoot Ni concentration, but increased shoot Ni yield due to the large increase in biomass yield. If a compost source is utilized, it should be tested with every species/accession used in the field program prior to application. Another factor to consider is a possible negative effect of compost biota on serpentine-endemic rhizobacteria, which can act to facilitate Ni uptake. However, growing hyperaccumulators on industrial waste material or mine spoils requires improvement of soil fertility, and even soil construction. Construction of a soil profile is based on the use of organic amendments derived from urban or industrial activities, avoiding clean topsoil (Séré et al. 2008; Chaney and Mahoney 2014). Furthermore, biochar (comprising of 50% coniferous and 50% hardwood chips pyrolyzed at *ca.* 650 °C) has been proved to considerably increase growth conditions for hyperaccumulators and subsequent accumulation of Ni in plant biomass (Rue et al. 2019). With appropriate soil conditioning, agromining could be conducted successfully on initially extreme substrates considering their metal contents.

5 Plant Management Practices

5.1 Weed Control and Co-cropping

Agromining employs plant management practices, beyond fertilizer treatment and pH adjustment, to enhance metal yield in ‘metal crops’ via a number of ways: (i) plant density is important to optimize biomass production per unit area, and we recommend an intermediate density for optimum Ni yield (Angle et al. 2001; Bani et al. 2015b); (ii) weed control reduces competition for essential nutrients and water between the ‘metal crop’ and weeds (Bani et al. 2015a; Chaney et al. 2007a); (iii) plant growth regulators may increase biomass production (Cabello-Conejo et al. 2014; Cassina et al. 2011) but can reduce their Ni yield (Cabello-Conejo et al. 2014), although a recent study shows positive effects on both biomass yield and Ni accumulation in two hyperaccumulator species (Durand et al. 2016); (iv) rhizobacteria may increase the phytoavailability of soil Ni (Abou-Shanab et al. 2003, 2006); and (v) mycorrhizae may be critical for those hyperaccumulator species that are strongly associated with mycorrhiza, such as *B. coddii* (Orłowska et al. 2011).

Increased yield of the mycorrhizal crop (due to higher phosphate uptake) greatly increased Ni yield ha^{-1} (Orłowska et al. 2011).

Co-cropping may not play any significant role in Ni agromining. Broadhurst and Chaney (2016) have shown in an experimental study that co-cropping neither increases yield nor uptake of Ni in hyperaccumulator species. The authors co-cropped *O. chalcidica* and ryegrass (*Lolium perenne*) and found that ryegrass interfered with both shoot and root systems of the hyperaccumulator plants, and this interference has a negative effect on overall Ni yield of *O. chalcidica*. It had been suggested that phytosiderophores secreted by the roots of grasses to obtain soil Fe could solubilize Ni and improve phytoextraction of soil Ni. Co-cropping *O. chalcidica* and *Lupinus albus* in natural ultramafic soils showed similar results in a study investigating whether co-cropping with a nitrogen-fixing plant could improve overall *O. chalcidica* phytoextraction of Ni (Jiang et al. 2015). This result suggests that monoculture of 'metal crops' may be sufficient to produce maximum metal yield when adequate inorganic fertilization is supplied. However, research is still needed in order to develop profitable agromining systems that take into account the principles of agro-ecology, with adequate plant associations, such as hyperaccumulators and legumes.

5.2 Harvest Method/Schedule

The management of propagation and harvest will necessarily be dependent upon the species being used for phytomining and climate. If seeds are readily available, and remain viable for some time, then sowing seeds will allow establishment of phytomining fields. If seeds are easy to work with, 'metal crop' management could follow that of crop plants. But if seeds are in short supply or it is difficult to establish a stand-by sowing in the field, and the plant is perennial, coppicing can be used to make annual harvests and cuttings can be rooted for transplanting to the field. Alternatively, seedlings raised under careful management in potting media during the dry season could be another possibility. So many factors influence these management choices that it is clear that planting and harvest management will be species-specific and location specific. Perennial hyperaccumulator crops are to be preferred as costs of plant establishment are a one-time expense.

For *Odontarrhena* and several other 'hypernickelophores' in southern Europe, the Mediterranean climate may drive operations. Seedlings could be established in an ultramafic soil-based potting medium during the dry summer using irrigation water, and the seedlings transplanted to the field in the desired density when rainfall returns in the autumn. This schedule could be performed by the farmer using local resources and would appreciably increase the Ni yield of the first year. During flowering at the beginning of the rain-free summer, the crop can be cut and dried in the field to make collection of the dried shoots (a hay) economic (Fig. 14). In tropical areas, the harvest might be scheduled during periods of low rainfall.

In the case of the tropical 'hypernickelophores', multiple patterns of management appear possible for different species. With woody species, some accumulate appreciable Ni in their trunk over years such that harvesting the trees for Ni could be effective for phytomining (e.g. *R.* cf. *bengalensis*). For most species evaluated to date, the Ni concentration in leaf biomass is much more than in the stem or whole biomass, so that for an annual harvest the collection of foliage before leaf fall will be most effective for phytomining. This approach appears to be possible for *Phyllanthus* species, but multi-year biomass collection has not yet been tested in the field. Collection of leaves from the soil surface could be conducted mechanically several times during the year if leaf fall is not seasonal. This latter approach to foliar biomass harvest has not been evaluated for phytomining or for other species, because 'normal' crops are not harvested in this fashion (e.g. tea and tobacco are hand-harvested).

Fig. 14 **a** Mechanical and **b** manual modes of harvesting *Odontarrhena chalcidica*, and subsequent bailing operations (**c**, **d**)

6 Pioneering Studies on the Agronomy of Tropical 'Metal Crops'

Most of the agromining trials have been undertaken in temperate regions mainly using *O. chalcidica* and *O. corsica* (Bani et al. 2015a; Li et al. 2003a, b). Until recently, the agronomy of tropical 'metal crops' has not been tested. Large agronomic trials have been undertaken recently in Sabah (Malaysia) to determine whether the trends observed in temperate regions could be confirmed in a wet tropical environment (Nkrumah et al. 2019a, b, c, d). The species used were *R.* cf. *bengalensis* and *P. rufuschaneyi*. The trial was undertaken in two phases: (i) extensive pot trials under controlled conditions to test the fertilization and agronomic systems (Fig. 15), and (ii) a large-scale field trial (Fig. 6) to incorporate the preliminary results from the pot trials (Nkrumah et al. 2019a, b, c, d). The results reveal that inorganic fertilization (N, P, Ca and S) significantly improved biomass of *R.* cf. *bengalensis* and *P. rufuschaneyi*, and ultimately Ni yield. However, K addition had a negative effect on the growth and Ni yield of *P. rufuschaneyi*, which may be related to the ecophysiology of this species, but this remains to be tested. The soil pH in the native habitats (Cambisol) of the selected 'metal crops' is *circum*-neutral, hence, reducing the soil pH below pH 6 negatively affects plant growth and Ni yield. Notably, the ultramafic substrates in Sabah vary strongly in physico-chemical properties (van der Ent et al. 2018, 2019). Therefore, in a field application employing

Fig. 15 Pot trials that were undertaken under controlled conditions to test the agronomic systems of two species of tropical 'metal crops'; *Phyllanthus rufuschaneyi* (small leaf blades) and *Rinorea* cf. *bengalensis* (large leaves)

a Cambisol substrate, it is not important to adjust soil pH. However, when employing substrates with pH outside of the optimum range (e.g. limonite soils, pH 5–6; young serpentinite soils, pH >7.4), then pH adjustment may need to be considered (Nkrumah et al. 2019d). Organic matter amendments reduced Ni extractability and strongly hindered growth of *P. rufuschaneyi*. Therefore, organic matter amendment may not be useful in field applications employing *P. rufuschaneyi* on Cambisol. However, some extreme substrates may require organic matter amendments in order to support normal plant growth (Nkrumah et al. 2016). Plant density and mass propagation methods are currently being trialled in Sabah. The agronomic system for recently discovered 'metal crops' (including *Antidesma montis-silam* and *Actephila alanbakeri*) (Nkrumah et al. 2018c, d; van der Ent et al. 2016) in Sabah should also be developed. Furthermore, the discovery of a potential 'metal crop' (*Blepharidium guatemalense*) in southeastern Mexico, with highly desirable traits for possible Ni agromining, opens up the possibility of developing an agronomic system for sustainable Ni agromining operations in this region (Fig. 16) (McCartha et al. 2019; Navarrete Gutiérrez et al. 2018). The agronomic system there could follow the approach developed in Sabah (Nkrumah et al. 2019a, b, c, d).

Fig. 16 *Blepharidium guatemalense*, a new nickel ‘metal crop’ in southeastern Mexico

7 Summary

A range of agronomic systems is available to increase the biomass and metal yield of ‘metal crops’ to be used in sustainable agromining. Our review has identified major factors in successful agromining. We have demonstrated that:

- Because hyperaccumulators show large natural variation for trace metal accumulation, the collection of diverse germplasm, followed by uniform evaluation of yield and metal accumulation, and then normal breeding techniques to improve the ‘metal crop’, is clearly a key step in developing agromining.
- Higher soil metal phytoavailability will always be a desired property of soils intended for commercial agromining. Subsoil metal increases shoot yield, shoot Ni concentration, and Ni quantity in shoots of Ni ‘metal crops’.
- Tilling soil to maximize root penetration, adequate inorganic fertilization, and appropriate plant densities are more important for developing efficient agromining approaches with *Odontarrhena* and *Phyllanthus* Ni hyperaccumulator species than organic soil amendments. However, extreme physical properties of some substrates (e.g. poor water-holding capacity) may require the application of amendments to improve local soil conditions necessary for normal plant growth.
- Management of propagation and harvest will necessarily be dependent upon the species being used for agromining and on the climate of the production site.
- The agronomy of a newly discovered ‘metal crop’ *Blepharidium guatemalense* in southeastern Mexico needs to be developed for sustainable Ni agromining operations in this region, and the approach could follow that used in Sabah.
- Feasibility of agromining for Ni is demonstrated by laboratory and field experiments, whereas the demonstration of its applicability to other strategic metals (e.g. Co, Mn, rare

earths) is underway and should follow the same general approach.

- Future developments of agromining should match the goals and constraints of agroecology, i.e. optimize the functioning of the agro-ecosystem and preserve or even improve a large range of ecosystem services (e.g. biodiversity, water quality).

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Processing of Hyperaccumulator Plants to Nickel Products

Baptiste Laubie, James Vaughan, and
Marie-Odile Simonnot

Abstract

Hyperaccumulator plants may contain valuable metals at concentrations comparable to conventional ore and can be significantly upgraded by incineration. There is an incentive to recover these metals as products in order to counterbalance the cost of disposing the contaminated biomass from contaminated soils, mine tailings, and processing wastes. Metal recovery has become an essential challenge as conventional ore grades decrease. Metal recovery is included in the agromining chain, which has been developed over the past two decades for Ni. More than 520 Ni hyperaccumulator species are currently known and some of these grow quickly providing a high-farming yield. Nickel recovery has been investigated at the laboratory scale and some processes have been upscaled. Most often, dry plants are burnt to produce ash, which has a very high Ni content, then the Ni in the ash is leached into aqueous solution. From there, the Ni is recovered by precipitation to obtain Ni salts or oxide. This hydrometallurgical route has now been scaled up. Other studies have aimed to obtain catalysts from ash or direct extraction of Ni from the plants. In the latter case, further processing requires complicated purification steps. Finally, the plants have also been treated by pyrometallurgical processes to produce Ni metal; initial studies have been carried out using pyrolysis. Interest in the production of carbon-supported Ni catalyst materials is increasing day by day, owing to the potential capacities of these products for use in bio-refineries. Finally, economic and environmental considerations are proposed here for supporting the interest of Ni recovery by agromining.

1 Introduction

Soil remediation was the first area of focus for the application of hyperaccumulator plant (HA) technology (Robinson et al. 1997a, b). Whilst this topic has been extensively investigated in terms of extracting metals from soil into plant tissue, the fate of contaminated biomass has seldom been considered. However, the topic of hyperaccumulator biomass processing is becoming increasingly important because large-scale application of this soil remediation technology (Chaney et al. 1998; Chaney 2019) would yield vast amounts of contaminated biomass that

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B. Laubie · M.-O. Simonnot (✉)
Laboratoire Réactions et Génie des Procédés, UMR 7274, Université de Lorraine - CNRS, 1, Rue Grandville, BP20451, 54001 Nancy Cedex, France
e-mail: marie-odile.simonnot@univ-lorraine.fr

J. Vaughan
School of Chemical Engineering, The University of Queensland, Banksia Building (1016), Long Pocket Precinct, Indooroopilly, QLD 4068, Australia

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_3

would need to be treated or disposed (Keller et al. 2005; Sas-Nowosielska et al. 2004). The pioneering work of Chaney and co-workers (Li et al. 2003) has shown how Ni could be commercially recovered from the biomass of *Odontarrhena chalcidica* (formerly *Alyssum murale*) and has introduced the concept of phytomining (Chapter "The Long Road to Developing Agromining/Phytomining"), which has recently been broadened to agromining (van der Ent et al. 2015).

Metal recovery from HA biomass is based on the principles of extractive metallurgy (Hayes 2003; Crundwell et al. 2011). Pyrometallurgy is the high-temperature chemical processing of metals, including unit operations such as calcination, roasting, smelting, and converting. In contrast, hydrometallurgy is the aqueous chemical processing of metals that is carried out at relatively low temperatures and which includes leaching, solution-phase upgrading, and purification followed by product recovery. Both of these processes are used to enable the production of metals, alloys, concentrates, salts, and intermediate compounds. The processes typically require chemical and energy input and become more challenging and expensive to run with increasing complexity and an ever-decreasing grade of the feedstock.

In the past, metals were considered abundant and easily extractable. In recent decades, the demand for raw materials has sharply increased, because of the economic growth of emerging countries and global demographic pressure. Many elements, including Ni and Co, have become strategic metals or even critical raw materials (Hagelüken et al. 2016). In this context, and also with the aim of creating a circular economy, metal extraction from secondary sources is increasingly important. Therefore, the development of new technologies and strategies to recycle metals has become a major economic, geopolitical, and environmental challenge (Dodson et al. 2012). In France, this topic has notably given rise to creation of the Laboratory of Excellence LabEx Ressources 21 'Strategic metals in the twenty-first Century' at the University of Lorraine (http://www.ressources21.univ-lorraine.fr/). In Australia, it is developed within the Sustainable Minerals Institute at The University of Queensland. Interest in this approach is the basis of a considerable amount of international collaboration, and in particular the Nancy-Brisbane axis has recently been highlighted (Li et al. 2020).

In this context, agromining technology may play an important role since it allows the recovery of metals and other compounds from soils or other secondary resources by taking advantage of the natural ability of hyperaccumulator plants to concentrate and purify elements. Processing the biomass to recover products also addresses the fate of metal-contaminated biomass in phytoremediation projects. Agromining feasibility depends on 'farming for metals' (van der Ent et al. 2015), that is to say, growing hyperaccumulator plants at large scale. This approach involves identifying the most promising hyperaccumulator plants and developing agronomic practices that enable a high yield of biomass and high concentration of the target metal to be recovered. These plants must be grown on suitable soils (e.g. those naturally containing metal concentrations such as ultramafic soils, mine tailings, secondary resources). The harvested biomass is then converted into a marketable product. Processes must be simple and robust with the ability to take into account biomass variability and have a low environmental impact. A further consideration is that the market for the final product, preferably a value-added product, must be established.

One of the most promising applications of agromining involves Ni. One reason for this is that >520 Ni HA plants have been identified (Reeves et al. 2017) and agronomic strategies have been developed, particularly with *O. chalcidica,* to reach yields of >100 kg of Ni per hectare in extensive conditions (Bani et al. 2009, 2015) or even up to 200 kg ha^{-1} under intensive agricultural conditions (Nkrumah et al. 2016). Efforts are also ongoing with tropical Ni hyperaccumulators that may be better suited to climates where considerable amounts of Ni-containing soils are available (Vaughan et al. 2016b). Another reason why agromining Ni is of interest is the relatively high value of Ni products compared to those of Cu, Zn and Cd, for example. As such, Ni agromining has received significant attention, and processes for Ni recovery by this technology have been designed (Li et al. 2003; Barbaroux et al. 2009, 2011, 2012; Losfeld et al. 2012a; Tang et al. 2012; Zhang et al. 2016).

This chapter begins with an overview of Ni products and conventional production processes, followed by a discussion of biomass processing more generally. The extraction of Ni from the biomass by thermal or chemical methods are described, as well as processes to recover the extracted Ni. The knowledge acquired in the laboratory has made it possible to scale up process technologies, which will be presented shortly. The chapter ends with a discussion on environmental and economic considerations.

2 Overview of Nickel Products and Production Processes

2.1 Main Production Processes for Nickel

According to the US Geological Survey (2020), an estimated 2.7 Mt of Ni was mined in 2019 with the main producers, in decreasing order, being Indonesia, Philippines, Russia, New Caledonia, Australia, Canada, China and Brazil. New demand is being driven by the need for Ni sulfate used in lithium ion batteries for the electric vehicle market; environmental aspects of Ni sulfate production was recently reviewed (Dry et al. 2019).

The production of Ni from sulfide ore exceeds that of laterites; about 60% of global Ni resources are contained in laterite deposits (Gleeson et al. 2003; Mudd and Jowitt 2014). Sulfide ores (0.7–2.2% Ni; e.g. pentlandite) are typically upgraded by mineral flotation and then smelted to take advantage of the exothermic reaction to provide heat to the reactor (Fig. 1). In laterite ores, Ni and Co substitute into the structures of oxy-hydroxide or hydroxyl-silicate host minerals (Hellman 2001). Two zones are recognized, as follows:

- Limonite zone (1–2 wt% Ni) near the surface where Ni is mainly hosted in ferric iron oxides and to a lesser extent in Mn-oxides; and
- Saprolite zone (1.5–2.5 wt% Ni), often found below the limonite zone, dominated by hydrous Mg-silicates.

Because of the exhaustion of large, high-grade, and accessible sulfide ore deposits,

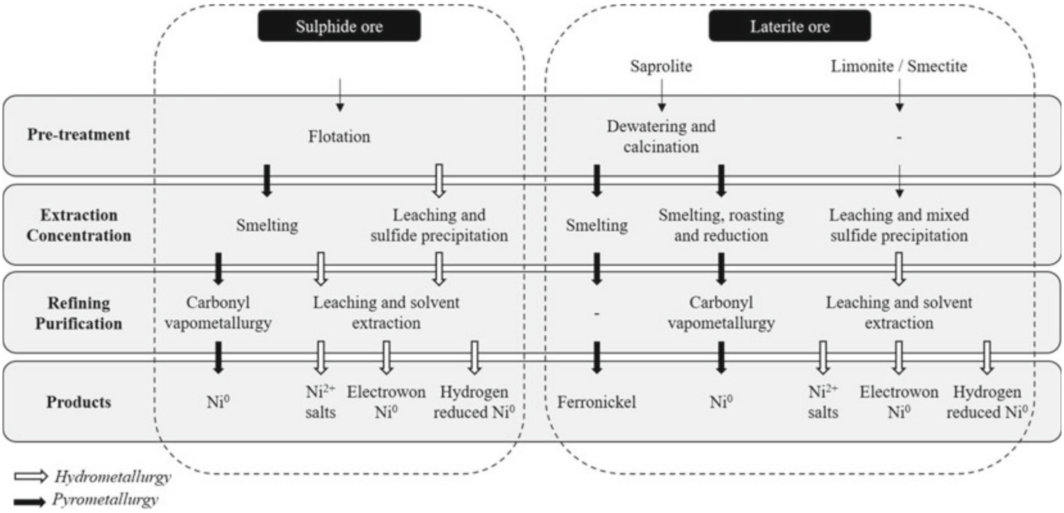

Fig. 1 Overview of the main processes of nickel ore (Adapted from Crundwell et al. 2011)

laterites are increasingly exploited, despite relatively costly Ni recovery processes in terms of high energy or reagent consumption and high capital equipment costs (Taylor 2013). In addition, such methods often fail to recover other valuable metals such as Co.

Saprolite ore is usually treated via pyrometallurgical reduction to ferronickel. Limonite is also increasingly being processed to low-grade ferronickel (Ni pig iron) by high-temperature reduction, typically in a blast furnace. Limonite is also processed hydrometallurgically via Pressure Acid Leaching (PAL) whereby the host mineral together with Ni and Co dissolve at high temperature (~250 °C) in sulfuric acid and the Fe largely re-precipitates as haematite or jarosite (Fig. 1). PAL plants have often proven to be a costly option and the first generation was plagued with technical challenges. Limonite ore is also treated through the Caron process that involves reduction roasting followed by leaching with an ammonia-ammonium carbonate solution (Caron 1924), as implemented at the Votorantim Macedo Refinery in Brazil and the recently closed Queensland Nickel Refinery in Australia (Fittock 1997). The Caron process suffers from high energy costs to dry and reduce the ore as well as low Ni and Co recoveries (70–85% Ni and 20–50% Co), relative to PAL. The cost and complexity of Ni processing is driven by the need to reject a wide range of impurities including Fe, Al, Si and Mn, amongst others.

2.2 Nickel Products

Nickel metal alloys tend to exhibit desirable mechanical and physical properties, corrosion resistance, durability, and ease of use (www.nickelinstitute.org). Manufactured Ni materials include stainless steel, a number of alloys, and Ni chemicals used for plating and electroforming, for catalysts, and for the production of coloured glass and ceramic glazes (e.g. Ni hydroxide, sulfate, carbonate). Therefore, Ni materials are used across many industrial sectors: metallurgy, architecture, building, and construction), automotive and aeronautics, petrochemical and chemical, energy and power, and food and beverage, as well as in electronics and battery production. The main primary Ni products are Ni and ferronickel metal, and Ni compounds such as chloride, sulfate, and oxide. Extensive processing is required to recover Ni salts of high purity as these are generally produced from processes that begin with use of a high-purity Ni metal feed.

2.3 Principles of Nickel Recovery from Hyperaccumulator Biomass

Similar to Ni production from ore, recovery from hyperaccumulator biomass is guided and constrained by elemental composition of the plants and the distribution of Ni-bearing phases. A general flow sheet of biomass processing is presented in Fig. 2.

The first approach is to extract Ni directly using high-temperature processing such as ferronickel smelting (Li et al. 2003). Biomass can then be integrated into existing smelter feed. Considerations for this option are that the natural purification afforded by the HA plant are being negated by recombining Ni with Fe, for example. Other practical aspects are propensity for the biomass to cause dusting issues or for the alkaline components of the biomass to react with the smelter refractory brick liners. In addition, the physical form of the biomass introduced into the smelter must be optimized. Granulation, briquetting, and compaction are required in order to avoid dispersion of the biomass in the flue gas.

The second approach is to char the biomass to produce catalysts used in the chemical industry, which appears to be quite promising. A third approach, which is well established now, is to ash the dry biomass. Once ashed, Ni is extracted and refined using hydrometallurgy by leaching, solution-phase purification, and then recovery of the desired product by precipitation or crystallization.

A fourth approach is to directly leach Ni from the dried biomass. However, the reaction is hindered by distribution of Ni throughout the organic matter and there remain questions about

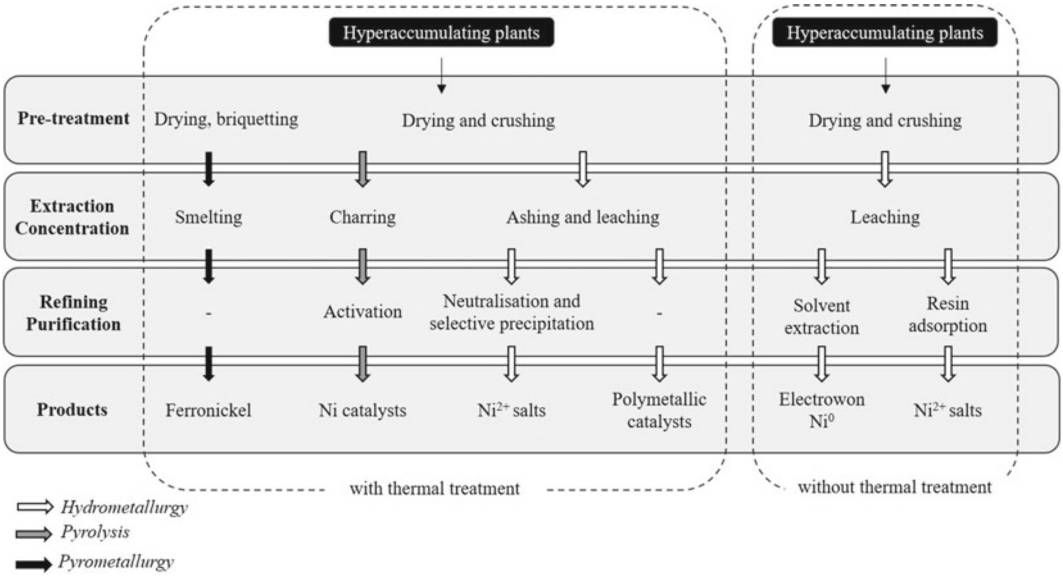

Fig. 2 General flow sheet of hyperaccumulator treatment to obtain Ni and Ni compounds

how to manage the dissolved organic compounds that remain.

Prior to the chemical processes, freshly harvested biomass is sun-dried to obtain a dry biomass containing 1–2 wt% Ni plus with other major elements such as Ca, Mg and K (Barbaroux et al. 2012; Zhang et al. 2014). Differences between Ni ore and hyperaccumulator biomass are that in HA biomass, there is a large amount of organic carbon and impurities such as Cl and P, whereas major concentrations of impurities in conventional ore such as Fe, Al, Si and Mn are substantially lower. These significant differences mean that the type of processing tailored for the bio-ore will not necessarily be the optimal route for hyperaccumulator biomass.

3 Thermal Treatment of Hyperaccumulator Plants

3.1 Investigation of Ashing

Biomass is ashed to remove organics, dispose of contaminated biomass, generate heat and energy, and to chemically liberate and concentrate elements (Robinson et al. 1997b; Li et al. 2003; Koppolu and Clements 2003; Koppolu et al. 2004; Boominathan et al. 2004; Sas-Nowosielska et al. 2004; Keller et al. 2005; Barbaroux 2010; Barbaroux et al. 2012; Zhang et al. 2014, 2016; Houzelot et al. 2017, 2018; Cassayre et al. 2020; Hazotte et al. 2020). Concentrating metal is important for minimizing costs of transporting the ashed bio-ore intermediate product, and size needed for the downstream processing equipment. Examples of major elements in the bio-ore ash are listed in Table 1. It is worth noting that Ni ash concentrations of 5–13% are much higher than grades of the mined Ni ore. Nickel ore grades of *ca.* 1 wt% are currently being processed by PAL and for production of Ni pig iron.

Biomass ashing has been extensively investigated at the laboratory scale using small amounts of *O. chalcidica* and other Brassicaceae such as *Bornmuellera emarginata* and *B. tymphaea* (Barbaroux et al. 2012; Zhang et al. 2014, 2016; Houzelot et al. 2017, 2018). Ashing is conducted in an electrical furnace. The ashing temperature is an important process parameter as temperatures >550 °C are required to effectively degrade organic matter, but if the temperature is too high then certain metals may be lost to volatilization. Ashing time is also an important variable

Table 1 The major elemental composition of Ni hyperaccumulator ash

g (kg-ash)$^{-1}$	Ni	K	Ca	Mg	References
O. chalcidica	126–168	126–128	131–172	42–71	Barbaroux et al. (2012)
					Zhang et al. (2014)
					Houzelot et al. (2017)
					Houzelot et al. (2018)
					Cassayre et al. (2020)
					Hazotte et al. (2020)
P. rufuschaneyi	127	72	108	14	Vaughan et al. (2016a)
R. bengalensis	55	85	254	12	

because a shorter time is desired to maximize furnace utilization, whereas a minimal period of time is required to ensure the organic residues have been combusted. From dry biomass to ash, the solid mass is decreased by a factor of about 15 with the Ni concentration increased from 1–2 wt% to 10–20 wt%. The ash contains a complex mixture of phases including oxides (NiO, MgO) and carbonates (K_2CO_3, $CaCO_3$, $K_2Ca(CO_3)_2$).

An original work (the first to our knowledge) was recently carried out on characterizing ashes and on predicting their composition at equilibrium by thermodynamic modelling (Cassayre et al. 2020). The combustion of three hyperaccumulator plants (*O. chalcidica*, *B. emarginata* and *R. bengalensis*) were experimentally studied at 550, 800 and 900 °C in a furnace at the lowest temperatures and in a boiler at the highest. Combustion was also studied by thermogravimetric analysis. The resulting ashes were characterized mineralogically and chemically by XRD and SEM-EDX. Their equilibrium composition was calculated using Factsage 7.2 software and the FactPS, FTOxid, FTSalt, and FTmisc databases (Bale et al. 2016). Experimental and calculated results concerning ash quantities and composition were compared. In summary, this work confirmed that Ni was present in the ash as Ni oxide (NiO) and as a solid solution (Ni,Mg)O. The high-temperature equilibrium modelling predicted the influence of temperature on the ash mass and on most crystallized phases. However, some behaviours are not yet sufficiently well predicted, for example the calculation under-estimates the ash mass and there are difficulties in interpreting the behaviour of carbonates that could be explained by incomplete data for the CaO-NiO-K_2O system, which is very important in the composition of plants.

Ash has also been characterized in terms of particle size distribution (Houzelot et al. 2017). Typically, it is quite fine, being less than a few hundred microns. This material needs to be sieved in order to remove unburnt carbon particles for improving leachate quality; ash cannot be treated by many physical sorting methods that are used with conventional ores.

The combustion of HA plants releases energy that can be recovered. This process has been studied in detail for three hyperaccumulators (*O. chalcidica*, *B. emarginata* and *Berkheya coddii*) (Hazotte et al. 2020). The higher and lower heating values (HHV and LHV) were measured using a calorimetric bomb, and the experimental results were in good agreement with results calculated using correlations designed to calculate these values from C, O and H contents of the dry plants. The three hyperaccumulator plants have an HHV on the order of 17 MJ kg^{-1}, lower than that of wood ($\sim$22 MJ kg^{-1}) but the same order of magnitude as other bio-resources or agro-wastes. Gaseous emissions were also monitored; these depend on plant composition and temperature. Moreover, attention must be paid to the chlorine content of the plants. While sulfur, which is susceptible to gas-phase SOx formation, remains largely in the ash (Cassayre et al. 2020), chlorine volatilizes as KCl, NaCl and HCl, which can lead to significant corrosion of the combustion device. Potassium volatilization is highly dependent on the molar ratios K/Cl and Si/K. In addition, the auto-ignition capacity of plants and ashes has also been investigated. It is known that fresh plants can degrade under the action of water and promote significant temperature increases. Dry plants and ashes are also susceptible to self-heating during storage due to effects of temperature. Standardized tests were conducted with plants and ashes subjected to temperatures up to 400°C, and the results were interpreted using the Frank-Kamenetskii model. These data clearly demonstrate that neither plants nor ashes are subject to self-ignition when stored at room temperature, even in summer (Hazotte et al. 2020). However, the presence of high Ni concentrations in the plant induces a decrease in self-ignition temperature of 20 °C, compared to the same plant without high Ni (Pacault et al. 2019).

3.2 Investigation of Charring

Pyrolysis of hyperaccumulator biomass is currently generating great interest. It is being studied from two distinct perspectives: (i) use of the contained Ni as a catalyst for conversion of the biomass to produce organic molecules (bio-refinery); and (ii) manufacture of carbon materials containing Ni^0 as a catalyst, for common reactions used in the chemical industry.

The pyrolysis of plants that hyperaccumulate Ni has been studied by microwave-assisted (MW) pyrolysis (Doroshenko et al. 2019). Nickel is considered a very promising transition metal for the thermal conversion of biomass into platform molecules. The role of Ni in MW-assisted pyrolysis has been studied, with plants having Ni hyperaccumulators (*Stackhousia tryonii, O. bertolonii*, *Phyllanthus balgooyi* and *Rinorea bengalensis*) as well as Ni-free or Ni-impregnated model plants. It has been shown that the presence of Ni during microwave biomass pyrolysis stabilizes hemicellulose. The cellulose is activated before hemicellulose, which is the opposite of normally occurs. In addition, the amount of biochar is increased (from 60 to 70% to more than 85%) and the bio-oil composition is largely modified. Bio-oil contains more N-containing compounds (e.g. triacetoamine) and levoglucosan (from cellulose). On the other hand, quantities of extractives (e.g. sterols and vitamins) are significantly reduced, a result that could be exploited for the bio-refinery.

It is interesting to note that the pyrolysis behaviour of hyperaccumulators is very different from that of Ni-impregnated plants, which behave like metal-free controls at low pyrolysis temperatures (280 °C) (Doroshenko et al. 2019). On the other hand, the rate of char gasification increases between 450 and 600 °C when wood is impregnated with Ni nitrate (Said et al. 2017), indicating significant catalytic activity of the metal as long as some carbon remains in the reactor (Said et al. 2018). The presence of Ni prevents sulfur volatilization, due to the formation of the stable sulfide Ni_3S_2.

Experiments with the hyperaccumulator tree *Pycnandra acuminata* showed that Ni is encapsulated with graphitic carbon layers and that the distribution of Ni nanoparticles is remarkably uniform in vessels of the xylem (Lerch et al. 2010). The presence of NiO is also observed. Important work on the catalytic properties of chars and their activation remains to be done.

4 Chemical Nickel Extraction

4.1 Ash Leaching

Ash leaching aims at transferring Ni from the solid to the aqueous solution phase. Acid leaching has been the focus of most reported studies, usually involving the use of hydrochloric or sulfuric acids (Barbaroux et al. 2012; Losfeld et al. 2012b). Sulfuric acid has advantages of being less expensive, less volatile, less corrosive to equipment materials of construction, and can easily be neutralized with limestone yielding gypsum. In order to minimize the amount of acid used, it is desirable to extract water-soluble salts such as potassium carbonate ahead of the leaching stage

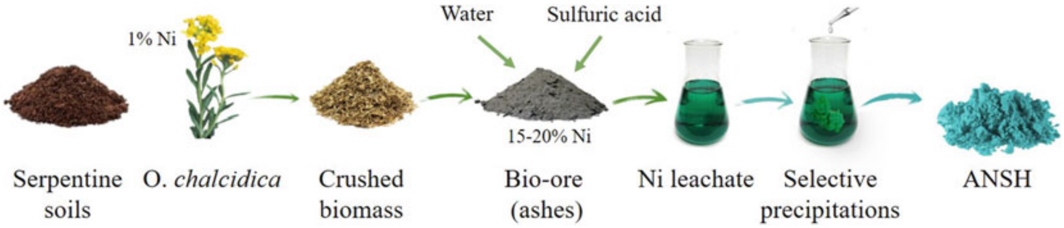

Fig. 3 General pattern of ANSH salt production from *O. chalcidica* hyperaccumulator

using a water wash. A potassium salt solution can be reused as fertilizer. Water washing of ash has been shown to be very effective for selectively solubilizing K salts (Barbaroux et al. 2012; Vaughan et al. 2016b; Zhang et al. 2016). From an acid leaching study of *O. chalcidica*, the optimal conditions were 10% solids in 2 M H_2SO_4, at 95 °C for 2 h, which enabled a nearly complete Ni extraction (Zhang et al. 2016). The leachate is then neutralized and partly evaporated to obtain a concentrated solution suitable for further purification and refining to a final product (Fig. 3). The unleached components of the ash and precipitated gypsum, free of Ni, can be re-used during metal farming. This operation is easy to scale-up following conventional methods of chemical engineering.

4.2 Direct Leaching

Another strategy is to extract the Ni from the plant without burning it, in order to avoid the ashing stage and drawbacks of combustion. Relatively severe conditions were tested experimentally in a hydrothermal treatment of HA in water over the temperature range of 200–350 °C using an extract of *Berkheya coddii* (630 mg Ni kg^{-1}) (Le Clercq et al. 2001). Later the extraction was tested under milder conditions, using different aqueous solutions. Barbaroux et al. (2009) showed that Ni could be extracted from the seeds of *O. chalcidica* using aqueous sulfuric acid solutions (typically 0.5 M, 90 °C, 120 min to extract 97%). Vaughan et al. (2016a, b) tested different aqueous solutions at 20 °C, containing acids or bases or carboxylic acids, for the extraction of Ni from a tropical HA, *R. bengalensis*, resulting in 43–59% Ni extracted under these conditions.

Initial water extraction trials gave encouraging results (Zhang 2014), therefore the water extraction of Ni from the plants *O. chalcidica* and *R. bengalensis* has been studied in detail (Guilpain et al. 2018a, b, c). Batch reactor experiments using solid contents of 4 and 8% showed that it was possible to extract up to 90% of the Ni contained in the tissues of *O. chalcidica*, but no more than 50% for *R. bengalensis* (Vaughan et al. 2016b). This result is explained by the different structures and difference in Ni storage organs between the two HAs (epidermal vacuoles for *O. chalcidica* and spongy mesophyll for *R. bengalensis*). Extraction of Ni from *O. chalcidica* was further carried out using laboratory columns in order to achieve a higher solid/liquid ratio than in a batch reactor. All of the direct extraction experiments clearly demonstrated that the other major elements (e.g. K, Ca, Mg) were extracted by water in the same way as Ni, as well as organic matter (Fig. 4a), notably low-mass carboxylic acids. Speciation calculations at thermodynamic equilibrium showed that Ni in aqueous solution did occur in free form (Ni^{2+}) but instead was bound to low-mass organic acids, as in the plant, and also to stronger ligands (Guilpain et al. 2018c). For this reason, while extraction with water at room temperature is easy, the recovery of pure Ni compounds can be complicated by the presence of many Ni-bearing organic and inorganic compounds in the leach solution.

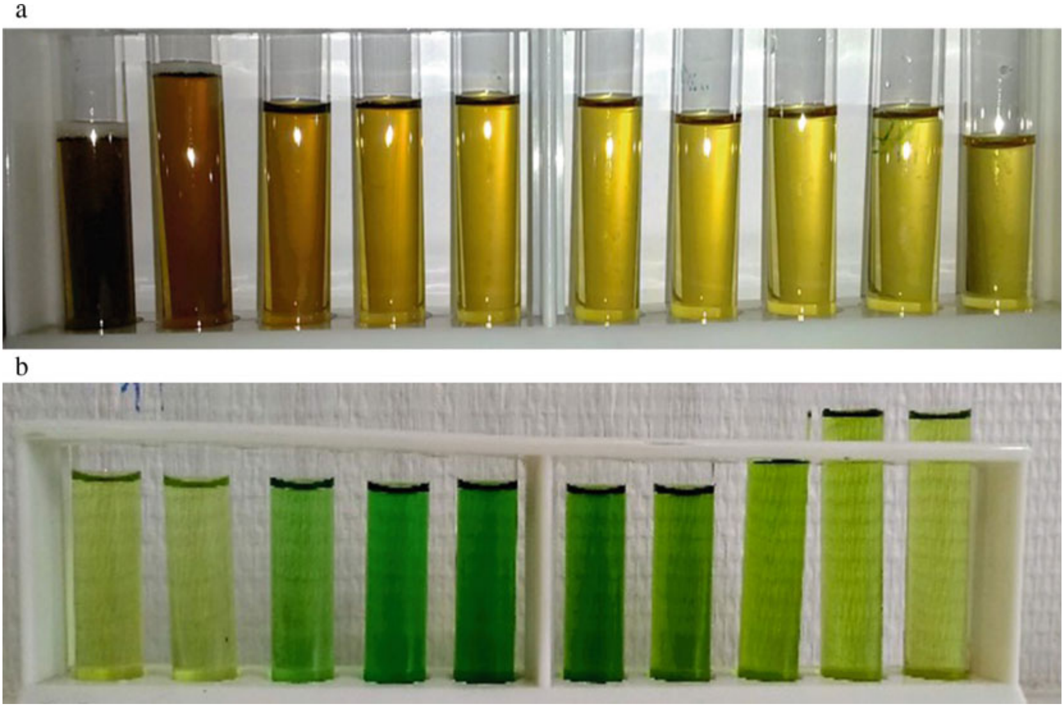

Fig. 4 Collecting fractions after laboratory column experiments with **a** *O. chalcidica* biomass with water, and with **b** acidic ash leachate purification and concentration with *O. chalcidica*

5 Recovery of Nickel Products

5.1 Nickel Metal

A method of recovering metallic Ni from HA ash was proposed by Chaney and co-workers (Li et al. 2003; Chaney et al. 2007) feeding the biomass or ash of *O. chalcidica* and *A. corsicum* directly into a smelter. Nickel metal can also be recovered from a leachate solution (Barbaroux et al. 2011). Direct leaching of *O. chalcidica* by a sulfuric acid solution has led to formation of a multicomponent leachate containing organic compounds. Selective precipitation or electroplating did not allow Ni recovery. Recovery of Ni from the *O. chalcidica* leachate was achieved by solvent extraction with bis (2,4,4-trimethylpentyl) phosphonic acid (Cyanex 272); however, loading and stripping were not selective over Mg. Versatic 10 solvent extractant, which was used at the Bulong Ni refinery, could be considered for this step (O'Callaghan 2003). Alternatively, ion exchange resin may be used and might be an easier process to implement for a small-scale operation (Littlejohn and Vaughan 2012). Following solvent extraction or ion exchange upgrading and purification, high-purity Ni can be recovered by electrowinning.

5.2 Nickel-Based Catalysts

Nickel-based catalysts have been produced at the laboratory scale from the ash of two HAs: *Psychotria gabriellae* and *Geissois pruinosa* harvested in New Caledonia (Losfeld et al. 2012a; Grison et al. 2013). HAs were firstly ashed at 500 °C, then leached with HCl. The leachates were dispersed on montmorillonite K10 to produce Lewis acid catalysts. These catalysts have been tested and compared to commercial $NiCl_2$ for use in the acetylation of anisole (Losfeld et al. 2012a) and green organic synthesis (Grison et al.

2013). Good performance has been attained, especially with *P. gabriellae*. The issue of performance *versus* plant variability should be addressed. Recently, a Ni metal catalyst was developed from the leaves of *P. gabriellae*. It was synthesized by pyrolysis of the formate obtained after extraction at 90°C for 18 h from the ash of the plant using formic acid (Escande et al. 2017). This catalyst showed high activity and selectivity for the transfer hydrogenation of carbonyl compounds.

All catalysts currently synthesized have been prepared by hydrometallurgical routes. The manufacture of carbon-supported Ni catalyst by direct pyrolysis is a world yet to be explored.

5.3 Nickel Compounds

The synthesis of ammonium Ni sulfate hexahydrate (ANSH) salt from the acid leachate of ash from *O. chalcidica* has been demonstrated (Barbaroux et al. 2012; Zhang et al. 2016). This method takes advantage of the very low aqueous solubility of ANSH at 0 °C. The process is presented in Fig. 3. The acid leachate is neutralized by $Ca(OH)_2$, in order to remove iron by precipitation of iron hydroxide. Magnesium is removed from solution by precipitation of MgF_2 using NaF. The solution is then evaporated before crystallizing ANSH by the addition of ammonium sulfate. Salt purity is increased by a second crystallization step; salt with purity of >99.1% was obtained (Zhang et al. 2016). Other process schemes have been developed from acid leachates, such as the use of resin to purify and concentrate Ni, limiting the waste produced (Fig. 4b). A variety of Ni-based compounds are obtained, particularly Ni oxide (NiO). This product is used as a pigment for colouring crystal (Fig. 5). A lower cost process yielding a relatively crude $Ni(OH)_2$ intermediate product has been demonstrated at the laboratory scale using ash of *R. bengalensis* (Vaughan et al. 2016b). An elegant aspect of the $Ni(OH)_2$ route is that a $Ni(OH)_2$ product is obtained by precipitation using a base which can be the K_2CO_3 containing ash water-wash solution from an earlier stage in the process. This resulting in significant savings in reagent costs and also yields a K_2SO_4 solution that could be recycled as a fertilizer at the metal farm.

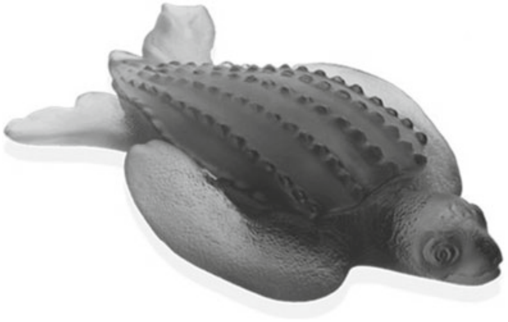

Fig. 5 A crystal leatherback turtle produced by Daum company (France) coloured by NiO produced from *O. chalcidica*

As explained above, it is difficult to synthesize Ni products from plant leach solutions because of the large number of compounds present. However, it has been shown that at least two strategies are possible: precipitation of Ni sulfide or hydroxide (Guilpain et al. 2018a), or separation of Ni from other compounds using a Ni-complexing resin, leading to the production of Ni salts such as oxalate or sulfate, for example (Guilpain et al. 2018a, b).

6 Upscaling of Plant Ashing and Nickel Recovery

Lab-scale research on the combustion of *O. chalcidica* and the recovery of Ni have provided the basis for the scale-up, especially in the context of the EU Life Agromine project (2016–2020) (Fig. 6). Importantly, ashing dry biomass at a large scale has some practical challenges (Fig. 6b). The simplest approach would be to burn the biomass in an open-air fire. However, this would be difficult to control and there would be no effective method for off-gas treatment and energy recovery. In addition, if the operation is not well controlled, oxygen-free zones may appear, in which case the Ni may be reduced to metallic form. Specialized equipment is available for biomass combustion, such as industrial biomass furnaces. For example, the KWB Multifire MF2 D/ZI has a continuous track and the combustion is monitored by the oxygen content of the exhaust fumes (Fig. 6d). This unit can process 7 kg h^{-1} of dry biomass. Temperature monitoring during the ashing process in this furnace indicated that the average temperature was ~900 °C. Properties of the ash obtained from this furnace can differ from that produced in a small-scale furnace but still meet process requirements (Houzelot et al. 2016). Other more basic equipment, such as bale furnace, are being tested (Fig. 6e). However, high levels of unburnt material limit the yields and purities of the ash-processing steps.

For the emission gases, the parameters to be taken into account are fly ash (which may contain Ni) and NOx (from N_2 in the injected air). The installation of gas filters (e.g. electrostatic precipitators) is essential (Delplanque et al. 2013), as is control of the air supply. Depending on the type of hyperaccumulators, pellets (for plants) (Fig. 6c) or wood chips (for trees) production are preferred, as they allow a homogeneous and complete combustion.

Recovery of Ni from the ashes (Fig. 6f) to make ANSH or Ni oxide was also scaled up to be conducted in a 60 L reactor equipped with a filtration system (Fig. 6g). Parallel-batch management increases the solution concentration by two and saves more than a third of sulfuric acid, producing similar yields (Kidd et al. 2018). The chemical analysis of the waste shows that a large part can be recovered, as fertilizer for ash washing solutions and as a substrate for Ni agromining via leaching of ashes.

7 Environmental and Economic Aspects

7.1 Green Products and Green Processes?

It is commonly believed that products made from plants are 'green' or at least 'bio-sourced' and therefore 'gentle'. However, metal products prepared by agromining have the same properties as products prepared by conventional technologies. In the case of Ni, the metal and metal compounds were selected for hazard and risk review by the EU in the 1990s and now are regulated by REACH and CLP, in terms of health and safety, worker and consumer protection, and protection of the

Fig. 6 Upscaling of Ni recovery from *O. chalcidica*: **a** bale of dry biomass, **b** ground biomass, **c** pellets prepared with water addition, **d** biomass furnace with a continuous track, **e** bale furnace, **f** ashes after combustion at 900 °C, **g** hydrometallurgical reactor to treat the ashes

environment (https://echa.europa.eu/documents/10162/13562/cmr_report_en.pdf). Risk management option analyses (RMOAs) have also been performed. Nickel metal is not classified. Concerning the other compounds, regulation may be enforced, particularly for worker protection. However, Ni substitution in most industries is difficult, and in some manufacturing, Ni is used to replace other hazardous metals (e.g. Cd, Cr). Therefore, the obligation of authorization would be more feasible than restriction (http://www.consultations-publiques.developpement-durable.gouv.fr/IMG/pdf/RMOA_NiO_PUBLIC.pdf).

Agromining is sometimes confused with ‘green chemistry’. Some of the 12 principles of green chemistry (https://en.wikipedia.org/wiki/Green_chemistry) are upheld but not all of them. Nevertheless, certain products made from plants, namely the catalysts, may open the way to green synthesis in organic chemistry.

In order to evaluate the environmental impacts of the agromining chain, multi-criteria and multi-stage assessment has to be conducted, mainly by Life Cycle Assessment (LCA). Recently, the first Life Cycle Assessment of an agromining chain was done and has proved the relevance of agromining for elaboration of the Ni salt ANSH produced from *O. chalcidica* grown in Albania (Rodrigues et al. 2016). This approach is fully described in chapter “Life Cycle Assessment and Ecosystem Services of Agromining”. Such evaluations should be conducted for all possible situations.

7.2 Agromining as a New Economic Activity

Aside from its scientific interest, agromining has led to new economic activity. Technical-economic evaluations of the process have been made for Ni (Angle et al. 2001; Li et al. 2003; Barbaroux 2010). For instance, from an order of magnitude economic study, consider the following scenario: a plant in Canada, 180 d yr^{-1}, transforming 5 t h^{-1} of *O. chalcidica* biomass to produce ANSH, the annual profit would be 8.5 million Canadian dollars. Clearly a dedicated economic study must be performed for each situation. Regardless, these calculations indicate the economic potential of processing HA biomass.

With a series of scientific, technical, economic, and environmental assessments, the entire chain of agromining is now implemented at the commercial scale as there are many applications for potential products. Initiatives have been taken to transfer this chain to include socio-economic activities. The newly created company, Econick (2016), is now producing Ni compounds from a large range of hyperaccumulator plants and brings to the market a series of high-value chemicals. This activity is designed for recovering metals from ultramafic soils, contaminated soils, and industrial wastes. In addition to the production of metal compounds, energy and by-products are produced.

8 Conclusions and Perspectives

The concept of agromining has been proposed for recovery of metals dispersed in soils or other resources at low concentrations that cannot be efficiently exploited by conventional metallurgical processes. Much of the effort has been focused on Ni agromining as many Ni HAs are known and vast areas of ultramafic soils and mine tailings are available. Furthermore, agronomic practices have been developed to produce high yields of Ni-containing plants, particularly *O. chalcidica.*

For Ni, several processes have been designed and upscaled to produce pure metal or a variety of Ni compounds. Life Cycle Assessment has proved that agromining to produce ANSH and recover energy has low environmental impacts. After an economic study proving the potential benefit of the approach, the start-up company Econick was launched in Nancy in 2016. Other developments are ongoing for Ni agromining, particularly with a focus on tropical hyperaccumulators.

Farming hyperaccumulator plants is an alternative to conventional mining of ores. High metal selectivity and concentration factors, together with the opportunity to recover energy and by products, are compelling reasons why agromining should be considered. Whilst agromining will not replace conventional mining practices, there appears to be scope for mainstream implementation of the technology.

Acknowledgements The authors acknowledge and thank the many researchers who have contributed to knowledge in processing of agromined bio-ore. We also acknowledge financers, especially Institut Carnot ICEEL, BPI France, the French Agence Nationale de la Recherche (Agromine project ANR-14-CE-04-0005, Labex Ressources 21 ANR-10-LABX-21), and EU (FACCE—Surplus: Agronickel project and LIFE: Life-Agromine project LIFE15 ENV/FR/000512).

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Processing of Plants to Products: Gold, REEs and Other Elements

Bastien Jally, Baptiste Laubie, Ye-Tao Tang,
and Marie-Odile Simonnot

Abstract

Nickel recovery by agromining has been widely studied because of the abundance of species that hyperaccumulate this element. But at the same time, in the context of the circular economy, there is growing interest in recovery of other elements for both economic and environmental reasons. Firstly, the recovery of gold from mine tailings appears increasingly promising, as does that of rare earth elements, which are strategic for numerous advanced technologies. In both cases, the economic value is a driver but also a need to remediate mine tailing by nature-based solutions. Secondly, despite being less valuable, the recovery of Cd and Zn has been much studied, because these two elements are contaminants of many industrial or agricultural soils and many plants are capable of accumulating them. It is therefore essential to find solutions for disposal of the biomass contaminated with metals and preferably by profiting from it. For this set of elements, processes based on thermal or chemical methods have been proposed on a small scale and some are on the verge of being scaled up. Other elements, such as metals of the platinoid family, Mn or molecules of pharmaceutical interest, have been the subject of pioneering work that remains to be developed. At the present time, it can be considered that in this area there is still a world to explore.

1 Introduction

The agromining chain, starting from the cultivation of plants to the recovery of products with commercial value, has been developed mainly for Ni (Chapter "Processing of Bio-ore to Products: Nickel") (van der Ent et al. 2015; Simonnot et al. 2018; Chaney 2019). There are two main reasons for this element focus. The first is that more than 520 hyperaccumulators have been identified (Reeves et al. 2017) and that agronomic processes have been developed for some, especially *O. chalcidica* (Bani et al. 2015). Several types of pyro- and hydrometallurgical processes have been developed to produce different Ni-based products (metal, salts, oxide) (Zhang et al. 2016). The second reason for the focus on Ni is the commercial value of the manufactured products, which are of interest to

B. Jally · B. Laubie · M.-O. Simonnot (✉)
Laboratoire Réactions et Génie des Procédés, UMR 7274, Université de Lorraine - CNRS, 1, rue Grandville, BP20451, 54001 Nancy Cédex, France
e-mail: marie-odile.simonnot@univ-lorraine.fr

Y.-T. Tang
Guangdong Provincial Key Laboratory of Environmental Pollution Control and Remediation Technology, Guangdong Provincial Engineering Research Center for Heavy Metal Contaminated Soil Remediation, Sun Yat-sen University, Guangzhou 510275, People's Republic of China

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_4

many sectors (stainless steel, catalysts, anti-corrosion coatings, pigments, etc.). As is the case with other metals, the price of Ni fluctuates (London Metal Exchange, https://www.lme.com), but the value of products such as salts and oxide are relatively stable depending on their purity.

Agromining is an attractive and promising method for recovering other metals, elements, and even chemical compounds from hyperaccumulator plants. For development to be considered, one must verify the existence and suitability of cultivable soils or other matrices (e.g. soils constructed from wastes, see Chapter "Agromining From Various Industrial Wastes") containing the target element, on which plants capable of hyperaccumulating them can be grown with a sufficiently high yield of both biomass and target element. The important criterion is not the concentration of the element in the plant, but instead the amount of element produced per hectare, calculated from the product of the biomass mass per hectare by the concentration of the target element in the biomass. The other essential point is the value of the products that can be synthesized. Calculations by Robinson et al. (1997), considering the price of the metal, provided an estimate of the biomass metal concentration required to make a US\$500 ha^{-1} return as a function of biomass production (t ha^{-1}). The concentration required increased with decreasing metal price for which the order at the time was: Au > Pd > Ag > Co > Tl > Ni > Sn > Cd > Cu > Mn > Zn > Pb. The table was updated in 1999 and the order shifted to: Pt > Au > Pd > Tl > Ag > Co > U > Sn > Ni > Cd > Cu > Mn > Zn > Pb, which reflects changes in metal price (Anderson et al. 1999). This metal order is 20 years old and has likely changed since then, and will in the future as well. Other important considerations are the cost of processing the biomass and the actual value to be obtained from the final product if not a metal. Not only the price and costs are to be taken into account, but also the existence of sectors with valuable applications and therefore of a potential market.

More than 700 hyperaccumulator plants have recently been identified and registered in a database (Reeves et al. 2017). Currently, the elements that are considered to be potentially hyperaccumulated are As, Cd, Cu, Co, Mn, Ni, Pb, rare earth elements (REEs), Se, Tl and Zn. Some of these are of relatively little commercial interest, such as As and Pb. In this case, the interest could be for detoxifying the biomass produced. Others are of definite interest, such as Cu, Co, Tl and Se, but either the relevant plants exist only under very specific conditions (e.g. Cu hyperaccumulators are mainly in the D.R. Congo) or soils containing the metals of interest are quite rare.

Currently, in the scientific literature, the elements that have been researched for recovery are essentially Au, REEs, Cd, Zn and Mn. As far as Au is concerned, there are no known hyperaccumulator plants, but Au can be phytoextracted from the soil if it is made soluble by use of (thio)cyanides. The processes developed for metal recovery from biomass include thermal processes, belonging to pyrometallurgy or pyrolysis, hydrothermal processes (such as supercritical water extraction), or hydrometallurgical processes. They should be designed as reagent-, water-, and energy-efficient processes to have the lowest possible impact on the environment and should be able to be considered as green chemistry processes.

This chapter presents the state of the art on the recovery of Au, REEs, Cd, Zn and Mn extracted from soils by plants. It also discusses the pioneering work that is beginning for other elements and opens up prospects for further developing the agromining industry.

2 Recovery of Gold

Gold occurs at relatively high concentrations in mine tailings and mine wastes, typically representing extremely large volumes, and can be recovered by phytoextraction as demonstrated by Anderson et al. (1998) with *Brassica juncea*. To overcome the poor solubility of Au in soil,

Table 1 Maximum gold concentrations measured in plants cultivated under greenhouse conditions

Plant species	Maximum gold concentration in plants (mg kg^{-1})
Brassica juncea	326
Brassica campestris	304
Chilopsis linearis	296
Raphanus sativus	220

Adapted from Wilson-Corral et al. (2012)

chemicals are often used to enhance phytoextraction (Wilson-Corral et al. 2012). Phytoextraction is generally performed with native plants of the parent mining sites. Data on plants, mechanisms of Au uptake, and field trials are given in review articles (Sheoran et al. 2009; Wilson-Corral et al. 2012). Table 1 lists the highest concentrations of Au found in plants to date.

Depending on the plants, Au concentrations in biomass range from ten to a few tens of mg kg^{-1}. The first route used for Au recovery from plant biomass was ashing, leaching by 2 M HCl, and solvent extraction into methyl isobutyl ketone, addition of a reducing agent (sodium borohydride) causing the formation of a black precipitate, and heating this precipitate at 800 °C (Lamb et al. 2001). This method has been simplified to save reagents and decrease costs. Recently, a field trial of Au phytomining has been conducted in Indonesia in order to assess the relevance of this technology for artisanal miners. Tobacco has been grown, harvested (100 kg) and ashed. Borax and Ag (used as collector metal) were added to the ash and smelted (Krisnayanti et al. 2016). Despite the low yield in this case, the trial has shown the technical feasibility.

The development of Au recovery pathways from hyperaccumulating plants can still be considered to be in the preliminary testing phase. At present, the small amount of available biomass and low concentration of this metal in biomass remain a challenge. However, the characterization of Au existing in the form of nanoparticles (Rodriguez et al. 2007) and its localization in the plant, given elements to develop new recovery processes. In addition, several economic evaluations have been performed (Anderson et al. 2005; Sheoran et al. 2009; Wilson-Corral et al. 2012), showing that Au phytomining could be valuable in many cases. For this reason, and also to contribute to the Au supply by adding value to unmined resources such as Au dispersed in the soils of mining areas, it is important to continue the development of Au agromining.

3 Recovery of Rare Earth Elements (REEs)

3.1 Potential for Agromining of REEs

The REEs are commonly defined as the 15 lanthanides in addition to Sc and Y. Their unique properties and wide range of application in modern technologies have resulted in substantial growth in production over the past two decades (Machacek and Kalvig 2016). However, most countries rely on imports as 90% of global production is located in China. Disparity between limited availability and high demand have increased prices, and several REEs are considered ‘critical’ strategic elements by the European Union and the USA (Bauer et al. 2010; Machacek and Kalvig 2016). Alternatively, in China, production has resulted in release of REEs in the environment and generation of hectares of abandoned mine tailings. In the Ganzhou county alone (Jiangxi Province), more than 100 km^2 of tailings were generated (Guo et al. 2014). Incentives from the Chinese government for the reclamation of these tailings benefitted the research on the agromining of REEs; the tailings are to date the only studied ‘natural’ substrate for REEs hyperaccumulator on a medium scale (Fig. 1). REEs are also used in some areas as fertilizers, which have generated contaminated fields to a certain extent. Industrial residues such

Fig. 1 Patches of wild *Dicranopteris linearis* growing on an eroded tailing bank in Ganzhou county, Jiangxi Province (China)

as metallurgical slags, coal ash, phosphogypsum (waste produced in the phosphoric fertilizer industry), or bauxite residue (red mud) all contain REEs in small concentrations but are available in vast areas (Binnemans et al. 2015). Such residues could be contemplated for soil construction for use in growing hyperaccumulators. Prices per ton vary greatly from one element to another. The following values are average transaction prices retrieved from the Association of China Rare Earth Industry website in March 2020 (http://www.ac rei.org.cn/portal.php) and converted from CNY (Chinese Yuan) to US$. The lightest REEs (pure oxides) are bought at fairly low prices (per ton): La_2O_3 ($1800), Ce_2O_3 ($2000), Sm_2O_3 ($2000), and Y_2O_3 ($3000). For these elements, the market is the largest in terms of quantities exchanged, and prices reflect natural abundance of the REEs. Alternatively, some REEs occupy niche markets with very low quantities exchanged and high prices, owing to a lack of technological applications coupled with element natural scarcity (e.g. Lu_2O_3 $6,200,000, Sc_2O_3 $1,268,000). In the middle of the price range lie the remaining REEs, the most notable being Pr_2O_3 ($47,000), Nd_2O_3 ($43,000) and Eu_2O_3 ($31,000).

Dicranopteris linearis (formerly known as *D. dichotoma*), one hyperaccumulator plant identified for REEs, is considered appropriate for REE phytoextraction because of its high contents of Nd and Pr (up to 900 and 300 $\mu g\ g^{-1}$, respectively), as well as other light REEs (La, Ce, and Sm), resulting in total concentrations of up to

3900 µg g^{-1} (Chour et al. 2020) (see Chapter "Element Case Studies: Rare Earth Elements"). Harvest of wild *D. linearis* collected at a mine tailings site in Ganzhou, Jiangxi Province, assumed that yields of 12–15 t ha^{-1} (dry weight) could be expected (Tang et al. unpublished data). Consistent values (12–20 t ha^{-1}) were found by a different team in a comparable mining area, in Changting county, Fujian Province (Li et al. 2013). With shoots containing an average of 0.2 wt% REEs, metal yields would be between 24 and 30 kg REEs ha^{-1}. However, varying yields are expected, depending on the strategy conducted for harvesting, as older ferns show higher REE concentrations (Liu et al. 2019). Additionally, a cultivation strategy needs to be established in order to maximize plant growth and metal yield. In Fujian Province, harvest experiments—without any other agricultural practices—were conducted with wild *D. linearis* in four comparable mining areas (Chen and Chen 2020). The best interval of time between each harvest for maximizing REE phytoextraction was determined to be two years, by clipping aboveground biomass. Availability of soil nutrients (particularly scarce on these types of soils) was identified as a critical parameter for phytoextraction yields, in boosting biomass growth without diminishing concentrations of REEs in the plant (Chen and Chen 2020). It is then expected that addition of amendments could greatly speed up the process, although to our knowledge this has not been applied to *D. linearis*. Moreover, and like for other hyperaccumulators, fertilization should be carefully applied in order to maintain bioavailability of REEs.

3.2 Processes for the Recovery of REEs

Efficient recovery processes are the last link in the agromining chain. In the same way, as for Ni recovery, several methods can be explored to recover REEs from the fern *D. linearis* (Fig. 2).

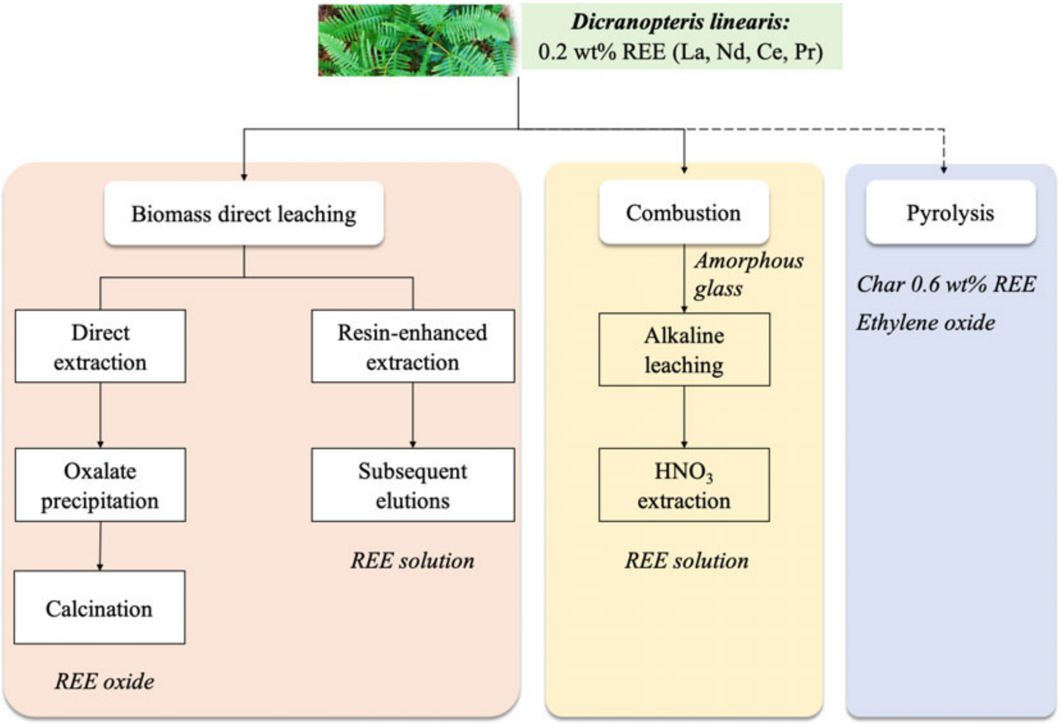

Fig. 2 Processes explored for the recovery of REEs from *Dicranopteris linearis* biomass

Direct Extraction

The REEs can be recovered in a three-step process, beginning by (i) direct biomass leaching using an aqueous extractant, (ii) precipitation of REEs in the oxalate form, and (iii) calcination of the oxalate to yield a mixed rare earth oxide (Chour et al. 2020). Satisfactory extraction yields (>80%) were obtained when using dilute H_2SO_4 (0.25 M) or ethylenediaminetetraacetic acid (EDTA) (0.05 M pH 4.5) to leach REEs from the plant at a solid:liquid ratio of 3 g L^{-1}. Alternatively, citric acid (1 M adjusted to pH 1.6 and 4), citrate (1 M adjusted pH 8) and water all gave inferior results and extracts were then discarded. After pH adjustment to 2.6, REEs are precipitated using oxalic acid at a molar ratio of 20:1. Immediate and quantitative (>90%) precipitation occurs under these conditions. However, the nature of the extractant from the previous step has a significant effect on solid purity: because EDTA is less soluble at lower pH it co-precipitates. Hence, H_2SO_4 was chosen as the best extractant. Ultimately, the oxalate is then dried and converted to oxide by calcination at 700 °C for 2 h. When using H_2SO_4 as the primary extractant, an overall yield of 72% is achieved and the oxide final purity is close to 95% (Chour et al. 2020).

Ion-Exchange Intensified Extraction

An original process based on an enhanced ion exchange leaching step has been developed (Chour et al. 2018). An HNO_3 solution (0.5 M) is used to leach the plant, while in contact with an ion exchange resin. The resin is set in a fiberglass sack to facilitate its removal, while ground biomass is dispersed in solution. The resin, a strong acid cation exchanger, adsorbs the REEs as they are leached, which displaces equilibrium and furthers extraction yield to its maximum (>98%). Once fully loaded, the ion exchange resin is transferred into a column, and purification of REEs is carried out by percolating successively three solutions through the resin bed. Two washing steps (water, followed by 0.75 M HNO_3) are used to remove impurities (e.g. K, Ca and Mg in the first step, and Al in the second step), which are more lightly bound than the REEs. During the second step, a small fraction of the REEs is lost during Al elution, which requires finding a compromise between purity and recovery by setting the volume and concentration of acid used. Eventually, REEs are eluted by using concentrated HNO_3 (3 M) that produces a solution having 81.4% REEs purity. At the same time, the adsorbent is regenerated to its initial state and is ready to reuse in further cycles. These operating conditions led to a global recovery yield of REEs of 78% (Chour et al. 2018).

Ash Extraction

Combustion of the plant is commonly used for organic matter removal and enrichment of the bio-ore (Hazotte et al. 2017; Simonnot et al. 2018). However, in the case of *D. linearis*, processes should be adapted to the specificity of the hyperaccumulating plant, and especially to the relatively high contents of Si (1.65 wt%) and Al (0.2 wt%) in *D. linearis*. Indeed, the resulting ashes mainly contain amorphous phases, stabilizing REEs, which make effective acidic leaching nearly impossible (Laubie et al. 2018). Importantly, this problem can be circumvented by pre-processing the ash through an alkaline leaching step (Chour 2018; Jally et al. submitted). This step aims at removing most of the Al and Si by using concentrated NaOH (20 wt%) and mild heating (*ca.* 80 °C). As in the Bayer process, Al is soluble as sodium aluminate ($NaAlO_2^-$). In a few minutes, the aluminosilicate structure is dissolved while REEs remain in their insoluble form, either as carbonates or hydroxide. The solid residue is later rinsed with deionized water to advance enrichment. Afterwards, REEs are available for extraction using a dilute HNO_3 solution (pH 2), yielding concentrations up to 330 mg L^{-1} as total REEs. The solution can then be purified from other cations (Ca, Mg and Mn) by either directly precipitating the carbonate form of the REEs, or by using an ion exchange resin, following the same strategy mentioned above. Additionally, vacuum-pyrolysis was investigated for the disposal of *D. linearis* biomass (Qin et al. 2019). When using a REE-rich biomass, an important fraction of the pyrolytic product (41%) was ethylene oxide, providing a key chemical resource. At the same time, it was observed that this method improved the total REEs concentration in the bio-ore from 1950 to 6160 mg kg^{-1}. Hence, the resulting char could be considered another source of REEs in the frame of a circular economy (Qin et al. 2019). Thus, the recovery of REEs has been considered using several approaches, and work is underway to assess which methods are relevant, with low environmental impact and low cost, for scaling up.

4 Recovery of Volatile Metals (Zinc, Cadmium and Lead)

Zinc, Cd, and Pb are metals with low volatilization temperatures (<1000 °C). Thus, pyrometallurgy is one of the preferred routes for their recovery (isolation, concentration). However, by controlling combustion conditions, it is possible to develop hydrometallurgical processes to recover these metals from the ashes of hyperaccumulating plants. The main issue is that the Zn hyperaccumulators tested all contain Cd. Cadmium can still find industrial applications: Ni-Cd accumulators, pigments, and anti-corrosion coatings in the aeronautics industry (International Cadmium Association 2017). But use of Cd is restricted by REACH regulation, Annex XVIII (Reach legislation), because of its toxicity (IARC 1993). The challenge for agromining is therefore to isolate the other metals (particularly Zn), by limiting traces of Cd in the finished products.

4.1 Thermal Treatment of Hyperaccumulators Containing Volatile Metals

The objective of the first thermal treatments of the plants was to provide a solution to decontaminate the metal-laden biomass, so that it would no longer be considered hazardous waste and could be stored safely and possibly used for other purposes (Sas-Nowosielska et al. 2004; Keller et al. 2005). More recently, studies have been carried out with the aim of separating and isolating metals from plants. Most experiments have been carried out with plants of the genus *Sedum*.

During incineration of *Sedum plumbizincicola*, Cd is mainly found (>70%) in fly ashes incinerated from 350 °C (Zhong et al. 2015a). At 950 °C, 20% of the metal is still left in the bottom ashes. Zinc and Pb behave differently, largely volatilizing from 650 °C. At 850 °C, Zn and Cd are in the form of ZnO and $Zn(OH)_2$ and CdO and $CaO_{0.67}Cd_{0.33}CO_3$, respectively, in bottom ash. Zn^0 and Cd^0 are mostly found in fly ash; Pb^0 oxidizes at *ca.* 750 °C to give PbO (Zhong et al. 2015b). It should be noted that some of the metals are found in the flue gas (Lu et al. 2012), requiring treatment (adsorption) in order to avoid toxic emissions.

Among the critical parameters, airflow increases the proportion of metals found in fly ashes (Zhong et al. 2015b). Oxygen content is also determining, when the biomass is pyrolyzed (gasified), the volatilization of metals is promoted. This result has been shown for *Noccaea caerulescens* (Keller et al. 2005) and for *Sedum alfredii* (Cui et al. 2018). However, by controlling the temperature, it is possible to partially separate Cd from Zn. Whereas Cd volatilizes at temperatures as low as 550 °C, Zn remains in the biochar at least until 750 °C. Lead volatilizes above 600 °C (Zhong et al. 2016; Du et al. 2019). However, it seems difficult to obtain Cd-free solids. Plant composition, combustion conditions, and furnace configuration appear to have a significant influence on metal volatilization temperatures.

4.2 Recovery of Volatile Metals by Hydrometallurgy

Catalyst synthesis has been explored with other metals than Ni using the same approach as for Ni (Losfeld et al. 2012a, b): ashing and leaching with HCl followed by deposition on montmorillonite K10, or another purification process.

Lewis catalysts for the Diels-Alder reaction have been prepared from the Zn hyperaccumulator plants *Noccaea caerulescens* and *Anthyllis vulneraria* (Escande et al. 2014a, b). These catalysts are very effective in organic chemistry for different types of reactions (e.g. Biginelli reaction). Their effectiveness is attributed to a polymetallic nature, particularly the presence of Fe in addition to Zn. Thus, these catalysts should be at the origin of exciting developments in green chemistry. Currently, the quantities that have harvested are 17 kg Zn ha^{-1} for *N. caerulescens* and 27 kg ha^{-1} for *Anthyllis vulneraria* (Grison et al. 2016). For industrial development, significantly higher harvest quantities would be required.

Aside from catalysts, Zn recovery has also been investigated. Leaching of the biomass of *S. plumbizincicola* (1.2 wt% Zn) (Yang et al. 2009a) and of *S. alfredii* (1.3 wt% Zn) (Yang et al. 2009b) in ammonia/ammonium chloride was effective. Following a systematic study of the leaching parameters, it was recommended to use a $NH_4Cl{:}NH_3$ molar ratio of 0.6 and a temperature of 60 °C. Another contribution has shown the possibility of recovering Zn and Cd from *N. caerulescens* biomass by cementation (Hazotte et al. 2017). Cementation is a simple and effective technique based on the difference in standard redox potentials of metals: ($E°(Cd^{2+}/Cd^0) = -0.40$ V, $E°(Zn^{2+}/Zn^0) = -0.76$ V relative to a standard hydrogen electrode). It consists of adding Zn powder to a solution containing Cd^{2+} cations, which gives Cd metal and Zn^{2+} cations (Eq. 1)

$$Zn^0 + Cd^{2+} \rightarrow Zn^{2+} + Cd^0 \qquad (1)$$

Noccaea caerulescens was burnt at 620 °C (to avoid metal volatilization) and ashes were leached by a 2 M H_2SO_4 solution. A series of cementation steps allowed the recovery of solids enriched in Cd and Zn separately. Cadmium pigments can then be synthesized (Fig. 3).

Fig. 3 Pigments synthesized from cadmium from the Zn/Cd hyperaccumulator *N. caerulescens*

ZnO nanoparticles were also produced using *S. alfredii* (13,721 mg kg^{-1} of Zn in shoots) from Liaoning Province, China (Qu et al. 2011). The chlorophyll and Zn present in the plant were extracted separately (in ethanol medium and with H_2SO_4, respectively), and then contacted to form $C_{32}H_{30}ON_4Zn(CO_2H)_2$. The addition of $NH_3{\cdot}H_2O$ resulted in the precipitation of $Zn(OH)_2$, which was dried at 550 °C for 2 h to produce nanoparticles of ZnO. Cadmium present in the plant material did not interact with chlorophyll. However, Cu traces were found in the particles. In a second paper, the authors showed that these particles have a $Cu_{0.05}Zn_{0.95}O$ composition and a diameter of about 97 nm (Qu et al. 2012). They were also able to manufacture carbon nanotubes from a synthesis residue. The advantages of this work are that it can be directly carried out on plants and the absence of combustion in the early stages of the procedure avoids the problem of metal volatilization.

More recently, subcritical hydrothermal liquefaction (HTL) was tested on *S. plumbizincicola* (Qian et al. 2018). Approximately 90% of the Zn contained in the plant was extracted at 220 °C. The hydrochar produced was then depleted in this element (concentration divided by about 8) and exhibited a good capacity of carbon dioxide capture. The presence of Zn (Zn^{2+}) in the aqueous phase allowed the production of long-chain fatty acids and acetic acid from the biomass of the plant. In this case, the metal was used directly without trying to isolate it. In contrast, Chen et al. (2019) showed that >85% of the Cd, Cu, Pb and Zn contained in *S. alfredii* remained in the solid phase at 320 °C after 30 min. This difference in results was attributed to the reduction of metals by the presence of substances in the biomass (e.g. glucose and vitamin C). The addition of an oxidizing agent (H_2O_2) did not change the result.

5 Recovery of Other Elements

The recovery of other elements, not only metals, from hyperaccumulators has also been investigated. However, research in this area is still very limited. Concerning Mn, the only means of recovery at present is the synthesis of polymetallic catalysts for use in organic chemistry. These catalysts were produced from *Grevillea exul* subsp. *rubiginosa*, a plant from New Caledonia (Escande et al. 2015, 2017; Garel et al. 2019; Bihanic et al. 2020). The first catalyst, known as Eco-Mn, prepared by the same method as for Zn catalysts, have shown effectiveness for olefin epoxidation, due to the action of Mn II and Fe III (Escande et al. 2015). For the second, called Eco-Mnox, the synthesis was followed by oxidation (H_2O_2–NaOH) that enabled the selective cleavage of activated 1,2 diol (Escande et al. 2017). The third, called Eco-CaMnOx, was prepared by the same method, replacing NaOH by $NaHCO_3$, allowing the epoxidation of various terpenes and lipids (Bihanic et al. 2020). Little information is available on the plant, except in Bihanic et al. (2020), which reports a Mn content of the leaves of *G. exul* ssp. *rubiginosa* of approximately 0.30%. Catalyst syntheses were made from a few tens of grams of leaves, but no data are given about the potential yield of Mn if this plant was cultivated.

Pioneering work has been done to recover Pd from the *Arabidopsis* plant, grown hydroponically in a Pd-spiked medium (Parker et al. 2014). Catalysts based on Pd nanoparticles, were synthesized from this plant by pyrolysis, characterized and tested by the Suzuki-Miyaura reaction (formation of C–C bonds). Excellent results were obtained, prompting a later study on the economic interest in Pd phytoextraction and analysis of the life cycle of the process, and demonstrating the potential of this approach (Harumain et al. 2017). Other work aimed at isolating As from ferns in the genus *Pteris* was done in order to find a safe disposal solution for the plant (da Silva et al. 2018; Li et al. 2018; Duan et al. 2017), and for As applications in medical, horticultural, and electronics industries (Eze and Harvey 2018). Thus, As^0 nanoparticles were synthesized from *Pteris cretica* (about 4500 mg As kg^{-1}). Particles of 45.5 nm were produced by reduction of $Mg_3(AsO_4)_2$. This intermediate was precipitated from fern biomass after solvent extraction with molybdic acid. It is also used to stabilize As with a yield of 98%, after extraction from the biomass with 35% ethanol and addition of $MgCl_2$ (da Silva et al. 2018). Some elements are by-products of the treatment processes described above and in Chapter "Processing of Bio-ore to Products: Nickel". For example, regarding the hydrometallurgical process to produce Ni salts or oxide from *O. chalcidica*, the K derived from ash washing can be valorized as a fertilizer. The solid obtained after evaporation contains mainly K_2SO_4 (nearly 83%), KCl, and $K_2CO_3{\cdot}1.5H_2O$ (unpublished data). The Ni concentration found in this solid is lower than the maximum concentrations authorized by the French certification for fertilizers NF U42-001 (22 *vs.* 120 mg kg^{-1}). Another example is the valorization of Al extracted by the fern *D. linearis* together with REEs (see Sect. 2). However, the interest in developing a specific Al purification process remains limited regarding the potential economic gain. By the same logic, elements such as P, whose resources are dwindling, can be accumulated by certain plants (e.g. *Pilea sinofasciata*, 16–30 g P kg^{-1}) and hence may be used for agricultural fertilization (Roy 2017).

6 Conclusions and Perspectives

At present, proof of concept for metal recovery through the agromining process has been established mainly for Ni. Indeed, this is so far the only metal for which hyperaccumulators have been cultivated on a large scale, allowing tonnes of plants to be harvested for pilot-scale demonstrations. In this chapter, we have shown that further research has been carried out or is underway for other elements, and for different reasons: for direct commercial value (Au, Pd), for manufacturing 'eco-catalysts' (Zn, Mn, Pd), for extraction from resources that would otherwise remain unexploited (Au, REEs), for extraction from soils for remediation purposes, and for detoxifying the biomass (As, Cd, Zn). In the years to come, agromining will be developed for the elements mentioned in this chapter, but also most certainly for others being phytoextracted from natural soils, from soils built with waste, and from solutions via hydroponics. Not only can the recovery of elements can be envisaged, but also the isolation of molecules of pharmaceutical interest, as demonstrated by Rue (2017) for the recovery of coumarines from Ni hyperaccumulators, and salidroside-type metabolite (described as anti-fatigue and anti-drug) from *S. alfredii* (Xing et al. 2012). For the recovery of elements or molecules of interest by agromining to become economically viable, the operation must be profitable. Firstly, there must be suitable plants that can be grown on soils or substrates for which it is possible to harvest large quantities of biomass having a high concentration of the desired compound. Secondly, clean, safe, and economical processes must be developed. Agromining is one separation technology among others, with the plant acting as an extractor and separator, albeit not a selective one. This technology is not necessarily adapted to all cases but is indicated when the compound of interest has a low concentration in the substrate. Finally, it must be kept in mind that agromining is a nature-based technology, but if valorization of biomass is pursued, the processes must have a low environmental impact in order to claim this as a member in the field of green chemistry.

Acknowledgements The authors acknowledge and thank the many researchers who have contributed to the knowledge in processing agromined bio-ore. We thank Institut Carnot ICEEL (REEcovery project), LabEx Ressources 21 and Sun Yat-sen University for financial support of the Ph.D. program of B. Jally.

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Life Cycle Assessment and Ecosystem Services of Agromining

Marie-Noëlle Pons, Jérémy Rodrigues,
and Marie-Odile Simonnot

Abstract

Starting from the concept of sustainability and the need for related metrics, this chapter presents the basics of the Life Cycle Assessment (LCA) methodology including different versions (attributional vs consequential, static vs dynamic). Key issues related to the application of LCA to agromining, either on natural soils (such as ultramafic soils) or on anthropogenically polluted areas (such as mine tailings), are highlighted. Land use impacts are more specifically described: these are linked to the ecosystem services rendered by land systems and characterized via indicators that attempt to quantify soil organic carbon content, biodiversity, and land erosion by rainfall and wind. In spite of the actual limitations on quantifying land use impacts, which are not specific to agromining projects, LCA offers a framework for discussion among agrominers regarding different aspects of their projects in terms of sustainability.

1 Introduction

In 1987, the Bruntland Report («Our Common Future») marked a turning point in sustainability awareness (World Commission on Environment and Development; WCED 1987). Different sustainability paradigms have been proposed (Neumayer 1999; Schlör et al. 2015), such as the weak sustainability concept developed by Hartwick (1977) and Solow (1993) or the strong sustainability concept proposed by Holling (1973, 1986). The Holling concept corresponds to a pessimistic vision of sustainability, whereby the loss or over-exploitation of natural capital, especially abiotic resources such as fossil fuels (Shafiee and Topal 2009) and minerals (Yellishetty et al. 2011; Boryczko et al. 2014), is considered as irremediable and cannot be compensated by human or human-made capitals. In contrast, the Solow-Hartwick concept is more optimistic, stating that it is possible to compensate the loss of natural resources by saving them, i.e. through improved efficiency of their usage and increased recycling (Habib and Wenzel 2014), or by developing new technologies (Moran et al. 2014; Middlemas et al. 2015). In the weak sustainability concept, the consumption of non-renewable resources can be kept on a non-declining path, that is, as long as resource use efficiency and recycling are both high. Agromining of natural ultramafic soils or of human-made tailings sites is part of this weak

M.-N. Pons (✉) · J. Rodrigues ·
M.-O. Simonnot (✉)
Laboratoire Réactions et Génie des Procédés,
Université de Lorraine, CNRS, 1 rue Grandville,
BP 20451, 54001 Nancy Cedex, France
e-mail: marie-noelle.pons@univ-lorraine.fr

M.-O. Simonnot
e-mail: marie-odile.simonnot@univ-lorraine.fr

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_5

sustainability strategic vision, as it is a new technology for efficiently using low-grade and secondary resources. During recent years, the concept of 'green growth', where it is assumed that environmental development and economic growth can progress in parallel, has been promoted by several international organizations (UNO, OECD, EU) (Fletcher and Rammelt 2017) and can inflect policies of development in various industrial sectors, such as in the metal industry (Huang et al. 2020).

Whatever the definition of sustainability, metrics are needed in order to assess the environmental impact caused by any human activity including agromining. Among the various environmental assessment methods that have been proposed (Jeswani et al. 2010) (Life Cycle Assessment (LCA) Environmental Impact Assessment (EIA) (Tukker 2000; Cashmore 2004), Strategic Environment Assessment (SEA) (Finnveden et al. 2003), Material Flow Analysis (MFA) (Brunner and Rechberger 2004), Substance Flow Analysis (SFA) (Yellishetty and Mudd 2014), Water FootPrint (WFP) (Hoekstra 2003) and Carbon FootPrint (ISO/TS 14067 2013; Wan et al. 2016), LCA is the most comprehensive (Guinée and Heijungs 1993; Guinée et al. 1993a, 1993b; Rebitzer et al. 2004; Pennington et al. 2004). Ecological footprints, which measure the human demand on nature, are attractive for exchanges with stakeholders, but these focus on only one issue, usually carbon (Muller et al. 2020), but also on water, land, and even metals (Liu et al. 2020); assessing globally the environmental impact of a complex system requires examining several impact categories simultaneously. To overcome this difficulty, the development of a framework for sound footprint metrics has been recently proposed (Ridoutt et al. 2016). In the meantime, LCA remains the most encompassing method for environmental impact assessment: without describing LCA in detail, the aim of this chapter is to summarize the issues for applying LCA to agromining, and to provide theoretical and practical recommendations for assessing agromining properly.

2 What Is Life Cycle Assessment?

Life Cycle Assessment is a standardized method (ISO 40040 2006; ISO 40044 2006) that allows quantification of the potential impacts of a product, a process, a service, or an activity on its environment (Heijungs et al. 2010). The method takes into account the entire life cycle, i.e. from the extraction of raw materials to its end (waste treatment and disposal) as well as necessary commodities (electricity, water, etc.). This cradle-to-grave (Fig. 1) vision allows anticipation of the possible transfers of impacts among life cycle stages. Energy generation (Sanz Requena et al. 2011; Turconi et al. 2013; Amponsah et al. 2014; Asdrubali et al. 2015), exploitation of abiotic resources (Moran et al. 2014), water (Duić et al. 2015), metals (Table 1), and manufacturing of food products (Roy et al. 2009; Cellura et al. 2012), but also contaminated land remediation (Witters et al. 2012; Visentin et al. 2019; Vocciante et al. 2019), are among the industrial sectors for which LCA has been largely developed.

Potential applications of LCA to agromining are, for instance: comparison with conventional supply chains, identification of potential hotspots, and assessment of potential environmental consequences of its massive development. These applications are discussed below.

LCA operates in general by comparing two or more products, processes, etc., one of which is used as a reference. In the case of Ni agromining, the reference process would be based on extractive metallurgy (pyrometallurgy or hydrometallurgy). Through the life cycle analysis of two scenarios for remediation of Pb-polluted soils in Spain, Vigil et al. (2015) concluded that for the phytoremediation scenario, biomass valorization of *Morus alba* (in the present case by anaerobic digestion) was necessary in order to obtain a sustainable benefit. Suer and Andersson-Sköld (2011) reached a similar conclusion in their comparison of phytoremediation of a former oil depot by use of *Salix viminalis* for biofuel production with a dig-and-dump scenario. Witters

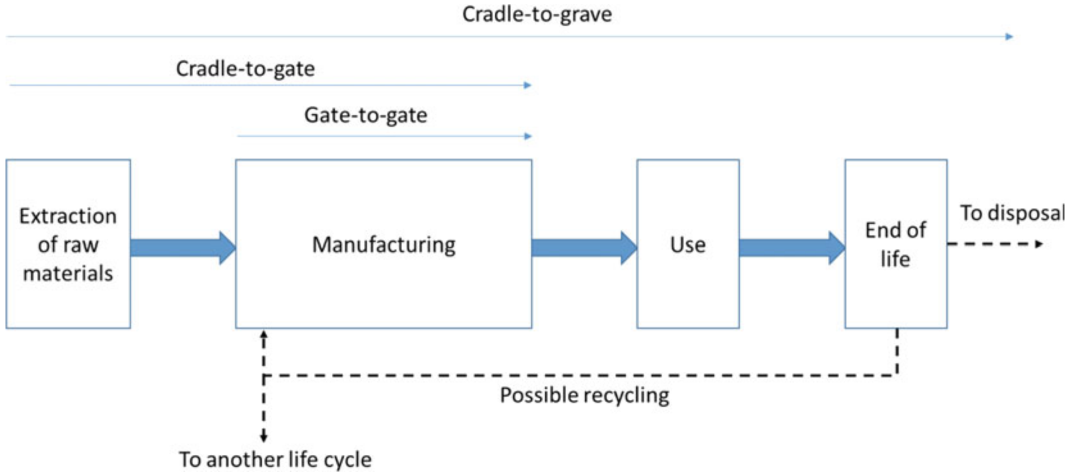

Fig. 1 Types of LCA according to the selected system boundaries

et al. (2012) have compared the cultivation of three energy crops (*Salix* spp., *Zea mays*, and *Brassica napus*) on soils hosting a diffuse pollution by Cd, Pb, and Zn: digestion of silage maize combined with combustion of the resulting contaminated digestate was the best scenario in terms of energy production and CO_2 abatement perspectives. The two other considered scenarios were willow combusted with electricity and/or heat production, and rapeseed combusted with biodiesel or oil production.

LCA can also be used on a single item (product, process, activity, etc.): the goal is then to compare stages of its life cycle (manufacturing, operation/use, end-of-life disposal, decommissioning, etc.) and to identify potential hotspots that may require improvements for minimizing environmental impacts (Nielsen and Wenzel 2002; Benini et al. 2015). This approach corresponds to the definition of classical or 'attributional LCA' (aLCA) and can be used as a management decision tool by companies willing to meet market expectations on the sustainability of their product (Buxel et al. 2015).

But what will happen if there is a high demand for agromined Ni products (Buxel et al. 2015)? If, in a given region, all of the ultramafic soils actually used for the production of food crops were devoted to Ni production, where would the food for local inhabitants and their cattle come from? This type of question is tackled in 'consequential life cycle assessment' (cLCA), which aims to help policy makers deal with large-scale and complex problems (Finnveden et al. 2009; Earles and Halog 2011; McManus and Taylor 2015). In aLCA, the models describing the processes are essentially static and do not take into account any variations in the market during the operation time (several decades sometimes for an industrial plant), either for commodities (change of the energy production mode, etc.), or for the products themselves (desire for the public to use more biosourced products; Meyerding 2016, etc.). Rebound effects, where increase in a process efficiency does not result in a decrease in energy or resource consumption because of an increase in product availability, of changes in user behavior and/or income, etc., are other aspects that can be addressed by cLCA (Benedetto et al. 2014; Vivanco and van der Voet 2014).

In cLCA, the system boundaries, i.e. limits between the technical system under consideration and its environment, are extended with respect to those in aLCA. The assessment is supposed to be more prospective than in aLCA. However, this is not always true in practice, because many aLCA studies are not restricted to a posteriori problems, i.e. those dealing with fully established industrial systems. aLCA has been applied to new

Table 1 LCA examples related to metals

Metal	Reference	Note
Gold (Au)	Mudd (2007)	Mining
	Norgate and Haque (2012)	Cradle-to-gate
Nickel (Ni)	Norgate et al. (2007)	Cradle-to-gate
	Mudd (2010)	Mining
	Norgate and Jahanshahi (2011)	Cradle-to-gate
	Schmidt et al. (2016)	Production route for Ni class 1
Copper (Cu)	Norgate et al. (2007)	Cradle-to-gate
	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Lead (Pb)	Norgate et al. (2007)	Cradle-to-gate
Zinc (Zn)	Norgate et al. (2007)	Cradle-to-gate
	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Aluminium (Al)	Norgate et al. (2007)	Cradle-to-gate
	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
	Liu and Müller (2012)	Cradle-to-grave
	Ciacci et al. (2014)	Cradle-to-gate
	Ding et al. (2012)	Cradle-to-gate, primary and recycled aluminium
	Paraskevas et al. (2016)	Cradle-to-gate, aluminium recycling
	Paraskevas et al. (2016)	Cradle-to-gate, primary aluminium
Titanium (Ti)	Norgate et al. (2007)	Cradle-to-gate
	Middlemas et al. (2015)	Cradle-to-gate
Steel	Norgate et al. (2007)	Cradle-to-gate
	Burchart-Korol (2013)	Gate-to-gate
Stainless steel	Norgate et al. (2007)	Cradle-to-gate
	Olmez et al. (2016)	Cradle-to-gate
Iron (Fe)	Yilmaz et al. (2015)	Gate-to-gate
	Olmez et al. (2016)	Cradle-to-gate
Cobalt (Co)	Norgate and Jahanshahi (2011)	Cradle-to-gate
	Schmidt et al. (2016)	Production route for Co class 1
Cadmium (Cd)	Fthenakis (2004)	Cradle-to-gate, for photovoltaics
	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Indium (In)	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Germanium (Ge)	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Gallium (Ga)	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Selenium (Se)	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Tellurium (Te)	Fthenakis (2004)	Cradle-to-gate, for photovoltaics
	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics
Molybdenum (Mo)	Fthenakis et al. (2009)	Cradle-to-gate, for photovoltaics

technologies that are only available in the laboratory or at pilot scale, and that are compared to established industrial approaches. For example, Vocciante et al. (2016) discussed the benefits of an electro-kinetic remediation process for removing metals from a polluted site. This is a rather new technology with few demonstration studies available and is compared by the authors to landfilling.

In cLCA, technology changes and maturation over the coming years with yield changes, market response times, etc., should be predicted. Alvarez-Gaitan et al. (2014) have examined the effect up to 2030 of increased potable water demand, due to population increase and availability of a better infrastructure, for chlor-alkali chemicals used in drinking water production: possible changes in the production mode of chlorine gas and of the electricity mix (taking into account energy prices), the effect of climate change on hydroelectricity production, and the social impacts (no nuclear power plants, no municipal solid-waste incinerators). cLCA includes also some economic information not needed in aLCA. However, the rules of how to apply cLCA are not yet fully defined. Marvuglia et al. (2013) proposed a framework to help practitioners implement cLCA, and described its application in the case of biogas production from crops. This framework includes indirect changes in land use as issues that are frequently addressed in cLCA involving agricultural resources (Kløverpris et al. 2008a, 2008b; Alvarenga et al. 2013; Vázquez-Rowe et al. 2014). The debate between aLCA and cLCA is not closed yet: it has been suggested recently that both approaches are necessary in order to manage the environmental responsibly (Brander et al. 2019; Yang 2019).

3 From Scope Definition to the Life Cycle Inventory

According to the ISO norm 14044, LCA is based on four steps (Fig. 2):

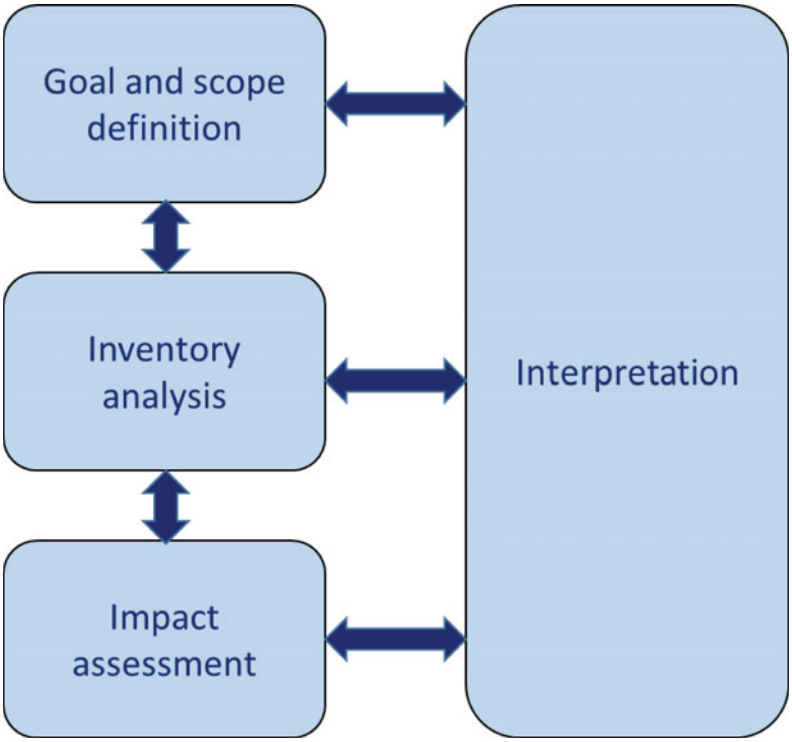

Fig. 2 Life cycle assessment framework

- *Goal and scope definition*: The goal and scope of the study are explicitly stated. The function (s) fulfilled by the system under study are identified and the functional unit defined: it is on the basis of the functional unit (surface of land to remediate, mass of metal to produce, etc.) that quantification of the impacts will be based. The system boundaries are set and the allocation method needed, when several co-products share the same process, is selected.
- *Inventory analysis*: Processes and activities required to produce the studied goods/services are described as thoroughly as possible. Input flows may originate from other processes/activities, which should be described as well, or come directly from the environment. Output flows are the studied goods, emissions of substances to the environment, and possibly co-products, by-products, or waste to be disposed. Data regarding these exchanged flows may either be collected during the study (and constitute the system foreground) or already be available within Life Cycle Inventory (LCI) databases such as EcoInvent (which would constitute the system background). Eventually, LCI sums up all of the flows exchanged with the environment, and is the basis by which potential impacts are calculated in the next step.
- *Impact assessment*: Potential impacts of the flows inventoried in the previous step are estimated. The quantities of these flows are multiplied by characterization factors, which express the potential impact of an elementary quantity of flow. These characterization factors are calculated using environmental models.
- *Interpretation*: This step allows comparisons among different systems, and for answering the questions formulated in the goal and scope definition. Processes and life cycle steps that contribute the most to a given impact are identified. The main hypotheses and control parameters of the studied system may be modified during a sensitivity analysis in order to test their influence on the results. Reliability of the results regarding data uncertainties may be tested through an uncertainty analysis, and their effect on the results documented.

The following sections discuss some methodological issues to bear in mind when doing an LCA.

3.1 Goal and Scope Definition

As an illustration, Fig. 3 summarizes the steps necessary to produce ammonium nickel sulphate hexahydrate (ANSH) by agromining on ultramafic soils in the Balkans (Bani et al. 2015; Zhang et al. 2016; Rodrigues et al. 2016). Figure 4 presents a simplified process tree for the conventional production of the same product, ANSH being obtained by mixing nickel sulphate hexahydrate with ammonium sulfate. Several steps are required in order to obtain nickel sulphate hexahydrate from Ni metal. The Class 1 Ni metal (>99% Ni) that is used in this process is produced mainly by pyrometallurgy of sulfidic ores (Norgate et al. 2007). For the sake of simplicity, not all of the inputs and not all of the emissions have been indicated. But the full process tree should contain all emission routes (to soil, atmosphere, and aquatic environment) and all inputs (water, fertilizers and pesticides for agricultural processes, reagents for chemical processes, energy).

In particular, waste treatment should be described in detail. Most mines around the world store their slurries (mixture of process water and tailings) in dams, with consequent risks of dyke failure or seepage of mine drainage. The effect of acid mine drainage (AMD) dispersion (e.g. the Aznalcóllar dam failure in Spain) can be severe to local environments, because of the extremely low pH and high concentrations of metals in the drainage that result in high environmental availability of the released metals. Reid et al. (2009) tested six scenarios for an underground hard rock Cu-Zn mine, based either on sending the tailings to a disposal area where they were submerged, or using these to partially backfill the mine galleries. According to these authors, there is no consensus on the best methods for describing land use and long-term impacts. As the rate of mining increases, due to economic development and decrease in ore grade (Mudd et al. 2007;

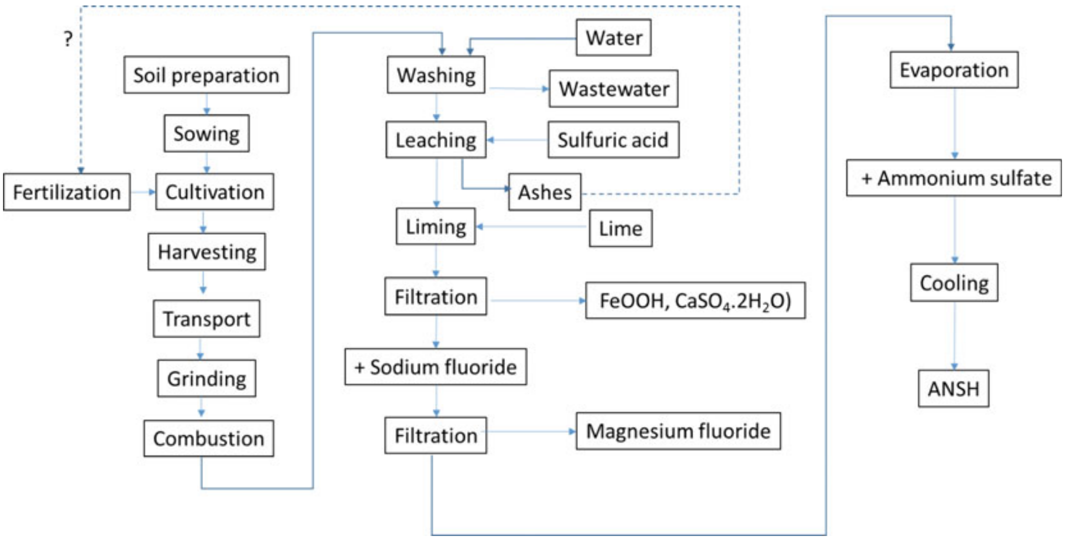

Fig. 3 Process tree for the production of ANSH based on agromining

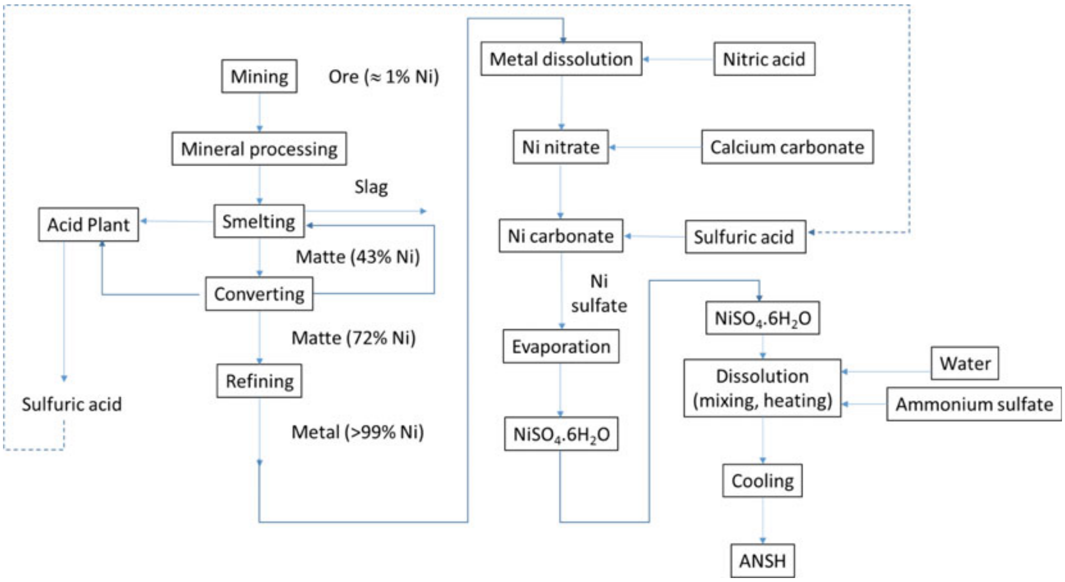

Fig. 4 Process tree for the production of ANSH from sulfidic nickel ore. Adapted from Norgate et al. (2007) for the metal production

2010), new technologies for reducing environmental impacts of tailings are developed (Edraki et al. 2014; Asif and Chen 2016). Tailings phytoremediation (or phytostabilization) has been proposed, mostly at the laboratory scale (Han et al. 2016; Yan 2016; Burges et al. 2016; Vargas et al. 2016) with some field-scale testing (Gil-Loaiza et al. 2016; Perkins et al. 2016; Davies et al. 2016), but phytomining (i.e. recovery of the metals from plants) is not an issue and no LCA seems to have been performed using phytoremediation technologies (Morais and Delerue-Matos 2010). In the Ecoinvent database, which provides process data for thousands of products to aid the data inventory phase in LCA, models for the treatment of sulfidic ore tailings (e.g. Ni, Cu, Pb, Zn, etc.) have been considered too uncertain, although effects of AMD on the environment (water and soil acidification, trace metal transfers in adjacent ecosystems and to humans) are recognized (Gutierrez et al. 2016; Romeiro-Freire et al. 2016; Taludkar et al. 2016). To date, only impacts related to land use are taken into account and not those related to effects on ecosystems (Althaus and Classen 2005).

Possible recycling should also be considered. In the conventional process for manufacture of ANSH, sulfuric acid is produced during the smelting/converting step and can be used, at least partly, later to transform Ni carbonate into Ni sulfate. In agromining, the recycling of elements (Ca, K, Mg and P) could be proposed for fertilization, but the concentrations of metals other than Ni should be taken into account and could prevent such a reuse. The production of sulfuric acid concomitantly to Class I Ni in the conventional process with sulfidic ore helps to illustrate a key issue in LCA: the allocation problem, which should be solved any time that an activity generates several co-products. There are basically three ways to deal with this problem:

- System subdivision: identify which subsystems generate the co-products and record only the flows related to the product under study.
- System expansion: do the full inventory of the system with all co-products and do the same inventory for the other ways to produce the co-products. In our specific example, the conventional manufacturing process for sulfuric acid is the contact process that combines sulfur, oxygen, and water in presence of a catalyst (vanadium oxide). In the agromining process, some elements can be recovered as fertilizers: conventional processes for the concerned fertilizers should be inventoried. Then theses flows will be subtracted from those of the process under study, as they will be avoided.
- Allocation: do the full inventory of the system with all co-products and use allocation factors to weigh the importance of the flows with respect to each co-product. The key issue is then the calculation of allocation factors: these are usually based on mass, economical value, or energy content, but there is no general consensus on the best allocation procedure (Reap et al. 2008; Schrijvers et al. 2016).

Localization of the process (and of the different stages of the process) should be specified for properly taking the transport modes and distances into account, as well as the local energy mix. The local energy mix is the recipe used locally to produce energy from different sources, such as coal, gas, etc. For the conventional route, ANSH is not necessarily produced at the mining site. In the agromining scenario, one option is to produce the plants and the ashes in one country and the metal salts in another country. In both cases, the mode of transport (ground, air, water) and the distances involved should be specified.

3.2 Life Cycle Inventory

Mass and energy balances are the core of the Life Cycle Inventory (LCI) and data collection is a key issue in LCA, on which will depend the accuracy of the results. LCI is in general a combination of generic information available in databases, especially for commodities (upstream of the process under study), waste treatment (downstream of the process under study) and transport, and information collected on specific sites (lab, pilot, or field/full scale).

In LCI lab-scale and pilot-scale processes are not optimized, and different technological choices may be made for the final industrial process, with different yields and recycling possibilities. These will influence the quality of the LCA results and should be considered during the sensitivity analysis.

LCA practitioners apply three main types of methods for LCI (Islam et al. 2016): process-based modelling, basic balances based on inputs and outputs to the system (IO LCI), and hybrid methods that combine to various degrees both previous approaches. LCI involving a full process-based model is very complex and time consuming, requiring mathematical expertise: in agromining such an approach will require a deep knowledge of all agricultural and chemical steps as well as those of ancillary processes (energy and reagents production, waste treatment). IO LCI is suitable for fast assessment, but its level of detail is low. The best solution is an integrated and hybrid approach, especially in the case of a new product or new technology development (Islam et al. 2016) such as agromining.

In LCI, not all flows are inventoried or characterized. Some flows are considered negligible, because they are consumed or produced in small volumes with respect to the main flows. However, it is not certain that fluxes below the cut-off value, i.e. the limit value under which the flows are not taken in account, will not ultimately have a major impact. In agromining, the mass flow of dust particles from biomass could be considered negligible and taken out of the inventory. But dust is a diffuse component of human toxicity (usually considered because of its concentration in heavy metals) and it would be better to evaluate dust contribution to final impacts before neglecting it (Lundie et al. 2007; Rodrigues et al. 2016).

It is important to define the localization of every process step as some impacts may depend upon it. Electrical energy is a good example of the importance of regionalization. Greenhouse gas emissions related to electricity generation depend upon the mode of production. Table 2 summarizes the emission factors for various sources of energy. Obviously, the emission factors for renewable energy (sun, wind, biomass, etc.) are much lower than those for fossil combustibles (coal, oil, natural gas) (Table 3). Therefore, depending on how the electricity is produced at the process location, the extent of greenhouse gas emissions can vary. In general, the electricity mix is country dependent but in some cases a company specific mix can be preferred, when it is largely different from the country mix (Gediga et al. 2015), or when the local distribution network is unreliable.

In practice, Fig. 5 summarizes the electricity mix applicable to different countries susceptible to developing agromining on ultramafic soils (World Bank 2016). However, these data do not take into account possible failure of the electricity grid. The loss index (World Bank 2016) is an indicator of the reliability of the electricity network. For example, it can be seen that if electricity in Albania is theoretically of hydroelectric origin (hence having very limited CO_2 emissions), the loss index is very high, and reliability of the network could be questionable. In such a case, another source of electricity, such as a portable generator running on diesel fuel,

Table 2 CO_2 emission factors per mode of electricity production

Nature	Emission factor ($kgCO_2$ $eq.kWh^{-1}$)
Hard coal	0.29 (France)
Brown coal	1.22 (Germany)
Crude oil	0.955 (France)
Natural gas (combined cycle)	0.57 (France)
Diesel (cogeneration)	0.69 (Switzerland)
Biogas (cogeneration)	0.03 (France)
Wood (cogeneration)	0.056 (France)
Hydroelectricity	0.0067 (French Alps)
Solar (ground solar panels, low voltage)	0.077 (France)
Wind (<1 MW, onshore)	0.014 (France)
Nuclear (pressurized water reactor)	0.013 (France)
Geothermal	0.075 (Germany)

Table 3 Some characterization factors for three categories of impacts

Warming potential (kg CO_2 eq 100 years)		Acidification (mol H^+ eq/kg)		Marine eutrophication (kg N eq/kg)	
CH_4	25	NH_3	3.02	NH_3	0.092 (air)
N_2O	298	NO_2	0.74	NH_3	0.824 (water)

Source EC-JRC (2011)

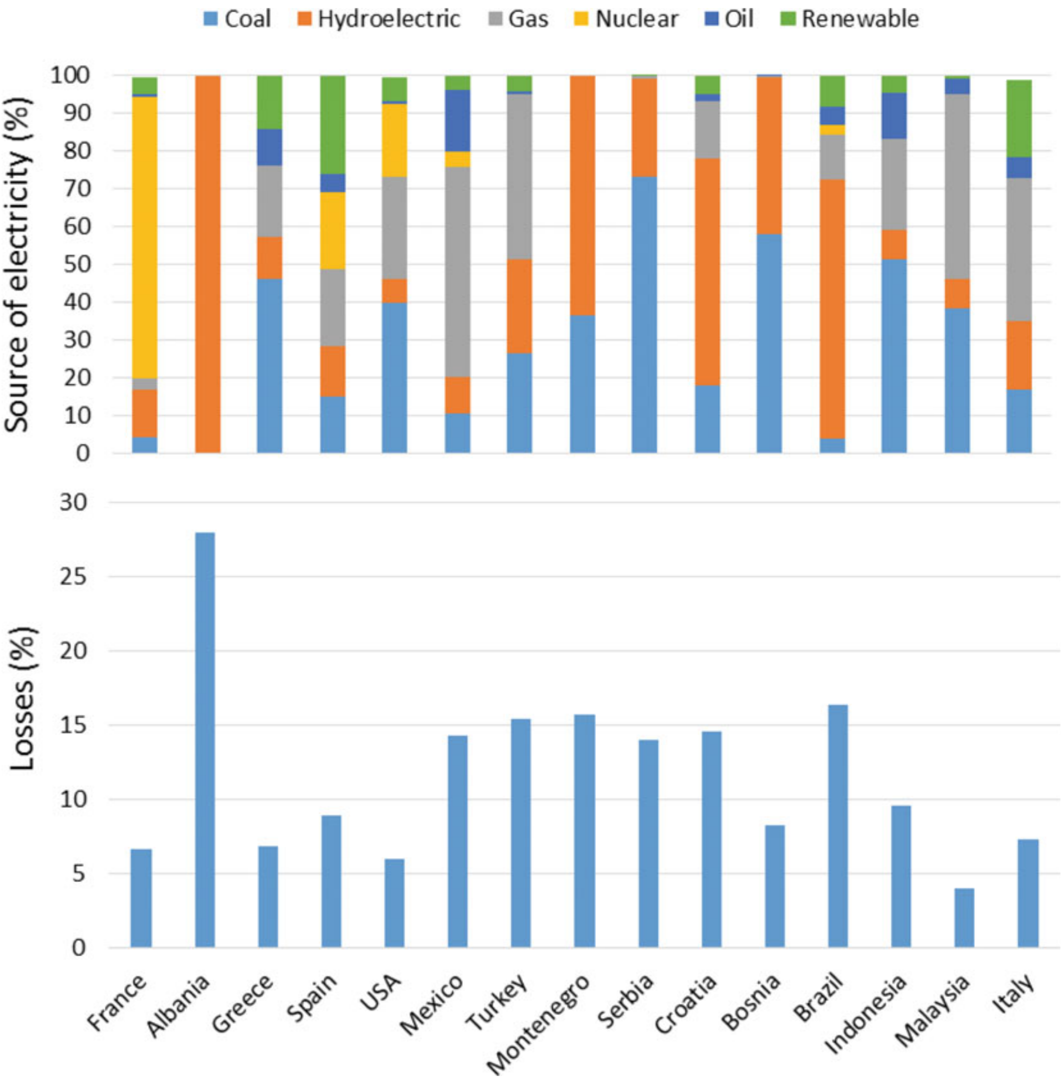

Fig. 5 Energy mix and electricity loss in the network for some countries susceptible to develop agromining on ultramafic soils. *Source of data* World Bank (2016)

would be used. This approach will increase locally the CO_2 emissions from electricity generation (Rodrigues et al. 2016).

4 Impact Assessment

In theory, the number of impacts that may be considered in LCA is not limited. The damages can be classified into areas of protection (depletion of natural resources, human health, and impact on ecosystems) and sub-categories, with for each, its relevant scale, an indicator and a model for characterization (Fig. 6). Three criteria are applied to select the most appropriate impacts for a given problem:

- Completeness: all environmental problems that can be linked to the goal to be achieved should be taken into account.
- Non-redundancy: the categories should not be redundant, and indicators should be independent of one another, in order to avoid double-counting.
- Validity: the environmental mechanism leading to each indicator should be clearly identified in its category. The characterization models should be accepted and validated by the scientific community.

All of the process flows are transformed into elementary flows (what is really taken from/emitted to the environment), such as CO_2 used for greenhouse gases or SO_2 for acidifying substances, owing to characterization factors.

Recommendations have been issued in the European context in 2011 to help identify the best practices for selecting the most relevant impacts and the most mature methods to evaluate them (EC-JRC 2011). In addition to problem-oriented methods aimed at midpoint impacts i.e. CML2002 (Guinée et al. 2002), EDIP (Hauschild and Potting 2005), TRACI (Bare 2002), or LUCAS (Toffoletto et al. 2007) and damage-oriented methods developed for the end-point areas of protection i.e. Eco-Indicator 99 (Goedkoop and Spriensma 2000), EPS2000 (Steen 1999a, b), or LIME (Itsubo et al. 2004), methods such as Impact 2002+ Jolliet et al. 2003), ReCiPe (Goedkoop et al. 2009), or Swiss Ecoscarcity 2013 (Frischknecht and Büsser Knöpfel 2013) cover the full spectrum of impacts. USETox exclusively deals with ecotoxicity and human toxicity (Westh et al. 2015).

Proper selection of the impact assessment methods remains a key issue in LCA because the results will depend upon the assumptions and models included in the methods. Up to now, the situation has been relatively clear for greenhouse gas emissions as the various methods are based on the IPCC recommendations. Since the 1990s, the 100-year Global Warming Potential (GWP100) has been used as the default emission metric. However, this simple metric does not encompass all subtleties or recent advances in climate science (Cherubini et al. 2016). Impacts

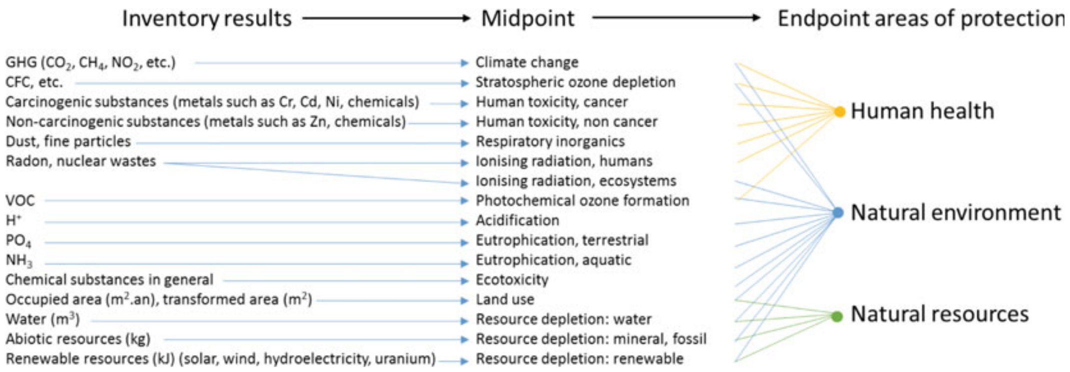

Fig. 6 Characterization framework linking the elementary flows from the LCI to indicator results at midpoint and end point. Adapted from Hauschild et al. (2013)

related to abiotic resource depletion (ADP), human toxicity, ecotoxicity, and land use are still subject to discussion (Renou et al. 2008; Owsianiak et al. 2013; Silva et al. 2019). These impacts are important for agromining, in its comparison with traditional mining of ores. Frischknecht et al. (2016) provided updated guidance for four impacts: global warming, fine particulate matter emissions, water use, and land use.

4.1 Abiotic Resource Depletion

The disputes related to ADP come primarily from definition of this impact. Some people argue that abiotic resource depletion is more an economic problem than an environmental one. However, ADP becomes an environmental problem when the energy demand required to extract abiotic resources from lower-grade ores or to recover them from recycled materials increases. Currently, the ADP of a given substance (Ni for example) is obtained by comparison with the depletion of a reference substance (Sb) and takes into account the element reserve, by the following formula:

$$ADP_{Ni} = \frac{DR_{Ni}/(R_{Ni})^2}{DR_{ref}/(R_{ref})^2}$$

where DR_{ref} is the extraction rate of the reference resource (here Sb), R_{ref} is the reserve of the reference resource, DR_{Ni} is the extraction rate of Ni, and R_{Ni} the reserve of Ni. But which type of reserve, ultimate or economic, should be considered? The ultimate reserve is estimated from the average element concentration in Earth's crust and the economic reserve is the part of the natural reserve base that can be economically extracted at the time of determination. The natural reserve base encompasses reserves that can effectively be extracted economically but also some for which extraction is only marginally economic or even sub-economic (van Oers and Guinée 2016). Finally, the extraction rate is time-dependent (van Oers et al. 2020): for Ni, ADP varied between 6.5×10^{-5} kg Sb eq/kg Ni in 1999 and 1.35×10^{-3} kg Sb eq/kg in 2015. Other means to assess the abiotic resources have been proposed, such as Cumulative Exergy Extraction from the Natural Environment or the ratio of industrial to ecological Cumulative Exergy Extraction (Alvarenga et al. 2016). Developments are on-going in order to clarify the different frameworks and to end with a shared vision of ADP (Drielsma et al. 2016).

4.2 Land Use (LU)

Impacts related to land use (LUI) consider land use changes (transformation of one land use to another due to human activity) and land occupation (use of a given land with a given activity). The land use changes (LUC) are further divided into either direct (dLUC) or indirect (iLUC) categories. Direct land use change refers, for example, to the creation of mine tailings deposits on natural lands or to agromining on natural lands. Indirect land use changes are induced when agromining is developed on a cropland or on forestland: new surfaces will be needed or yields would be improved (intensification by increased use of fertilization or irrigation) to compensate for the loss of actual production (food, wood). Land use changes can affect several other impact categories such as GreenHouse Gas (GHG) emissions and ecosystem health (Garraín et al. 2016).

LUIs are defined not only in terms of surface used for a given activity but also in terms of functions of the land, i.e. ecosystem services rendered by the land. A set of nine land use functions (LUF), divided into three categories, has been proposed by Pérez-Soba et al. (2008). These cover the following issues: (i) societal (provision of work, human health and recreations, and culture), (ii) economic (residential and land independent production, land-based production and transport), and (iii) environmental (provision of abiotic resources, support and provision of biotic resources, and maintenance of ecosystem processes). Anthropogenic land use affects supporting processes, including soil formation, primary production, photosynthesis, nutrient and water cycling, and regulating processes such as maintenance of biodiversity, pollination, water purification, waste treatment, and regulation of climate, erosion, air quality, water, diseases, and natural hazards (Taelman et al. 2016). The type of land cover is one parameter to consider, as well as its geographical position. The ecosystem services should not be considered individually: these form a 'cascade' (Potschin-Young et al. 2017).

In LCA, LUI is generally viewed as negative. In order to evaluate impacts related to land use change for natural land and compare different situations, Koellner et al. (2013) proposed an indicator related to several factors including duration of the damage, intensity of soil degradation, and extent of surface damage. As depicted in Fig. 7, in their model the land use change supposedly induces an immediate loss of quality, related to the land functions. During new land use, the quality of the ecosystem would remain constant, but at a level lower than that of the reference state. After the end of the land use, the ecosystem would return naturally and at a constant rate to its initial (reference) state. The reference state is defined as the natural land cover existing under the prevailing soil and climate conditions. The characterization factors are equivalent to the areas depicted in Fig. 6: the impact of soil transformation is proportional to the loss of quality relative to a reference state and to the duration of natural regeneration of the ecosystem.

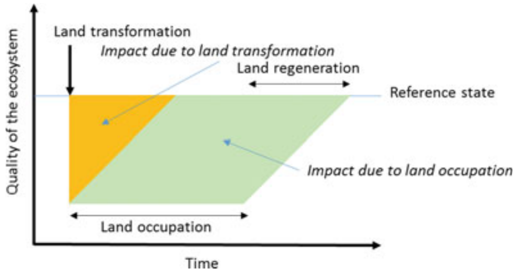

Fig. 7 Schematic representation of the evaluation of LUC according to Koellner et al. (2013)

The duration of natural regeneration of the ecosystem is the time needed for the ecosystem to recover properties analogous to those of the reference state. It can be very short (a few years) for the natural reforestation of old agricultural parcels, to centuries (for grasslands, swamps, etc.), or kiloyears (for peatlands, primary forest, tropical forest) in the case of severely disturbed sites (Koellner and Scholz 2007; Brandão and Milà I Canals 2013; Saad et al. 2013). Altitude and latitude are among the factors that influence the natural regeneration (Lindeijer et al. 1998; Saad et al. 2013).

Several indicators including emitted GHG, soil organic carbon (SOC) content, biodiversity, visual landscape quality (Schüpbach et al. 2020), etc., have been proposed to quantify LUI but there is no consensus yet on the most adequate metric (Perminova et al. 2016; Taelman et al. 2016). SOC promotes plant productivity and increases water retention. Depending upon the land management, SOC can increase or decrease during land use. It is also influenced by the type of soil as well as by climatic conditions. If LCA impacts are often calculated at the country level, this level of detail is not well adapted to LUI, because limits on ecosystems (characterized by soil type, climate conditions, etc.) do not always correspond to political boundaries (Saad et al. 2013). Morais et al. (2016) have calculated characterization factors for SOC depletion in Europe at three spatial scales: climate region, ecoregion (i.e. the main terrestrial natural regions as defined by Olson et al. (2001) and NUTS II regions), but a higher degree of scale might be needed for local projects. This is even more critical for large countries such as Brazil (Pavan and Ometto 2016). In Europe, ultramafic soils can be found in several biomes, each biome comprising a number of ecoregions: (i) 'Deserts and Xeric Shrublands' and 'Mediterranean Forests, Woodlands and Scrub' for the Balkans (Greece, Albania, etc.) and Italy; and (ii) 'Temperate Broadleaf and Mixed Forests' for Galicia (Spain), Austria, or Poland. The sequestration of carbon in soil also affects impacts on climate change due to delayed CO_2 emissions in the atmosphere: the time horizon depends upon the climate, from 20 years in a tropical climate to as much as 100 years in cold climates (Goglio et al. 2015).

Biodiversity is not easily measured by a single indicator. de Baan et al. (2013) have tested the relative change of species richness (SR), i.e. the ratio between the specific species richness in a natural reference and in the used land, for assessing LUI but recognized that this is a rough approximation, first of all because it records only the number of species and not the number of individuals per species. Furthermore, some species are more tolerant to pollution than others. Lindqvist et al. (2016) applied the method proposed by de Baan et al. (2013) to spruce monoculture in Sweden and emphasized the constraint of data availability and critical choice of the reference. Reflecting on the inability of SR to reflect all complexity of biodiversity, de Souza et al. (2013) have added functional diversity of three taxonomic groups (birds, mammals, and plants) based on functional traits such as leaf area, height, fruit type and length, foliage, growth form, etc. for plants. In their LUI assessment of the production of kiwifruit in New Zealand, Coelho and Michelsen (2014) preferred to combine naturalness and ecosystem scarcity and vulnerability, but these indices are also difficult to obtain, especially at a regional level. Moreover, there are large data gaps required for properly evaluating the effect of land use activities; more information on the regeneration time of ecosystems are especially needed. In the future, land surface phenological traits obtained by the analysis of remotely sensed data provided by satellite platforms may help to monitor spatial and time variations of vegetation biodiversity (Viña et al. 2016) and habitat fragmentation (Kuipers et al. 2019). The difficulties in assessing biodiversity are well recognized among decision makers, LCA practitioners, scientists, and conservationists: several authors have recently proposed a unifying approach (Lindner et al. 2019; Maier et al. 2019), making use of the naturalness concept.

If there are still many discussions related to LUI, the situation is not better for assessing LUC. de Rosa et al. (2016) have reviewed the different models, and have classified them as follows:

- Economic Equilibrium Models (EEMs), based on fluctuations between supply and demand until an equilibrium point is reached (Starr Ross 1997): modification of any activity (increase or decrease) requiring land use will induce LUC.
- Causal-Descriptive Models (CDMs), which forecast future supply and demand patterns based on the predicted trajectories for goods and agriculture supply/demand.
- Normative models, which are simpler than EEMs and CDMs, are based on statistics such as how much GHG are emitted and how much land is necessary, on average, to produce a given food product. A good example is given by Audsley et al. (2009) on the reduction of GHG emissions for the UK food system.
- Schmidt et al. (2015) have proposed a conceptual model to relate the impact of iLUC to an increase of GHG emissions based on land markets: this model includes effects related to deforestation and cultivation intensification. One soil property that can be modified with a new land use is its organic carbon content (Njakou Djomo et al. 2015; Bosco et al. 2016).

Land erosion, by rain or wind, is one of the ecosystemic regulating processes affected by land use. There are still large gaps in the modelling of soil erosion by wind: it depends on many factors such as climatic erosivity (resulting from the stress due to wind and weather conditions), soil erodibility (i.e. ability of a soil to be eroded), vegetation cover, and surface roughness (Borrelli et al. 2016). Importantly, however, recent research brings promises: land erosion could be incorporated in the near future for European studies (Borrelli et al. 2014). Loss of elements (heavy metals and phosphate) through erosion related to runoff on soils can be calculated using the Revised Universal Soil Loss Equation (Wischmeier and Smith 1978; Renard et al. 1997), as follows:

$$A_{soilloss} = R \cdot k \cdot LS \cdot C \cdot P$$

This equation calculates $A_{soil\ loss}$, the potential amount of soil lost through erosion, as the product of different factors describing the erosive force of rainfall (erosivity R), the type of soil (erodibility k), and field slope and length (LS), implemented cropping and anti-erosion practices (C and P). The last are further described by Panagos et al. (2015a, b) as the product of different parameters, characterizing for example land cover and tillage practices. As underlined by Rodrigues et al. (2016), it is important to be able to decrease land erosion during metal agromining in order to limit the dissemination of heavy metals into the aquatic environment. According to Nemecek et al. (2014), heavy metal emissions to groundwater due to infiltration of rainwater through the soil are assumed to be constant: the contribution is calculated with an allocation factor accounting for both direct anthropogenic inputs and atmospheric deposition.

In the case of agromining, the framework depicted by Koellner et al. (2013) is more complex. Metals that are naturally present in soils and can constitute a health risk, either by transfer to edible crops or to groundwater (Tang et al. 2012; Rivera et al. 2016) will be slowly removed (Harris et al. 2009). Therefore, the quality of the soil will be theoretically better after agromining and will never go back to its initial (i.e. reference) state (Fig. 8). When some decrease of soil quality can be foreseen during the initial crop installation, a global improvement will result from the decrease in metal concentrations. However, the question remains open in terms of biodiversity: it will be probably modified but it is difficult to say whether it will be improved or not.

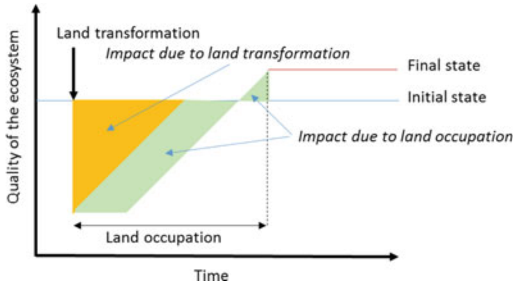

Fig. 8 Schematic representation of a possible scheme for LUC evaluation in case of agromining on ultramafic soils

In the case of phytomining of mine tailings, the reference state should be carefully selected, depending on the system frontiers, including:

- Either phytomining is limited to the treatment of tailings, without considering how the tailings were produced and in such case the framework will be similar to the case of agromining on ultramafic soils (Fig. 8);
- Or the frontiers encompass the whole mining implementation by which the reference state would be the pristine natural ecosystem (Fig. 7). As a consequence, it can be foreseen in such a case that the regeneration time will be very long.

4.3 Human Toxicity

The most recent method to calculate human toxicity impact in LCA is the USeTox method (Rosenbaum et al. 2008). Since then, USeTox has been continuously upgraded (Saouter et al. 2017). In USeTox, the characterization factor of a substance is the result of the matricial product of fate factors, exposition factors, and human toxicological effects. The case of metals should be considered carefully in phytomining and agromining processes, as their transfer from soils to plants is well-known. For this reason, it has been advocated to cultivate energy crops and not food crops on metal-contaminated agricultural land (Van Slycken et al. 2013; Pandey et al. 2015; Vigil et al. 2015). Depending upon the metal under concern, models used to assess their behaviour should take into account the metal speciation, in soil (Plouffe et al. 2015) as in water (Hedberg et al. 2019). Some metals have biologically significant chemical properties and a dose effect that should be considered. As oligo-elements, Co, Cr(III), Cu, Se, and Zn are among the nutritionally essential metals. Nickel has a possible beneficial effect (Goyer 2004) on human health but can be also very detrimental, although Ni-induced toxicity mechanisms are still not well established (Genchi et al. 2020). Because large uncertainties still exist concerning human toxicity to certain metals, a sensitivity analysis should be conducted to compare the outputs of different impact assessment methods (Pizzol et al. 2011).

The metal transfer chain might not be direct. *Odontarrhena chalcidica* is a Ni-hyperaccumulating plant that grows naturally in Albania and in northern Greece, and is a good candidate for agromining in those regions. It has been noticed that the yellow flowers of this plant attract a large number of honeybees: beekeepers have taken advantage of this observation to install beehives very close to or even within *O. chalcidica* fields. As honeybees are critical contributors to pollination, their presence is highly valuable in terms of ecosystems service provision, although a clear framework should still be defined for pollination impact assessment within LCA (Crenna et al. 2017; Arzoumanidis et al. 2019). Importantly, the question arises as to whether the Ni, which accumulates in the flowers (Zhang et al. 2014), can contaminate the honey. Although the honeybees use the flower nectar for honey production, they also collect pollen grains that can be incorporated into various beehive products and into the food chain.

5 Space and Time Dependence

The regionalization of impacts or of their source similar to the electricity mix is now a well-accepted idea in LCA (Verones et al. 2017). However, it takes time to build the relevant databases, especially for toxicity related emissions (Huijbregts et al. 2003), including for Europe (Sleeswijk et al. 2008), for Canada and USA (Lautier et al. 2010), and for Australia (Lundie et al. 2007), as well as for developing countries. The transfer of pollutants from one region to another, either by air (transport by wind) or water, should be better considered (Belyanovskaya et al. 2019). Freshwater ecotoxicity depends upon the chemistry of the local receiving waters (pH, suspended matter, dissolved organic carbon, etc.): if the characterization factor (CF) for Ni appears to be site-independent, this is not the case for all metals, such as Cr or Cu (Dong et al. 2014). In contrast, Ni terrestrial ecotoxicity is highly variable with respect to geography: its three orders-of-magnitude variability has been explained by pore-water concentrations of Mg^{2+} that compete with Ni^{2+} (Owsianiak et al. 2013). The IMPACT World+, proposed in 2019, is a globally regionalized impact assessment method that combines the latest developments in terms of regionalization (Bulle et al. 2019).

Another need for improvement in impact evaluation is considering time dependence, which is not yet taken into account. In the case of greenhouse gas emissions, the classical approach is to model the effect of GHG emissions occurring along the time horizon considered in a project as if these were taking place at the beginning of the time horizon. Such a time-distributed emission of CO_2 occurs in systems involving agriculture (including agromining) and forestry. Time-adjusted warming potentials have been proposed to solve this problem. By taking into account the year of emission, Kendall (2012) demonstrated that CO_2 emissions from a commercial building were significantly reduced (by 30%) for a 100-year horizon, but that the decrease became less significant when the time-horizon was increased. A similar dynamic LCA has been developed by Levasseur et al. (2010) in their comparison of GHG emissions (including those related to LUC) between fossil fuels and biofuels: under some conditions, use of corn ethanol was less favorable, compared to gasoline, when the dynamics of the emissions were taken into account compared to classical (static) LCA.

Factors influencing plant growth such as temperature, water availability, or CO_2 concentration in the atmosphere are expected to vary in the future owing to climate change, inducing possible decreases in yields (Ingvordsen et al. 2015; Niero et al. 2015). It is not straightforward to model the effect of climate change on any plant, but time-adjusted LU characterization factors could be developed in the future to better analyze the impact on land functions.

As the goal of agromining is to export toxic metals out of the soil, the soil metal content is expected to decrease over time. This is another aspect that should encourage the use of dynamic LCA for the assessment of agromining implementation (Shimako et al. 2017; Cardellini et al. 2018). However, the choice of the length of the time horizon used for the assessment is still a topic of dispute (Lueddeckens et al. 2020).

6 Conclusions

The concept of Life Cycle Assessment has been introduced as a tool for the environmental assessment of agromining, either on natural ultramafic lands or on mine tailings. LCA is a powerful tool that has been successfully used in studying various supply chains. In particular, in the case of site phytoremediation, this tool has highlighted that valorization of the produced biomass is crucial to reducing environmental impacts. Metrics related to land use and land use change need refinement in terms of selecting the most appropriate indicators of ecosystem services, of developing models to evaluate these indicators, and of access to regionalized and time-dependent databases required to feed these models. More recently, and more specifically to agromining, LCA demonstrates that ensuring soil erosion control could be a key advantage of agromining—plant growth ensuring soil cover and structure as opposed to leaving soils bare. Considering the potential toxicity of metal-rich soil particles, this feature is of crucial importance. However, the still-pioneering state of agromining yields many uncertainties. Co-product valorization, cropping practices, biodiversity integration, and local social and economic consequences of land used for agromining, remain unknowns that need to be dealt with. This chapter gives an overview of how these features can be assessed in LCA, as well as some current methodological issues and considerations that one should take into account while assessing agromining supply chains using this methodology. Nevertheless, even with the actual limitations, the LCA methodology gives to the developers of agromining solutions a framework for assessing the environmental sustainability of their projects. There is an incentive to better include ecosystem services in LCA (Liu et al. 2018), especially their benefits and values (Pavan and Ometto 2018): an integration of the ecosystem services cascade framework within LCA is highly desirable (Rugani et al. 2019). This approach would be very good for stressing the environmental sustainability of agromining. The final decision should also encompass economic and social impacts, which are not taken into account in LCA (Jiang et al. 2015; Cappuyns 2016).

Acknowledgements The authors acknowledge the financial support of Région Lorraine (AME) and EU (ERDF) through the LORVER project (www.lorver.org) and the French Agence Nationale de la Recherche (Agromine project ANR-14-CE04-0005). This research was also conducted as part of the EU LIFE-AGROMINE Project (LIFE15 ENV/FR/000512); we gratefully acknowledge this support.

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Exceptional Uptake and Accumulation of Chemical Elements in Plants: Extending the Hyperaccumulation Paradigm

Antony van der Ent, A. Joseph Pollard,
Guillaume Echevarria, Farida Abubakari,
Peter D. Erskine, Alan J. M. Baker,
and Roger D. Reeves

Abstract

Hyperaccumulation of elements in plants has been previously defined as an element-specific behaviour resulting in concentrations in foliar dry mass that are at least two orders of magnitude greater than the average concentrations of the same element in typical plants. The recognition of trace element hyperaccumulation of Ni, Co, Cu, Zn, Cd, Pb, Mn, Se, As and Tl has recently been clarified. In this review, instances of unusual accumulation of other elements in plants are assessed, including both common soil elements (such as Al and Si) and non-essential (ultra-)trace elements (such as Ba, Sr, Sb, W, Pd and rare earth elements). When sufficient data are available, threshold criteria for recognition of hyperaccumulation are proposed, but in other cases there is insufficient knowledge to make such a judgment. The physiological basis for hyperaccumulation is also discussed. Most of the elements described here are non-essential, with no known selective advantage for hyperaccumulation. It is therefore likely that these elements accumulate as an incidental side-effect of nutrient acquisition, either because they are chemical analogues of mineral nutrients, or because of shared uptake pathways.

Keywords

Contamination · Hyperaccumulator · Extreme accumulation · Ultra-trace elements · Phytoextraction · Soil elements

1 Introduction

The term 'hyperaccumulation', describing a highly unusual pattern of trace-element uptake and concentration in plant shoots, was first applied by Jaffré et al. (1976) in the title of their paper describing the concentration of Ni by the tree *Pycnandra* (formerly *Sebertia*) *acuminata* (Sapotaceae). A more precise definition of Ni hyperaccumulation was proposed by Reeves (1992, p. 261): "*a hyperaccumulator of Ni is a*

A. van der Ent (✉) · G. Echevarria · F. Abubakari ·
P. D. Erskine · A. J. M. Baker
Centre for Mined Land Rehabilitation, Sustainable
Minerals Institute, The University of Queensland,
Brisbane, QLD 4072, Australia
e-mail: a.vanderent@uq.edu.au

A. Joseph Pollard
Department of Biology, Furman University,
Greenville, SC, USA

A. van der Ent · G. Echevarria · A. J. M. Baker
Laboratoire Sols et Environnement, Université de
Lorraine – INRAE, UMR 1120,
Vandoeuvre-lès-Nancy, France

A. J. M. Baker
School of BioSciences, The University of
Melbourne, Melbourne, VIC 3010, Australia

R. D. Reeves
Palmerston North, New Zealand

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_6

plant in which a Ni concentration of at least 1000 μg g^{-1} has been recorded in the dry matter of any above-ground tissue in at least one specimen growing in its natural habitat." In attempting to broaden the definition of hyperaccumulation to elements other than Ni, van der Ent et al. (2013) proposed that the threshold criteria for hyperaccumulation be based on elemental concentrations (i) 2–3 orders of magnitude greater than in leaves of plants growing on uncontaminated soils, and (ii) at least one order of magnitude greater than the usual range in plants on metalliferous soils. These nominal thresholds provide a practical operational framework for recognizing hyperaccumulators, and when used sensibly, can guide identification of extreme physiological behaviour as well as allowing recognition of hyperaccumulators in the field without experimental cultivation (van der Ent et al. 2013). Based on this concept, nominal threshold criteria were proposed as 100 μg g^{-1} for Cd, Se and Tl, 300 μg g^{-1} for Co, Cr and Cu, 1000 μg g^{-1} for As, Ni and Pb, 3000 μg g^{-1} for Zn and 10 000 μg g^{-1} for Mn, as measured in dry leaf tissue of plants grown in their natural habitats. Approximately 700 taxa have been recorded as hyperaccumulators of one or more metals based on these criteria. Thus, hyperaccumulation as currently defined is a rare phenomenon known in little more than 0.2% of the world's inventory of vascular plants (Reeves 2003; Reeves et al. 2018).

The fate of trace elements in soil is dependent on various physical, mineralogical, chemical, and biological processes that control their speciation, retention, solubility, transport, and bioavailability (McBride 1989; Ross 1994). Plants are known to selectively accumulate or exclude certain elements, resulting in large differences between their concentrations in soil and biomass (Baker 1981). Excessive uptake has been described as 'inadvertent' (Boyd and Martens 1992) if it serves no adaptive function in the plant. Inadvertent uptake may result from particular conditions of soil pH and redox potential that regulate heavy-metal solubility and bioavailability (Wenzel et al. 1999), where protons compete at the exchange sites with metal cations and OH^{-} with oxyanions of metals and metalloids (Sposito 1995). Metal oxyanions (e.g. of Cr, V, Mo, U) are more soluble in acidic soils, whilst metalloids such as As and Se are more soluble in alkaline environments (Adriano 2001). The soil distribution coefficient K_d for a chemical species relates to its partition between soil and the water with which it is in contact, i.e. the soil/water concentration quotient for the species. Mobility of an element is also influenced by the K_d value, e.g. for Na and Cl (Sheppard 2011), and can lead to greater uptake of low-K_d elements compared to others having high K_d values. Hydrous oxides of Al, Fe and Mn are effective reservoirs of heavy metals, and their chemisorption depends on the degree of crystallinity, co-precipitation, surface precipitation, particle diffusion, and surface morphology (McBride 1989). Iron oxides are effective in immobilizing metalloids such as As, whilst Mn-oxides mediate the oxidation of various metals and metalloids, resulting either in decreased mobility (e.g. Co, Fe and As) (Oscarson et al. 1981; Dillard and Schenck 1986) or increased mobility (e.g. Cr) (McBride 1989). Metal carriers involved in forming soluble complexes such as citric, malic, oxalic, tartaric, and many other organic acids common in soil solution (Stevenson and Ardakani 1972) can influence the availability or solubility of elements such as Zn, Fe, Mn, Cu and P (Marschner et al. 1987), which are commonly retained in solution as anions or neutral species. This process results in greater uptake by the root at a given concentration of free ions (Minnich et al. 1987; McBride 1989), and the pH, redox status, cation exchange capacity, quantity and quality of organic matter, oxides, and clay minerals can largely influence the rhizospheric processes (McBride 1989; Adriano 2001).

Plants may also inadvertently take up elements via pathways with structural or chemical similarities to those used for the uptake of essential elements. The essentiality of certain micronutrients (e.g. Cu, Mn, Zn, Ni, Mo) for plant growth and development is well established, and plants suffer nutrient deficiency symptoms when the supply of the essential element(s) is insufficient (DalCorso et al. 2014).

In contrast, other elements such as Na, Si, Al and Co are not essential, but are known to be beneficial (Pilon-Smits et al. 2009). Higher concentrations of non-essential elements are needed by plants for osmotic roles, and low concentrations can act as enzymic co-factors for beneficial effects (Pilon-Smits et al. 2009). Many plants have the ability to accumulate high concentrations of metal ions in shoots, while maintaining a low concentration in roots (Krämer 2010). The ability to accumulate a particular element more than a chemically analogous one, when both are given at the same time and at equal concentration, could be due to higher mobility of one than the other or a result of deficiency of the first element that leads to inadvertent uptake. This effect has been observed with Se and S where exposure to high Se levels affects protein synthesis, causing symptoms that mimic S starvation, including chlorosis and stunted growth, together with prematurity, withering, and drying of leaves (Terry et al. 2000).

We have reviewed the current state of knowledge on hyperaccumulators of Mn, Ni, Co, Cu, Cr, Zn, Cd, Pb, As, Se and Tl (van der Ent et al. 2013), aiming to clarify the conditions appropriate for recognizing such plants. Hyperaccumulation in many cases concerns trace elements that are essential micro-nutrients, with the notable exceptions of As, Cd, Pb, Tl and the rare earth elements (REEs). Apart from the elements mentioned in our earlier review, the rest of the Periodic Table has received far less attention in the literature. An overview of the uptake of less-common elements by plants, with a focus on toxicity, has been presented by Babula et al. (2008).

This chapter attempts to outline and systematise the current state of knowledge regarding uncommon accumulations of elements that have previously received little attention, including abundant soil elements (Al and Si) and non-essential (ultra-)trace elements (such as Ba, Sr, Sb, W, Pd and the REEs). Where sufficient data exist, we propose threshold values to recognize the species that achieve hyperaccumulation of these elements, in a similar way to those surveyed by van der Ent et al. (2013). Major plant nutrients (N, P, K, S, Ca and Mg) are not considered here, because the regulation of these macronutrients is a common requirement for all plants. As there is a very extensive literature on Na-accumulating plants (halophytes), these are not considered further here.

2 Pitfalls and Caveats

Contamination of plant samples either by their substrate or extraneous materials commonly enriches the sample in certain elements that can lead to a false indication of hyperaccumulation. Many of the plants discussed in this paper show highly abnormal behaviour, some of which could be due to contamination of plant materials directly from soil or aerial fallout. This contamination is a strong possibility where the soil contains particles of nearly pure minerals of the metal being investigated, such as carbonates, silicates, or sulfates of Pb, Zn or Cu. Plants sampled adjacent to smelters could have metal vapours and very fine dusts deposited directly on them, producing contamination even more concentrated than that provided by dusts from the soil, and such sources of contaminants may pass easily into solution under the conditions used for plant analysis (Reeves 2006). Similar reservations apply to studies based on aerial deposition onto plants of sub-micrometre particles emitted in urban environments: there may be no strong evidence produced that the element studied has actually reached the upper plant parts via the soil and then the root system. This situation applied in former times to the deposition of lead from motor vehicles when the use of tetra-alkyl Pb compounds was common in motor fuels; it also applies to more recent studies of deposition of platinum group elements from catalytic converters in vehicle exhaust systems in urban environments. In such cases, the plant analysis may be useful simply as a pollution monitor regardless of whether the plant root system has been involved. One way of avoiding surface contamination is thorough washing of leaf samples, which has been discussed in detail elsewhere (Faucon et al. 2007; Reeves and

Kruckeberg 2018). The most certain way of confirming genuine accumulation involves growing plants in their natural soil in a glasshouse, climate room, or environmental chamber, avoiding airborne contamination and other uncontrolled irregularities of natural conditions in the field (van der Ent et al. 2013, 2015).

Criteria for hyperaccumulation are typically expressed as elemental concentrations in leaf dry mass (van der Ent et al. 2013). Confusion may arise through the tendency in the geochemical literature, especially for ultra-trace elements, to report elemental concentrations as a fraction of ash weight, which will always produce a much higher value because ashing reduces the plant mass by combustion of organic matter. Typically, dry wood of various trees has as little as 0.4–1.8% ash implying a conversion factor of 55–250, whereas the leaves of trees, shrubs, and grasses typically yield 2.5–15% ash, i.e. a conversion factor of 7–40. In our survey of elements, we have reported both ash- and dry-matter-based concentrations where both are given in the literature. Where the only available data are based on ash mass, we have used the approximation (based on analytical experience of the authors) that concentrations in ash will generally be 5–40 times higher than those based on dry mass, although this factor undoubtedly will vary from species to species.

Many of the elements reviewed here present challenges in application of the proposal that hyperaccumulation thresholds should be chosen to represent foliar concentrations at least two orders of magnitude greater than those occurring in plants on uncontaminated soil, or one order of magnitude greater than in most plants on metalliferous soil (van der Ent et al. 2013). Because ultra-trace elements are often neither essential nor toxic, there is little published literature to establish what concentrations occur in ‘normal’ plants. Thus, although there may be reports of unusually high accumulation, it may be problematic to establish the boundaries of hyperaccumulation. Below we attempt to point out cases where broader surveys are needed.

In describing exceptional uptake of elements, we have continued to use the well-established term ‘hyperaccumulator’. However, it should be understood that this single term is a broad umbrella to cover what must be a very wide range of interactions among various inorganic elements and phylogenetically diverse groups of plants. It would be a mistake to assume that all plants labelled as hyperaccumulators share common physiological mechanisms, ecological interactions, or evolutionary pathways. That was not true for the previously recognized hyperaccumulators (van der Ent et al. 2013), and it is probably even less true as the concept of hyperaccumulation is extended to common and ultra-trace elements. Although it is tempting to invent new terms to describe each situation, the truth is that most of these examples have not been studied thoroughly enough to justify a complex and potentially confusing array of new descriptors.

3 Instances of Unusual Accumulation Grouped by Elements

3.1 Alkali Elements

Lithium (Li), rubidium (Rb), and caesium (Cs) are alkali elements that have common characteristics of a single electron in the outermost energy level, resulting in highly reactive chemical behaviour. Although they are not considered essential nutrients in plants, these elements play important roles in cell physiology, including transport into and out of the cell (Ariño et al. 2010). The plant species *Tridax procumbens* (Asteraceae) accumulates up to 158 $\mu g\ g^{-1}$ Li in the Mangampeta barite mining area in Andhra Pradesh, India (Raghu 2001). More than 1800 $\mu g\ g^{-1}$ Li has been found in leaf tissues of *Apocynum venetum* (Apocynaceae) collected from sites in northern Xinjiang, China (Jiang et al. 2014). In six locations within the northern Mojave Desert in California, USA, the shrub *Lycium andersonii* (Solanaceae) has a mean Li concentration of 29 $\mu g\ g^{-1}$ and maximum of 166 $\mu g\ g^{-1}$ (Romney et al. 1977) (Table 1).

Accumulation of Rb with levels up to 98 and 44 $\mu g\ g^{-1}$ dry weight in *Medicago sativa* and

Table 1 Unusual accumulation of Alkali Elements (Li, Rb and Cs)

Foliar (µg g^{-1} DW)	Species (family)	Region of origin	Location of record	Life form	References
Li					
158	*Tridax procumbens* (**Compositae**)	Tropical America	Mangampeta barite mining area, India	Perennial herb	Raghu (2001)
>1800	*Apocynum venetum* (**Apocynaceae**)	Northern Asia and South East Europe	Northern Xinjiang, China	Perennial herb	Jiang et al. (2014)
166	*Lycium andersonii* **(Solanaceae)**	USA	South of Nevada Test site (Mojave Desert), USA	Deciduous shrub	Romney et al. (1977)
Rb					
98	*Medicago sativa* (**Fabaceae**)	Asia Minor, Transcaucasia, Iran and the higher Turkmenistan	–	Perennial herb	Kabata-Pendias and Pendias (1979)
44	*Trifolium repens* **(Fabaceae)**	North-western Africa, Europe and Central Asia	–	Perennial herb	Kabata-Pendias and Pendias (1979)
413	*Hoslundia opposita* (**Lamiaceae**)	sub-Saharan Africa	Gatumba Sn-Ta mining area, Rwanda	Perennial herb	Nieder et al. (2014)
Cs					
26	*Hoslundia opposita* (**Lamiaceae**)	sub-Saharan Africa	Gatumba Sn-Ta mining area, Rwanda	Perennial herb	Nieder et al. (2014)

Trifolium repens (Fabaceae), respectively, have been reported (Kabata-Pendias and Pendias 1979). The species *Agropyron repens* (Poaceae) and *Cirsium arvense* (Asteraceae) accumulate Rb when growing in polluted areas (Alda et al. 2015). *Hoslundia opposita* (Lamiaceae) accumulates up to 413 µg g^{-1} Rb and up to 26 µg g^{-1} Cs in the Gatumba Sn-Ta mining district in Rwanda (Nieder et al. 2014).

The alkali trace metals are chemical analogues to K. Lithium and K^+ share similar transport carriers, and Rb may partly substitute for K sites in plants owing to their similar properties, but cannot replace the metabolic roles of K, which makes Rb toxic to plants at high concentrations. Despite the chemical similarity of Rb to K, uptake and transportation of Rb within plants are reported to be different from those of K (Souty et al. 1975; Wallace 1970). With Cs, a similar distribution pattern to that of K suggests that this metal can compete with K uptake and K binding sites in cells (Isaure et al. 2006). Apart from the few studies cited above, little information has been published regarding the concentrations of Li, Rb and Cs in either typical plants or anomalous accumulators. It thus would be premature at this time to recognise criteria for hyperaccumulation of these elements.

3.2 Alkaline Earth Elements

Barium (Ba), beryllium (Be), strontium (Sr), and radium (Ra) share many chemical properties with Mg and Ca and are members of the alkaline earth chemical group. Barium and strontium are not essential to plants (Pais and Jones 1997), but both have the potential to influence plant growth directly or indirectly through competition with, and replacement of, essential elements such as Ca and Mg (Memon et al. 1983). In a survey of plants from eastern European forests, the mean Ba concentration in most plants ranged from 2 to 13 μg g^{-1}, with the exception of *Vaccinium cyanococcus* (Ericaceae) in which there have been reports of highly elevated levels of Ba (Markert and Vtorova 1995). A study of a barite-mineralized area near Kato Potamia, northern Greece, showed Ba concentrations of 180–760 μg g^{-1} in *Minuartia verna* and 57–325 μg g^{-1} in *Rumex acetosella* on soils with 4000–17 400 μg g^{-1} Ba (Reeves et al. 1986). *Miscanthus floridulus* (Poaceae) is reported to contain Ba up to 179 μg g^{-1} Ba where growing on uranium mill tailings in South China (Li et al. 2011). *Indigofera cordifolia* (Fabaceae) was found to accumulate Ba in its shoots up to 3550 μg g^{-1} in the Vemula barite area in Andhra Pradesh, India (Raghu 2001). The Brazil nut tree *Bertholletia excelsa* (Lecythidaceae) from South America was reported by Seaber (1933) to accumulate Ba in the nut at concentrations up to 4300 μg g^{-1}; but more recent measurements by Parekh et al. (2008) give the range of 96–1990 μg g^{-1} (Table 2).

The absorption of Sr is influenced by plants' metabolic requirements for Ca and is related to both the mechanisms of mass flow and exchange diffusion (Elgawhary et al. 1972), and hence Sr and Ca can compete with each other. However, Sr cannot replace Ca in its important biochemical functions. Typical concentrations of Sr in plants were reported in an older survey to range between 5 and 39 μg g^{-1} (Bowen and Dymond 1955). Unpublished analytical data amassed by one of the current authors (R.D. Reeves) includes more than 750 Sr concentrations measured in several hundred plant species growing on a variety of soil types (ultramafic, sulfide-rich, and non-metalliferous) in Turkey, Queensland, and New Zealand. The data approximate a lognormal distribution covering the concentration range 1–963 μg g^{-1}, with a median of 54 μg g^{-1}. The highest published Sr concentration found in a naturally growing plant is 17 650 μg g^{-1} in *Ulmus glabra* (Ulmaceae); up to 1762 μg g^{-1} Sr occurs in the leaves of *Arabis stricta* (Brassicaceae), both growing in strontium-rich soils (Bowen and Dymond 1955). *Parthenocissus quinquefolia* (Vitaceae) contains up to 190 μg g^{-1} Sr in its shoot where growing on uranium mill tailings (Li et al. 2011). Sr concentrations up to 8484 μg g^{-1} have been recorded in the roots of *Cassia angustifolia* (Caesalpiniaceae) in the Vemula barite area of Andhra Pradesh, India (Raghu 2001).

Small traces of radium (α-emitting ^{226}Ra derived from the decay of ^{238}U and β-emitting ^{228}Ra derived from ^{232}Th) have also been detected in Brazil nuts. These traces of radiogenic isotopes are still much higher than those found in most other plant materials, and the uptake is considered to parallel that of the high Ba accumulation observed for this species. Radium activities reported by various workers include, for ^{226}Ra: 1.8 pCi g^{-1} (Turner et al. 1958) and up to 6.6 pCi g^{-1} (Smith 1971), and for ^{228}Ra: 0.16–3.6 pCi g^{-1} (Penna-Franca et al. 1968). Parekh et al. (2008) found activities of each isotope in nuts from a range of sources to be in the range of 17–31 mBq g^{-1}, which is equivalent to 0.46–0.84 pCi g^{-1}; it was noted that the exposure from consumption of 28 g of nuts was still insignificant compared to the total doses of radiation received from all other sources.

Beryllium is easily absorbed by plants where it is mobile in soil. For Be^{2+}, mechanisms of absorption by plants seem to be similar to those involved in the uptake of major divalent cations Mg^{2+} and Ca^{2+}. However, these two elements reveal competitive interactions, whereas Be is known to substitute for Mg (Kabata-Pendias 2010). The mean concentrations of Be in plants vary widely from 0.001 to 59 μg g^{-1}, and toxic effects have been observed at soil concentrations of 10–50 μg g^{-1} (Kabata-Pendias 2010). However, an increase in Be levels in plants over baseline concentrations could be an indicator of pollution from industrial sources (Sarosiek et al. 1997). Beryllium in tissues of trees grown in a polluted region of Bohemia in the Czech Republic showed the highest concentrations in

Table 2 Unusual accumulation of Alkaline Earth Elements (Ba, Be, Sr)

Foliar ($\mu g\ g^{-1}$ DW)	Species (family)	Region of origin	Location of record	Life form	References
Sr					
17 650	*Ulmus glabra* **(Ulmaceae)**	Ireland to Urals	Se-rich soil, Regilbury, Somerset, UK	Tree	Bowen and Dymond (1955)
1762	*Arabis stricta* **Brassicaceae**	United Kingdom	Se-rich soil in Avon Gorge, UK	Perennial herb	Bowen and Dymond (1955)
477	*Carex humilis* **(Cyperaceae)**		Se-rich soil in Avon Gorge, UK	Sedge	Bowen and Dymond (1955)
8484	*Cassia angustifolia* (**Caesalpiniaceae**)	India and South China	Vemula barite mining area, Andhra Pradesh, India	Annual herb	Raghu (2001)
Ba					
2600	*Juglans nigra* **(Juglandaceae)**	North America	Ba-rich soil,Virginia, USA	Tree	Robinson et al. (1950)
1700	*Fraxinus pennsylvanica* (**Oleaceae**)	North America	Ba-rich soil,Virginia, USA	Tree	Robinson et al. (1950)
3550	*Indigofera cordifolia* (**Fabaceae**)	India	Vemula barite mining area, Andhra Pradesh, India	Annual herb	Raghu (2001)
Be					
310	*Picea mariana* **(Pinaceae)**	North America	Polluted region of Bohemia	Tree	Vesely et al. (2002)
230	*Fagus sylvatica* **(Fagaceae)**	Europe	Polluted region of Bohemia	Tree	Vesely et al. (2002)

Picea mariana (Pinaceae) needles (310 $\mu g\ g^{-1}$) and in *Fagus sylvatica* (Fagaceae) leaves (230 $\mu g\ g^{-1}$), whereas the concentrations in stem wood and bark varied within the range of 3–78 $\mu g\ g^{-1}$ (Vesely et al. 2002).

Uncertainties regarding typical or baseline concentrations of Ba, Sr, Be and Ra create difficulties in assigning hyperaccumulation thresholds. Based on the data above, threshold criteria of 1000 $\mu g\ g^{-1}$ for Ba and 3000 $\mu g\ g^{-1}$ for Sr are tentatively suggested. Data are insufficient at this time to evaluate hyperaccumulation criteria for Ra and Be.

3.3 Halogens

The halogens fluorine (F), bromine (Br), and iodine (I) can form very reactive chemical compounds. The ability of certain seaweeds, particularly in the Phaeophyceae (brown algae), to accumulate iodine has been known for many centuries. An extensive review of halide metabolism in the brown algae has been provided by La Barre et al. (2010). The remainder of this review will focus on true plants (Embryophytes) (Table 3).

Fluorine is considered non-essential to plants and the concentration of F in plants is typically <10 $\mu g\ g^{-1}$ (Davison et al. 1983). This value might suggest a hyperaccumulation threshold of 1000 $\mu g\ g^{-1}$; however, there are few known examples with concentrations that high. Fluoride is the most phytotoxic of the common air pollutants (Weinstein and Alscher-Herman 1982), and F pollution can result in fluorosis in cattle due to ingestion of contaminated plant material (Arnesen et al. 1995). Unusually high concentrations of F have been found in several plant species from different parts of the world,

Table 3 Unusual accumulation of Halogens (F, Br and I)

Foliar ($\mu g\ g^{-1}$ DW)	Species (family)	Region of origin	Location of record	Life form	References
F					
3060	*Camellia japonica* (**Theaceae**)	China, Korea and Japan	–	Tree/shrub	Zimmerman et al. (1957)
455	*Palicourea marcgravii* (**Rubiaceae**)	Brazil	Brazil	Shrub	Hall (1972)
5450	*Minuartia verna* (**Caryophyllaceae**)	Europe	Fluorspar mine waste, United Kingdom	Herb	Cooke et al. (1976)
175	*Gastrolobium bilobum* (**Fabaceae**)	Australia	Western Australia	Shrub	Hall (1972)
Br					
22 000	*Metasequoia glyptostroboides* (**Cupressaceae**)	Central and Western China	Polluted sites, Japan	Tree	Takada et al. (1993)
9200	*Pinus luchuensis* (**Pinaceae**)	Japan	Polluted sites, Japan	Tree	Takada et al. (1993)
2300	*Pinus densiflora* (**Pinaceae**)	China, Japan and Korea	Polluted sites, Japan	Tree	Takada et al. (1993)
I					
285	*Carex* sp. (**Cupressaceae**)	Canada	Iodine contaminated peat bog, Manitoba Canada	Sedge	Maillant et al. (2007)

including Africa, Australia, and Asia. Accumulation of F in the form of fluoroacetate has been recorded from the South African *Dichapetalum cymosum* (Dichapetalaceae) (Marais 1944) containing 60–235 $\mu g\ g^{-1}$ dry weight monofluoroacetate in its leaves (Peters 1960), and the seeds of the related *D. toxicarium* have been found to contain monofluorocarboxylic acids such as ω-fluorooleic and ω-fluoropalmitic (Peters et al. 1960; Ward et al. 1964; Hall 1972). *Acacia georginae* (Fabaceae) from Australia also contains F as fluoroacetate (Oelrichs and McEwan 1961; Peters and Shorthouse 1964), as does *Palicourea marcgravii* (Rubiaceae) from Brazil (De Oliveira 1963; Hall 1972). It has been stressed that many of these occurrences of high plant F have been found in plants growing on soils with fluoride concentrations that are normal or lower. The chemical analogy between F and Cl was observed in a study comparing the absorption of both elements by *Hordeum vulgare* (Poaceae). This research showed that F ion was markedly discriminated against in favour of Cl ion, with a 100-fold difference found in amounts of Cl and F absorbed in 3-h periods from solutions of initially equal F and Cl concentrations (Venkateswarlu et al. 1965).

Fluoride is present as fluoride ion in some plant tissues, including the leaves of ornamental camellias (*Camellia japonica*) (Theaceae), at 790–3060 $\mu g\ g^{-1}$ dry weight (Zimmerman et al. 1957), and in commercial tea (*C. sinensis*) at 140–300 $\mu g\ g^{-1}$ dry weight (Venkateswarlu et al. 1965). Several species of *Gastrolobium* (Fabaceae), such as *G. grandiflorum* from Australia, have F concentrations up to 120 $\mu g\ g^{-1}$ dry weight and also convert absorbed fluoride ion to fluoroacetate (McEwan 1964). These examples of F accumulation from soils of normal F status can be regarded as accumulation against a concentration gradient. This contrasts with the situation of plants colonising soils derived from fluorspar mine waste in England, as reported by Cooke et al. (1976). The substrates studied in this work, located in the Peak District (Derbyshire) and Weardale (Co. Durham), contained F at concentrations of 2.3–17.4 wt%, together with high concentrations of Pb and Zn. From six sites studied, and 38 plant species noted, 14 species (five grasses, eight forbs and one tree) were found with foliar F concentrations attaining 320–5450 $\mu g\ g^{-1}$, depending on the species. In this case, there was no evidence of fluoroacetate synthesis, nor was much of the F in simple ionic form. Instead, it was suggested that the F was mainly present as insoluble compounds with Ca, Mg, Al and/or silicates, which would also explain the apparent lack of phytotoxicity.

Bromine and iodine have similar chemical properties to chlorine but are not essential elements for plants (Yuita et al. 1982). The mean Br content of plants does not exceed about 50 $\mu g\ g^{-1}$; some higher values are related to pollution (Kabata-Pendias 2010). High concentrations of Br were also recorded in the needles of *Picea rubens* (Pinaceae) (up to 1120 $\mu g\ g^{-1}$) and *Abies fraseri* (Pinaceae) (up to 1300 $\mu g\ g^{-1}$) collected from the Blue Ridge Mountains in the southeastern United States (Robarge et al. 1989).

In a peat bog that was contaminated with I for one decade in the boreal forest of central Canada, *Carex* sp. accumulated more I than all other species by two orders of magnitude (Maillant et al. 2007). The concentrations in the leaves of this species ranged from 55 to 285 $\mu g\ g^{-1}$ I with a geometric mean of 125 $\mu g\ g^{-1}$. For all other species I concentrations in leaves did not exceed the 3.8 $\mu g\ g^{-1}$ found in an unknown species of willow (*Salix*). The predominant molecular species of I in the peat bog, and therefore the species that might have been taken up by plants, was believed to be iodide ion. Despite these anomalously high levels of Br and I, we are not aware of any records of species accumulating concentrations two orders of magnitude above typical baseline values.

3.4 Transition Metals

Tungsten (W), vanadium (V), and niobium (Nb) have different chemical properties but are considered together here because of their limited ionic mobility in the soil in a form that is available for plant uptake. Tungsten is not essential to plants, and normal levels are reported as 0.07 $\mu g\ g^{-1}$ in dry matter (Bowen 1966) or 0.5 $\mu g\ g^{-1}$ in ash (Brooks 1972). Therefore, 100 $\mu g\ g^{-1}$ on a dry weight basis might represent a conservative threshold for hyperaccumulation. The species *Eucalyptus melanophloia* (Myrtaceae) near an active wolframite mine in North Queensland, Australia, has been reported to contain high concentrations of W in its leaves (13.6 $\mu g\ g^{-1}$), young stems (2.9 $\mu g\ g^{-1}$), and old stems (4.3 $\mu g\ g^{-1}$) (Pyatt and Pyatt 2004). *Pinus sibirica* (Pinaceae) and a *Salix* sp. (Salicaceae) have been found to bioaccumulate W at concentrations up to 1500 $\mu g\ g^{-1}$ dry weight over W-rich fissures (Kletzin and Adams 1996). *Nothofagus menziesii* (Fagaceae) from New Zealand can accumulate up to 220 $\mu g\ g^{-1}$ in leaves (Quin et al. 1972; 1974), and *Leonurus cardiaca* (Lamiaceae) growing near a W-related industrial site accumulated 600 $\mu g\ g^{-1}$ in plant tissues (Kletzin and Adams 1996). WO_4^{2-} was found to substitute for MoO_4^{2-}, which leads to an over-expression of the nitrate reductase structural gene (Deng et al. 1989) (Table 4).

Vanadium is not essential to plants (Pais and Jones 1997). For most plants, typical V concentrations are reported to range from 12 to 30 $\mu g\ g^{-1}$ V ash weight (or approximately 1–3 $\mu g\ g^{-1}$ on a dry weight basis) (Cannon 1963). Based on this range, a hyperaccumulation threshold of 300 $\mu g\ g^{-1}$ might be appropriate; however, it is unclear whether any species attain this concentration. *Astragalus confertiflorus* (Fabaceae) from U-V deposits in the Colorado Plateau (USA) has been reported to accumulate up to 900 $\mu g\ g^{-1}$ V ash weight ($\sim$45–90 $\mu g\ g^{-1}$ dry weight) in the form of $Ca_3(VO_4)_2$ (Cannon 1963). *Astragalus* species have been noted to tolerate high amounts of V that they utilize in nitrogen fixation. It is believed that VO_4^{3-} replaces MoO_4^{2-} for nitrogen fixation in those species of *Astragalus* (e.g. *A. pattersonii*) (Fabaceae) from the same region where those species are known to accumulate MoO_4^{2-} (Cannon 1963). The ability of plants to tolerate high

Table 4 Unusual accumulation of Transition metals (W, V, Nb, Re, Tc and Hg)

Foliar ($\mu g\ g^{-1}$ DW)	Species (family)	Region of origin	Location of record	Life form	References
V					
90^{*}	*Astragalus confertiflorus* (**Fabaceae**)	USA	Colorado Plateau, USA	Herb	Cannon et al. (1968)
Nb					
10	*Rubus arcticus* (**Rosaceae**)	Poland	Nb mineralized area, Komi, Poland	Perennial herb	Kabata-Pendias and Pendias (1999)
W					
13.6	*Eucalyptus melanophloia* (**Myrtaceae**)	New South Wales and Queensland, Australia	Active wolframite mine, Queensland, Australia	Tree	Pyatt and Pyatt (2004)
1500	*Pinus sibirica* (**Pinaceae**)	USA	W-rich fissures, USA	Pine tree	Kletzin and Adams (1996)
220	*Nothofagus menziesii* (**Fagaceae**)	New Zealand	W-contaminated soils, New Zealand	Tree	Quin et al. (1972, 1974)
1500	*Salix* spp. (**Salicaceae**)_	USA	W-rich fissures, USA	Tree/shrub	Kletzin and Adams (1996)
Re					
30^{*}	*Astragalus pattersonii* (**Fabaceae**)	North America	Se deposits on the Colorado Plateau, USA	Perennial herb	Myers and Hamilton (1960)
Hg					
1278	*Polypogon monspeliensis* (**Poaceae**)	Southern Europe	Almaden mining district, Spain	Annual grass	Molina et al. (2006)
30	*Betula papyrifera* (**Betulaceae**)	North America	Hg metallogenetic province in British Columbia, Canada	Tree	Warren et al. (1966)
14	*Cytisus scoparius* (**Fabaceae**)	Western and central Europe	Active Hg mining and smelting site on Mount Amiata, Italy	Perennial shrub	Siegel et al. (1987)

* converted from original ash value

contents of V in soils is probably due to partial exclusion and to immobilization of the element as an insoluble product in the root (Lepp 1981).

Vanadium occurs as vanadate ion (VO_4^{3-}) in Ca, Mg, Cu, Zn, Pb, U and ferric Fe compounds, and can replace Mo as a specific catalyst for N fixation (Bortels 1936, 1937; Burk and Horner 1935). In the search for U-V deposits on the Colorado Plateau, Cannon (1963) observed two species of *Astragalus* (*A. pattersonii* and *A. preussii*) taking up high concentrations of VO_4^{3-} instead of MoO_4^{2-}. However, the concentration of VO_4^{3-} in *A. pattersonii* was higher than in *A. preussii* because it contains nearly twice as much Ca. This result suggested that the V in *A. pattersonii* may precipitate in the root as $Ca(VO_3)_2$, whereas the solubility and mobility of the Mo as $CaMoO_4$ may increase (Cannon 1963).

Tyutina et al. (1959) found that the perennial herb *Rubus arcticus* (Rosaceae) accumulates as much as 10 μg g^{-1} Nb dry weight from a Nb-mineralized area of Komi, Russia. More than half of the species studied had less than 0.6 μg g^{-1} Nb but several showed elevated levels up to 10 μg g^{-1}. Other studies have reported very poor solubility of Nb in soils and subsequent low uptake by plants, although up to 8.4 μg g^{-1} Nb has been reported in an unidentified species (Echevarria et al. 2005). Apparently, low accumulation of Nb by plants is related to the low solubility of this element in soils, i.e. Nb is mostly present as $Nb(OH)_5$, which is insoluble at most soil pH values (Echevarria et al. 2005). Most crops are reported to have Nb concentrations of a few ng g^{-1} in leaves, fruits, or seeds (Sheppard et al. 2010). Data are currently insufficient to recognise hyperaccumulators of this element.

Rhenium (Re) and technetium (Tc) are two elements that form oxyanions in oxidised environments (Echevarria et al. 1997). Rhenium is most commonly associated with Mo minerals, but is not essential to plants (Kabata-Pendias 2010). It is mobile in soil as perrhenate (ReO_4^-), which is readily accumulated in green leaves and plants in amounts that can exceed many times its sub-ng g^{-1} occurrence in Earth's crust (Kalinin et al. 1986). Although a normal concentration of Re in plant ash is stated to be about 0.005 μg g^{-1} (Brooks 1972), attention was drawn by Myers and Hamilton (1960, 1961) to unusual Re accumulation by some plant species from soils rich in U and Mo in Utah, Colorado, and New Mexico. From a deposit in Utah containing schroeckingerite (a Na–Ca-uranyl carbonate-sulfate-fluoride mineral) they found 300 μg g^{-1} Re in the ash of *Atriplex confertifolia* (Chenopodiaceae), and up to 150 μg g^{-1} in *Astragalus preussii* (Fabaceae), *Ephedra viridis* (Ephedraceae), and *Grindelia fastigiata* (Asteraceae). From a site in Colorado they reported up to 300 μg g^{-1} Re in *Astragalus pattersonii* and 150 μg g^{-1} in *Eriogonum inflatum* (Polygonaceae). A sample of *Oenothera caespitosa* (Onagraceae) from New Mexico also had 150 μg g^{-1} Re. Accumulation of Re by leaves of an unnamed *Acacia* sp. (Mimosaceae) growing close to a copper concentrate from a mine in Bulgaria has also been reported (Bozhkov et al. 2008). Rhenium concentrations in the ash of four specimens from various locations were 0.59, 8.76, 13.2, and 1686 μg g^{-1}, from which it can be inferred that the concentrations measured on a dry-matter basis might have spanned a broad range from 0.03 to 168 μg g^{-1}. The 'cementation copper concentrate' itself contained 135 μg g^{-1} Re.

Technetium, which is chemically similar to Re, has no stable isotopes; the longest-lived (half-life 211,000 yr) is ^{99}Tc, a radionuclide produced from the spontaneous fission of uranium. The predominant form of Tc in oxidised environments is pertechnetate, i.e. TcO_4^- (Echevarria et al. 1997), which is extremely mobile and can be taken up instead of NO_3^- by roots with soil-to-plant concentration ratios that can reach 371 (Echevarria et al. 1998, 2003). It was clearly shown that the presence of NO_3^- inhibits pertechnetate uptake by plants, and that only full depletion of soil nitrate ions allows substantial—and almost total—uptake of all TcO_4^- ions present in the soil by roots (Echevarria et al. 1998).

These cases of Re and Tc raise many questions because there does not seem to be any genetic specificity of Re and Tc hyperaccumulation in all plant species tested to date (from different families). The Re accumulation ability in nature depends on Re concentration in the soil (Bozhkov et al. 2008). It is extremely likely that the same mechanism that allows the extraordinary uptake of pertechnetate by plants (Echevarria et al. 1998) also allows the extremely high perrhenate uptake, i.e. inadvertent uptake of ReO_4^- instead of NO_3^-. As for Tc, it is the only explanation for non-specific (genetic) adaptation, and it would explain the extremely high concentration ratios observed for both elements. If this mechanism is confirmed, it could mean that Re phytoextraction is possible by any type of crop provided that nitrogen supply (i.e. nitrate concentration levels in soils) is managed properly. On the other hand, it seems inappropriate to designate any taxon as a hyperaccumulator if high foliar concentrations are driven primarily by environmental factors.

Mercury (Hg) is a non-essential element and can be toxic to plants at high tissue concentrations (Shah et al. 2010). The accumulation of Hg in terrestrial plants has been attributed to both the concentration of the element in the soil and in the plant species (Crowder 1991). The concentration of Hg in plants has been reported to be 0.001 µg g^{-1} (Malyuga 1964). If this is valid and representative, then a conservative threshold for Hg hyperaccumulation might be 1 µg g^{-1}. The annual grass *Polypogon monspeliensis* (Poaceae) was found to accumulate Hg in the roots, stems, and leaves with levels up to 1095 µg g^{-1}, 710 µg g^{-1} and 1278 µg g^{-1}, respectively, in the Almaden Hg mining district in Spain (Molina et al. 2006). In an active Hg mining and smelting site on Mount Amiata (Siegel et al. 1987), *Equisetum arvense* (Equisetaceae) contains up to 5.5 µg g^{-1} Hg, with up to 8.8 µg g^{-1} Hg present in *Pinus nigra* (Pinaceae) and 14 µg g^{-1} Hg dry weight in *Cytisus scoparius* (Fabaceae). *Betula papyrifera* (Betulaceae) accumulates up to 30 µg g^{-1} Hg in a Hg metallogenetic area in British Columbia (Warren et al. 1966).

3.5 Post-transition Metals

Aluminium (Al) is the third most abundant element in Earth's crust, constituting over 8% (Jones and Bennett 1986). Aluminium is not essential and is generally present in higher plants in the range of 100–1000 µg g^{-1} (Kabata-Pendias 2010). In acidic soil, the soluble forms of Al, $Al(OH)^{2+}$ and $Al(OH)_2^+$, are the most phytotoxic in most plants, with toxicity depending on the speciation, concentration, and ionic strength of the solution (Blamey et al. 1991). In contrast to most elements considered in this review, there is an abundant literature on the interactions of plants and Al. Plants have been categorized according to their sensitivity or resistance to Al toxicity: plants with low resistance survive only in soils having low Al contents, whereas those with moderate or high resistance are able to grow in soils having higher concentrations of Al (Jansen et al. 2002a). Aluminium toxicity can hinder root elongation and thus interfere with nutrient and water uptake in plants (Bulanova et al. 2001; Mohanty et al. 2004). However, evidence of a physiological function comes from the observation that low levels of Al can have a beneficial effect on plant growth, especially in Al-tolerant plant species (Clark 1977; Foy et al. 1978) (Table 5).

Aluminium accumulation is rather common among plants from numerous phylogenetic lineages around the world (Chenery 1955; Chenery and Sporne 1976; Jansen et al. 2000, 2002b), and the ability to accumulate Al is a competitive advantage for plants growing on soils with high Al availability (Haridasan 1988). High Al concentrations in the tissues of some plants were recognised over 300 years ago, as extracts of *Symplocos* spp. (Symplocaceae) and of *Memecylon edule* (Melastomataceae) were used in India and Southeast Asia instead of alum as a mordant for fabric dyes, as noted by Hutchinson (1943).

Extreme Al accumulators in the genus *Symplocos* are listed by Schmitt et al. (2016). These include the following species with reported Al concentrations >10 000 µg g^{-1}: *S. spicata* (72 300; Faber 1925), *S. myrtacea* (35 400; Chenery 1948), *S. crassipes* (33 880; Metali 2010), *S. microcalyx* (23 050; Watanabe et al. 2007), *S. coreana* (14 770, Watanabe et al. 2007), *S. odoratissima* (23 380, Schmitt et al. 2016) and *S. ophirensis* (21 350; Schmitt et al. 2016). Up to 50 000 µg g^{-1} was also found in *S. tinctoria* (syn. *S. tinctoria* var. *tinctoria*) (Hutchinson 1945). Lower contents of Al, in the range 1000–10 000 µg g^{-1}, were recorded in other *Symplocos* species (Schmitt et al. 2016).

Although high Al concentrations are common in the Symplocaceae, it is a relatively small family having only about 260 species (Christenhusz and Byng 2016). The greatest numbers of Al accumulators have been found in the much larger families Rubiaceae (Jansen et al. 2000,

Table 5 Unusual accumulation of Post-transition metals (Al, Bi and Sn)

Foliar (μg g^{-1} DW)	Species (family)	Region of origin	Location of record	Life form	References
Al					
72 300	*Symplocos spicata* (syn. *S. cochinchinensis*) (**Symplocaceae**)	Southeast Asia to Australia	Java, Indonesia	Tree	Faber (1925)
35 400	*Symplocos myrtacea* (**Symplocaceae**)	Japan	–	Tree	Chenery (1948)
33 883	*Symplocos crassipes* (**Symplocaceae**)	Peninsular Thailand and Malaysia into Borneo		Shrub	Metali (2010)
24 180	*Symplocos odoratissima* (**Symplocaceae**)	Indonesia	Central Sulawesi, Indonesia	Tree	Schmitt et al. (2016)
66 100	*Miconia acinodendron* (**Melastomataceae**)	Colombia to Martinique and Brazil		Tree	Chenery (1948)
18 000	*Faramea marginata* (**Rubiaceae**)	South and North America	Swamp forest in the llha do Mel, Brazil	Tree	Britez et al. (2002)
32 100	*Faramea axillari* (**Rubiaceae**)	South and North America	–	Tree	Chenery (1948)
36 800	*Rudgea justicioides* (**Rubiaceae**)	Brazil	–	Shrub	Chenery (1948)
15 400	*Psychotria brachiata* (**Rubiaceae**)	West Indies, Trinidad and Central America	–	Shrub	Chenery (1948)
36 202	*Craterispermum laurinum* (**Rubiaceae**)	Tropical Africa	10 miles north of Monrovia, Liberia	Shrub	Jansen et al. (2003)
10 535	*Coptosapelta olaciformis* (**Rubiaceae**)	South East Asia	Surigao, Philippines	Shrub	Jansen et al. (2003)
10 357	*Coccocypselum canescens* (**Rubiaceae**)	Mexico, Central America, West Indies and South America	Kappesberg, Montenegro, Brazil	Shrub	Jansen et al. (2003)
40 000	*Faramea insignis* (**Rubiaceae**)	South America	–	Shrub	Chenery (1946)
36 900	*Faramea anisocalyx* (**Rubiaceae**)	South tropical America	–	Shrub	Chenery (1946)
>30 000	*Camellia sinensis* (**Theaceae**)	Japan	Kyoto University farmland, Japan	Shrub	Matsumoto et al. (1976)
Bi					
2.9	*Buddleja davidii* (**Scrophulariaceae**)	China	Xikuangshan mining and smelting area, Hunan province, China	Shrub	Wei et al. (2011)

(continued)

Table 5 (continued)

Foliar (µg g^{-1} DW)	Species (family)	Region of origin	Location of record	Life form	References
Sn					
1990	*Cyperus rotundus* (**Cyperaceae**)	Africa, Southern and Central Europe	Sn mining catchment Bestari Jaya, Malaysia	Perennial plant	Ashraf et al. (2011)
704	*Festuca pratensis* (**Poaceae**)	Eurasia	Metallurgic industrial area of Târgovişte city, Romania	Perennial grass	Elekes et al. (2010)
631	*Cynodon dactylon* (**Poaceae**)	sub-Saharan Africa	Metallurgic industrial area of Târgovişte city, Romania	Grass	Elekes et al. (2010)
310	*Phragmites australis* (**Poaceae**)	Cosmopolitan	Sn mining catchment Bestari Jaya, Malaysia	Perennial grass	Ashraf et al. (2011)

2003) and Melastomataceae (Jansen et al. 2002b). Chenery (1946) reported 40 000 and 36 900 µg g^{-1} Al in the leaves of *Faramea insignis* and *F. anisocalyx* (Rubiaceae), respectively. Further studies of *Faramea* in Brazil (Britez et al. 2002) showed that 30 samples of *F. marginata* (syn. *F. latifolia*) accumulated 13 000–23 000 µg g^{-1} Al with a mean of 18 000 µg g^{-1}. *F. axillaris*, *Psychotria brachiata,* and *Rudgea justicioides* (Rubiaceae) were recorded with Al concentrations up to 32 100, 15 400 and 36 800 µg g^{-1}, respectively (Chenery 1948). The last of these species has recently been transferred to *Palicourea* as *P. parajusticioides* (Taylor et al. 2015). *Rudgea viburnoides* was shown to possess foliar Al concentrations exceeding 15 000 µg g^{-1} in the Brazilian Cerrado (Malta et al. 2016). Leaves of *Craterispermum laurinum*, *Coccocypselum canescens*, and *Coptosapelta olaciformis* (Rubiaceae) accumulate up to 36 200 µg g^{-1}, 10 360 µg g^{-1} and 10 540 µg g^{-1} Al, respectively (Jansen et al. 2003).

In the Melastomataceae, Chenery (1948) found up to 66 100 µg g^{-1} in the leaves of *Miconia acinodendron.* Jansen et al. (2002b) carried out semiquantitative colorimetric (aluminon reagent) tests on 166 species in the Melastomataceae, finding evidence of Al accumulation (>1000 µg g^{-1}) in nearly half of these. They also summarized similar results from earlier workers and quoted examples of plants in this family with quantitatively measured Al in the range 4300–66 100 µg g^{-1}. Among data from one of the present authors (RD Reeves, unpublished) on plants from relatively low-Al ultramafic environments in Goiás State, Brazil, the local ultramafic-endemic *Microlicia macedoi* (Melastomataceae) stands out, with Al contents in the range 5790–8260 µg g^{-1}. Specimens of other members of the Melastomataceae (*Pterolepis haplostemona, Pt. trichotoma*, and *Tibouchina* spp.) from the same area also had high Al concentrations, in the range of 1500–4060 µg g^{-1}.

Since the ability of the economically important tea bush, *Camellia sinensis* (Theaceae) to accumulate Al was noted by Bertrand and Levy (1931), this species has been the subject of many studies (see, for example, Sivasubramaniam and Talibudeen 1971; Konishi 1992; Carr et al. 2003). Under natural conditions, it can accumulate up to at least 30 000 µg g^{-1} in the epidermal cells of mature leaves (Matsumoto et al. 1976). The interaction between Al and fluoride is of special interest, as this plant is known to accumulate both elements, involving Al-F complex formation (Yang et al. 2016).

Although many of the extreme records of Al accumulation are found in the families Symplocaceae, Rubiaceae, and Melastomataceae, values >1000 μg g^{-1} have been reported in species from at least 52 families (Jansen et al. 2002b). Aluminium accumulators as defined by Hutchinson (1945) and Chenery (1948) are plants capable of concentrating >1000 μg g^{-1} of Al in their leaves. Justification for using this threshold can be found in the study of Metali et al. (2012) in which the distribution of reported Al concentrations in a variety of floras, and in a tropical collection of their own, was reviewed. A bimodal distribution of the global dataset (1044 species) was identified, with a statistically determined separation occurring at *ca.* 1280 μg g^{-1}. Separate consideration of temperate ($n = 602$) and tropical ($n = 442$) species showed Al thresholds at 1050 and 2680 μg g^{-1}, respectively.

In the extensive literature on anomalously high Al concentrations in plants, various authors have used the terms accumulation and hyperaccumulation interchangeably. We concur with Jansen et al. (2002a) that for clarity and consistency with other elements, plants having Al concentrations >1000 μg g^{-1} should be designated as hyperaccumulators. However, based on this criterion, Al hyperaccumulators can be considered relatively common (almost 25% of the species surveyed by Metali et al. 2012), contrasting sharply with the estimate that approximately 0.2% of all vascular plants hyperaccumulate heavy (transition) metals and metalloids (Reeves et al. 2018).

Tin (Sn), like Hg, is non-essential and potentially toxic. Brooks (1972) gives a typical normal soil concentration of 10 μg g^{-1} Sn and a concentration in plant ash of 1 μg g^{-1}, which corresponds to a dry weight Sn concentration of *ca.* 0.1–0.2 μg g^{-1}. This range is similar to values reported for standard plant materials such as Bowen's kale and NBS orchard leaves, which are in the range 0.16–0.3 μg g^{-1} (Peterson and Girling 1981). Higher concentrations have been observed in a variety of plant species growing on soils abnormally enriched in Sn, particularly where the Sn is in easily available forms, such as the cations Sn^{2+} and Sn^{4+} (Romney et al. 1975). On soils with Sn concentrations above 50 μg g^{-1}, several plant species were found with 12–84 μg g^{-1} Sn on an ash weight basis (average 46 μg g^{-1}), corresponding to *ca.* 5–10 μg g^{-1} dry weight (Sarosiek and Klys 1962). A similar Sn concentration, up to 6.9 μg g^{-1} dry weight, was found by Peterson et al. (1979) in leaves of the mangrove *Rhizophora conjugata* (Rhizophoraceae) at sites close to Sn smelters and industrial pollution in West Malaysia; this concentration was regarded as being near the top end of the range for normal plant uptake. Somewhat higher concentrations, up to 32 μg g^{-1}, were reported by Peterson et al. (1976) in the fern *Gleichenia linearis*, also from a Malaysian Sn mining area. More recent work (Ashraf et al. 2011) on metal-contaminated former Sn mining sites of Bestari Jaya, Malaysia, has shown Sn concentrations of up to 1990 μg g^{-1} in *Cyperus rotundus* (Cyperaceae) and 310 μg g^{-1} in *Phragmites australis* (Poaceae). Average Sn concentrations in leaves of nine dominant species were 110–670 μg g^{-1}.

Kovalevsky (1987) noted Sn accumulation by several species of sedge (*Carex* spp.), as well as by *Artemisia stolonifera* and *Abies nephrolepis*, growing over Sn orebodies. In the last of these, the ash-weight concentration of Sn reached 3000 μg g^{-1}, compared to a background of 10 μg g^{-1}, perhaps equivalent to dry weight concentrations of 300 and 1 μg g^{-1}, respectively. Up to 704 μg g^{-1} has been recorded in *Festuca pratensis* (Poaceae) and up to 631 μg g^{-1} in *Cynodon dactylon* (Poaceae) grown in the metallurgical industrial area of Târgovişte city in Romania (Elekes et al. 2010). These data lead to our tentative suggestion that 300 μg g^{-1} could be regarded as a threshold for Sn hyperaccumulation in plants.

Bismuth (Bi) is not essential to plants and the Bi content of plants has not been studied extensively. Brooks (1972) gave a typical concentration in plant ash as 1.2 μg g^{-1}, implying a dry weight concentration of *ca.* 0.1 μg g^{-1}. The species *Buddleja davidii* (Scrophulariaceae) was reported to accumulate Bi up to 2.9 μg g^{-1} at the site of the Xikuangshan Sb mining and smelting area in Hunan province in China (Wei et al. 2011).

3.6 Metalloids

Boron (B) is the only element in this survey that is generally regarded as essential for plant growth and survival. Boron is a non-metal and its functions interrelate with those of N, P, K and Ca in plants (Rasheed 2009). Plants take up B in the form of boric acid or borate (BO_3^{3-}) (Goldbach 1997); B is normally present in plant leaf tissues at concentrations of 10 to 50 µg g^{-1} (Aydın and Çakır 2009). Some species show severe toxicity symptoms at tissue levels of *ca.* 50 µg g^{-1} and such concentrations can be found in tissues where the available soil B exceeds 3 µg g^{-1} (Hakki et al. 2007) (Table 6).

Much of the literature on B in plants relates to crop plants or to a very limited variety of species growing in a particular soil environment. Extensive data acquired by one of the present authors (R.D. Reeves), covering more than 1600 analyses and more than 500 species from a variety of geological environments in ten countries, show a rather wider range of plant B concentrations. The plants were from a variety of soils (ultramafic, sulfide-rich, and non-metalliferous) and lacked abnormal soil B concentrations. Plant B ranged from 1 to 667 µg g^{-1} with a median of 43 µg g^{-1}. In no case was there evidence of adverse plant health, even in those samples having more than 100 µg g^{-1} B in the above-ground dry matter. It is noteworthy that B levels in monocotyledons tended to be lower than in dicotyledons: all 100 specimens from the Poaceae and Cyperaceae had B concentrations below 16 µg g^{-1} and most were <10 µg g^{-1}. On the other hand, none of the dicotyledonous specimens had B concentrations below 10 µg g^{-1}. Among the species with consistently higher B concentrations were *Thymus sibthorpii* (Lamiaceae) and *Rumex acetosella* (Polygonacae) from a variety of (mainly sulfide) mineralized soil sites in northern Greece (Reeves et al. 1986). Although the soils had unremarkable total B concentrations (6–93 µg g^{-1}; median 26 µg g^{-1}), various populations of these species were found with median foliar B concentrations of 72–271 µg g^{-1} (R.D. Reeves, unpublished data). These observations indicate that plant B concentrations that can be regarded as 'normal' should perhaps be extended to cover the range of about 1 to as much as 400 µg g^{-1}.

Studies of areas with strongly B-enriched soils have shown much higher B concentrations in some of the B-tolerant plant species. The grass *Puccinellia frigida* (Poaceae) growing under extremely B-rich conditions near hydrothermal springs in Chile can accumulate foliar B up to 4930 µg g^{-1} (Rámila et al. 2015), and *Gypsophila sphaerocephala* var. *sphaerocephala* (syn. *G. laricina*) (Caryophyllaceae) was found to contain high B concentrations in its above-ground parts (2093 µg g^{-1} in seeds; 3345 µg g^{-1} in leaves) in an active borate mine area of Kırka (Eskişehir Province) in Turkey (Babaoğlu et al. 2004). These values are clearly separated by at least one order of magnitude from the median and extended range of 'normal' B concentrations noted above, and therefore can be regarded as examples of hyperaccumulation. We tentatively propose the use of 3000 µg g^{-1} as a threshold for B hyperaccumulation, while acknowledging that further research is needed to 'fine-tune' this value.

Silicon (Si) is the second most common element in Earth's crust, exceeded only by oxygen. Silicon is not regarded as essential to plants in general but is considered 'quasi essential' for certain plants involving secondary beneficial effects, and there is no doubt that Si makes up a large proportion of the inorganic ash in many grasses and horsetails (Epstein 1972, 1994, 1999). Silicon is mainly available to plants in the form $Si(OH)_4$, at typical concentrations of 0.1 to 0.6 mM in soil water (Epstein 1994). An effect of Si on soil fertility has been observed where an increase in PO_4^{3-} availability leads to displacement of PO_4^{3-} by SiO_4^{4-} (Okuda and Takahashi

Table 6 Unusual accumulation of Metalloids (B, Si, Sb)

Foliar ($\mu g\ g^{-1}$ DW)	Species (family)	Region of origin	Location of record	Life form	References
B					
4930	*Puccinellia frigida* (**Poaceae**)	Chile	Extremely B-enriched soils near hydrothermal springs, Chile	Perennial grass	Rámila et al. (2015)
3345	*Gypsophila sphaerocephala* (**Caryophyllaceae**)	Turkey	B mining area of Kırka, Eskiflehir, Turkey	Shrub	Babaoğlu et al. (2004)
Si					
8480	*Faramea marginata* (**Rubiaceae**)	South and North America	Swamp forest in the llha do Mel, Brazil	Tree	Britez et al. (2002)
22 075	*Emmeorhiza umbellata* (**Rubiaceae**)	Northern Trinidad and tropical South America	Brazil	Shrub	Jansen et al. (2003)
13 438	*Craterispermum laurinum* (**Rubiaceae**)	Tropical Africa	10 miles north of Monrovia, Liberia	Shrub	Jansen et al. (2003)
12 100	*Danais fragrans* (**Rubiaceae**)	Madagascar	Toliara, Madagascar	Shrub	Jansen et al. (2003)
24 300	*Picea* sp. (**Pinaceae**)	Northern, central and eastern Europe	Bedgebury Pinetum, United Kingdom	Tree	Hodson et al. (1997)
Sb					
4029	*Boehmeria nivea* (**Urticaceae**)	China	Xikuangshan Sb mining area, China	Perennial herb	Okkenhaug et al. (2011)
1367	*Achillea ageratum* (**Asteraceae**)	Europe-Greece	Old Sb mining area, Southern Tuscany, Italy	Perennial herb	Baroni et al. (2000)
1600	*Dittrichia viscosa* (**Asteraceae**)	Southern Europe	Sb-mining areas in Extremadura, Spain	Perennial shrub	Murciego et al. (2007)
1164	*Silene vulgaris* (**Caryophyllaceae**)	Northern Africa	Old Sb mining area, Southern Tuscany, Italy	Perennial herb	Baroni et al. (2000)

1965). Silicon has a high affinity for Al and can alleviate Al toxicity in vascular plants (Pontigo et al. 2017). Once absorbed by plants, the role of Si is to help overcome numerous biotic and abiotic stresses (Ma 2004).

After uptake by roots, Si is deposited as amorphous silica ($SiO_2 \cdot nH_2O$) throughout the plant (Currie and Perry 2007). Concentrations of Si in plants range from 0.1 to 15% of dry weight depending on the species (Epstein 1994; Hodson et al. 2005), although even higher concentrations have been recorded, as mentioned below. Silicon accumulation is mainly found in bryophytes, pteridophytes, gymnosperms, and monocots, but is rare in dicots (Ma and Takahashi 2002). Up to 24 300 $\mu g\ g^{-1}$ Si was found in the needles of *Picea* species (Pinaceae), being the highest Si accumulation reported for this medium from 18 genera of conifers collected from Bedgebury Pinetum in the UK (Hodson et al. 1997). Among dicots, most records of high Si concentration are from the family Rubiaceae. Thirty samples of

Faramea marginata (Rubiaceae) contained Si ranging from 6500–14 000 μg g^{-1} with a mean of 8480 μg g^{-1} (Britez et al. 2002). Among other Rubiaceae, *Emmeorhiza umbellata*, *Craterispermum laurinum*, and *Danais fragrans* accumulated up to 22 075, 13 438 and 12 100 μg g^{-1} Si, respectively, in foliage (Jansen et al. 2003).

Ma et al. (2001) defined Si accumulators as plants having high concentrations of Si in their aerial parts, without identifying a specific threshold value. In the absence of formal statistical analyses of Si concentrations across a wider range of taxa, we propose a working definition of Si hyperaccumulation of 10 000 μg g^{-1} (i.e. 1 wt%).

Antimony (Sb) is not essential to plants. Brooks (1972) gave 1 μg g^{-1} as a normal value for Sb in plant ash (equivalent to 50–100 ng g^{-1} in dry matter); various other studies and summaries report the normal Sb contents of terrestrial vascular plants as 2–30 ng g^{-1} (Bowen 1979), or 50 ng g^{-1} for grasses and herbaceous vegetable and grain products (Coughtrey et al. 1983). Plants readily absorb soluble forms of Sb (Coughtrey et al. 1983; Baroni et al. 2000) and a soluble fraction of Sb is present as antimonate (Sb^{V}), especially under oxidizing and basic conditions. Antimonate can be adsorbed by the same soil constituents that bind phosphate and arsenate (Adriano 2001). Antimony is an analogue to P (i.e. it also forms stable antimonate ions, SbO_4^{3-}, in the environment), and also strongly accumulates in the roots/rhizomes of four ferns of the genus *Pteris* (Feng et al. 2013), but apparently there is no translocation of Sb to fronds as for As. This is due to the fact that Sb was taken up by the fern as Sb^{V} (antimonate) and not as Sb^{III} (antimonite). It was demonstrated by Tisarum et al. (2015) that the addition of P reduced Sb^{V} uptake, but increased Sb^{III} uptake by *Pteris vittata*. The uptake of Sb^{III} by *Pteris* may be related to its trait of As hyperaccumulation whereby the fern also takes up As^{III} as arsenite.

The uptake of Sb can lead to plant damage and interfere with uptake of certain essential elements. However, Sb-tolerant and Sb-accumulating plants have evolved a defensive mechanism to alleviate Sb toxicity using an efficient antioxidative system and the ability to immobilize Sb in the cell wall (Feng et al. 2013). *Boehmeria nivea* (Urticaceae) from an active Sb mine in China accumulates up to 4029 μg g^{-1} Sb (Okkenhaug et al. 2011), although other plants in the same environment had much lower concentrations, e.g. 91 μg g^{-1} Sb in *Hippochcaete ramosissima* (Equisetaceae) (Wei et al. 2011). In studies of an abandoned Sb mine in Italy where the soil contained >9000 μg g^{-1} Sb with an extractable concentration of 793 μg g^{-1}, Baroni et al. (2000) reported Sb concentrations of up to 1367 μg g^{-1} in basal leaves and 1105 μg g^{-1} in inflorescences of *Achillea ageratum* (Asteraceae), up to 1150 μg g^{-1} in roots of *Plantago lanceolata* (Plantaginaceae), and up to 1164 μg g^{-1} in shoots of *Silene vulgaris* (Caryophyllaceae). *Dittrichia viscosa* (Asteraceae) accumulates up to 1600 μg g^{-1} Sb in foliage collected from the San Antonio Sb mine in Spain (Murciego et al. 2007). Based on the typical values summarized at the beginning of this section, a tentative hyperaccumulation threshold for Sb of 1000–3000 μg g^{-1} can be proposed, and the highest reported concentrations could exceed that threshold. Further work is needed in order to quantify typical Sb concentrations on both normal and Sb-enriched soil.

3.7 Rare Earth Elements

The REEs are a group of 17 elements comprising Sc, Y and the lanthanoid series in the Periodic Table and are generally considered neither toxic nor essential to plants (Tyler 2004). Accumulation of REEs was briefly discussed in van der Ent et al. (2013) but is updated and expanded here. The majority of plants suggested as REE accumulators are pteridophytes (ferns) (Liu et al. 2018). The REEs, especially La, share similar physiological functions with Ca, with respect to ion uptake and translocation in plants (Brown et al. 1990). Several ferns in the genera *Polystichum* and *Dryopteris* in Dryopteridaceae, *Diplazium* in Woodsiaceae, and *Asplenium* in Aspleniaceae have been reported to accumulate La and Ce (Ozaki et al. 2000). *Dicranopteris*

Table 7 Unusual accumulation of Rare Earth Elements (Sc, Y, REEs)

Foliar (µg g^{-1} DW)	Species (family)	Region of origin	Location of record	Life form	References
REEs					
7000	*Dicranopteris linearis* (***Gleicheniaceae***)	China	REE contaminated mined soil, China	Perennial fern	Shan et al. (2003)
1022	*Blechnum orientale* (***Blechnaceae***)	China	REE mining area, China	Perennial fern	Xiao et al. (2003)
3000	*Pronephrium simplex* (***Thelypteridaceae***)	China	Nanjing National Natural Reservation, China	Fern	Lai et al. (2006)
1350	*Carya tomentosa* (**Juglandaceae**)	USA	Atomic Energy Commission's Oak Ridge Reservation, Tennessee, USA	Tree	Thomas (1975)
2300	*Carya glabra* (**Juglandaceae**)	USA	Pegmatite vein of the Moorefield mine, Va, USA.	Tree	Robinson et al. (1938)

dichotoma, (syn. *D. linearis*) (Gleicheniaceae), a perennial fern native to acidic soil in South China, is reported to hyperaccumulate several of the light REEs (LREEs, lanthanum to europium), including La, Ce, Pr, and Nd up to *ca.* 7000 µg g^{-1} in its dry leaf biomass (Shan et al. 2003), and a separate study reported up to 3358 µg g^{-1} total REEs occurring in the same species (Xiao et al. 2003). Up to 3000 µg g^{-1} LREEs are found in *Pronephrium simplex* (Thelypteridaceae) (Lai et al. 2006). *Blechnum orientale* (syn. *B. orientale* var. *orientale*) (Blechnaceae) accumulates up to 1022 µg g^{-1} REEs in foliage (Xiao et al. 2003) (Table 7).

One of the few published reports of high REE concentrations in flowering plants involves *Phytolacca americana* (Phytolaccaceae), which was found to have total foliar REE concentrations of 1040 µg g^{-1} where growing naturally as an exotic weed in an REE-mining area in China (Yuan et al. 2017b). Leaf concentrations exceeded those in roots and stems. Physiological studies in hydroponic culture showed that LREEs appear to enter the roots of *P. americana* through Ca^{2+} ion channels, whereas heavy rare earth elements (HREEs) may share pathways with Al^{3+} (Yuan et al. 2017a). Hence this appears to be an example of inadvertent uptake, comparable to the hyperaccumulation of Mn in *P. americana* as a side-effect of enhanced phosphate uptake through rhizosphere acidification (Lambers et al. 2015; DeGroote et al. 2018). It is known that La^{3+} can be used as a Ca^{2+}-channel blocker in the root cell membranes of plants (Lewis and Spalding 1998), by which La^{3+} replaces Ca^{2+} sites in enzymatic reactions (Squier et al. 1990). A high correlation between foliar Al and La has been found in *Rhynchanthera grandiflora* (Melastomataceae) (Olivares et al. 2014). La^{3+} can compete for the Al^{3+}-binding sites on the plasma membrane of *Fagopyrum esculentum* (Polygonaceae) (Ma and Hiradate 2000). Some REEs, such as La^{3+}, Gd^{3+} and Sc^{3+} have been used as Al^{3+} analogues to study Al^{3+} toxicity in plants (Clarkson 1965; Kopittke et al. 2009; Reid et al. 1996), but different effects were found depending on the REEs involved (Kopittke et al. 2009).

There are difficulties in applying the concept of hyperaccumulator thresholds to the REEs, including the tendency for some researchers to report concentrations of total REEs whereas others focus on individual elements. Nonetheless, some publications have referred to hyperaccumulation of REEs despite the lack of a

Table 8 Unusual accumulation of Platinum group and Noble elements (Pt, Pd, Rh, Ir and Au)

Foliar (µg g^{-1} DW)	Species (family)	Region of origin	Location of record	Life form	References
Pd					
0.71	*Berkheya coddii* (**Asteraceae**)	South Africa	Nickel-rich outcrops near Agnes Gold mine, Barberton, South Africa	Perennial herb	Nemutandani et al. (2006)
Pt					
0.03	*Taraxacum officinale* (**Asteraceae**)	Europe	Along highways in Germany	Perennial herb	Djingova et al. (2003)
0.025	*Nerium oleander* (**Apocynaceae**)	Southern Europe	Palermo area, Italy	Shrub	Orecchio and Amorello (2010)
Au					
0.5	*Equisetum arvense* (**Equisetaceae**)	North America		Perennial herb	Cannon et al. (1968)
0.78	*Pinus pinea* (**Pinaceae**)	Mediterranean region	Palermo area, Italy	Tree	Dongarra et al. (2003)
Ir					
0.0004	*Taraxacum officinale* (**Asteraceae**)	Europe	Along highways in Germany	Perennial herb	Djingova et al. (2003)
Rh					
0.0046	*Nerium oleander* (**Apocynaceae**)	Northern Africa, southern Europe and Western China	Palermo area, Italy	Shrub	Orecchio and Amorello (2010)

formal definition (e.g. Shan et al. 2003; Lai et al. 2006). It appears that this has been based on an arbitrary criterion of 1000 µg g^{-1} for the sum of all 17 REEs, but this could potentially be lowered to 100 µg g^{-1}. We suggest that further research is necessary in order to confirm that this is an appropriate threshold, and to eliminate the possibility of airborne REE contamination in samples from the vicinity of mines and smelters.

3.8 Platinum Group and Noble Elements

Platinum (Pt), palladium (Pd), rhodium (Rh), and iridium (Ir) are part of the group of platinum group elements (PGEs), which also includes Ru and Os. They are grouped here with gold (Au) because both PGEs and Au are known as precious metals due to their economic value, rarity of occurrence, and stability of the metal form of the elements (Table 8).

A very comprehensive review of information on plant uptake of PGEs and Au has been provided by Dunn (1992), in which the focus is on the location of ore deposits. Other sources of soils enriched in PGEs include mine tailings and processing plant waste. During recent decades, PGEs have also been emitted by automobile traffic, and contamination is noticeable in some cities and along major highways (Lyubomirova and Djingova 2015), all leading to research on plant uptake of the metals from these sources.

Dunn (1992) noted that in dry plant tissue, background levels of Pd (the most abundant PGE), are in the sub-ng g^{-1} (parts per billion) range and that the other five PGEs occur at levels of a few ng kg^{-1} (parts per trillion). Kothny (1979) studied Pd concentrations by a colorimetric method in various species growing on soil derived from sedimentary rocks having 40 ng g^{-1} Pd, in which the plant tissues had 15–110 ng g^{-1} on an ash weight basis (converted to 1.2–6.6 ng g^{-1} on a dry weight basis). Later work (Kothny 1987) using solvent extraction, followed by atomic absorption analysis with a graphite furnace, showed up to 43 ng g^{-1} Pt and 18 ng g^{-1} Pd in the dry matter of various trees, shrubs, and grasses. It was suggested that Pd may form part of a metalloenzyme or may be taken up in place of Mn^{2+}, an element with a similar ionic radius. It was also noted by Kothny (1979) that some Mn ores are also enriched in Pd. From the point of view of plant uptake, the data of Dunn (1992) on all of the PGEs and Au are most relevant. Measurements were made on several tree species as well as Labrador tea (*Ledum palustre*) and marsh grass growing over a platiniferous sulfide deposit in Saskatchewan. Platinum and Pd concentrations were both in the range of 10–1660 ng g^{-1} in the ash. As the given ash percentages were all below 6.5%, these data convert to 0.05–60 ng g^{-1} in the dry matter for both Pt and Pd. Concentrations of the other four PGEs (where detected) were all below 80 ng g^{-1} in ash or below 2 ng g^{-1} in dry matter.

A study of tree species from a mafic/ultramafic complex in Minnesota (Buchheit et al. 1989) gave maximum values for both Pt and Pd of <10–52 ng g^{-1} in the plant ash, probably corresponding to <1 ng g^{-1} in dry matter in most cases. Dunn (1992) reported details of other Pt and Pd analyses carried out on plants of platiniferous areas in Montana (USA) and British Columbia (Canada). Maximum ash values of 56 ng g^{-1} (Pt) and 285 ng g^{-1} (Pd) were recorded in limber pine (*Pinus flexilis*) from the Stillwater Complex (Montana) by Fuchs and Rose (1974), but older twigs of Douglas fir (*Pseudotsuga menziesii*) were found with much higher concentrations of 3 µg g^{-1} (Pt) and 15 µg g^{-1} (Pd) in the ash (Riese and Arp 1986).

Berkheya coddii (Asteraceae), a hyperaccumulator of Ni, has been reported to accumulate Pd and Pt from a naturally enriched soil. Mean (dry mass) concentrations recorded were 0.71 µg g^{-1} Pd in the leaves and 0.18 µg g^{-1} Pd in roots, and 0.22 µg g^{-1} Pt in the leaves and 0.14 µg g^{-1} Pt in roots, from a soil that contained 0.04 µg g^{-1} Pt (Nemutandani et al. 2006). These values might conceivably be regarded as hyperaccumulation relative to the low concentrations measured in most plants, although the absolute values are still quite low.

Gold (Au) is not essential to plants but can be absorbed in soluble forms, and where Au enters root vascular systems, may be easily transported to the shoot. A comprehensive compilation of early data from many literature sources was provided by Brooks (1982), which includes a listing of Au concentrations found in more than 130 plant species, together with soil concentrations where these were also measured. It is clear that in nearly all cases, the Au concentrations (dry matter basis) are in the range <1–1000 ng g^{-1}, i.e. less than 1 µg g^{-1}. Claims of Au concentrations much greater than this have rarely been replicable, for a variety of reasons associated mainly with faults in the analytical procedures or contamination from a number of possible sources. Particularly noteworthy was the long-running myth about the very large accumulation of Au (to 610 µg g^{-1} in ash, which might correspond to over 100 µg g^{-1} in dry matter) by the swamp horsetail *Equisetum palustre*, deriving from reports from Slovakia by Nemec et al. (1936) and Babička (1943). Importantly, such high Au concentrations were not substantiated by Cannon et al. (1968) in studies in the USA, nor by Brooks et al. (1981) who reported on six *Equisetum* species from various sites in Canada, including some with auriferous mineralization. In all of these cases, Au levels were below 1 µg g^{-1}. The earlier claims were based on a wet-chemical procedure that would have included the final weighing of As sulfide in addition to any gold that was present; it was further shown that most *Equisetum* specimens from auriferous areas contained

40–738 μg g^{-1} As on a dry weight basis (Brooks et al. 1981). Because of the typical co-occurrence of Au and As in sulfide mineralized areas, presence of the *Equisetum* species may nevertheless act as an indirect indicator (but not accumulator) of elevated Au. Gold concentrations in a variety of plants from an auriferous area in the Canadian Arctic were also all below 0.1 μg g^{-1} (Reading et al. 1987). A tabulation by Dunn (1992) of Au concentrations in the ash of a wide variety of plant tissues from auriferous sites of western Canada shows nearly all to be <1 μg g^{-1}, probably corresponding to dry matter concentrations below 0.1 μg g^{-1}. The highest value found, 3 μg g^{-1} in the ash of twigs of *Tsuga heterophylla*, is probably equivalent to about 0.3 μg g^{-1} in dry matter. Gold hyperaccumulation can be defined as accumulation to >1 μg g^{-1}, this lower limit being based on a nominal 'normal' Au concentration of 0.01 μg g^{-1} in dry matter (Anderson et al. 2003). Further critical examination is probably needed for any claims relating to Au concentrations exceeding about 3 μg g^{-1} in the dry matter of wild plant material, even in auriferous environments. Except where the plants themselves are cyanogenic, as in *Phacelia sericea* (Girling and Peterson 1978; Girling et al. 1979), or perhaps in cases of gold dissolution mediated by micro-organisms (Brooks and Watterson 1992), more significant transfer of Au from soil to the upper parts of plants relies on external application of complexing agents such as cyanide or thiocyanate to the soil (Anderson et al. 1999).

3.9 Radioactive Elements

The overall crustal abundances of uranium (U) and thorium (Th) are estimated to be 2.6 and 9.7 μg g^{-1}, respectively (Taylor 1964). Brooks (1972) gives typical soil concentrations as 1 μg g^{-1} U and 13 μg g^{-1} Th. These two elements have different chemical and biological behaviours, but are grouped here because of their concomitant geological occurrences, and hence potential exposure to plants. Naturally radioactive areas where uraniferous mineralization exists provide habitats where plants are exposed to anomalous U, Ra and Th concentrations. Comprehensive discussions of natural and anthropogenic radionuclides in plants have been given by Momoshima (1997) and Gupta and Clemens (2014). Discussion of normal and anomalous plant concentrations of U and Th (and some other elements) reported in the literature is often made difficult by the frequent failure of authors to specify whether their data have been presented on an ash-weight or dry-weight basis. In some cases, especially where the analysis has been carried out radiometrically, the concentrations of particular isotopes of U and Th (U^{238}, U^{235}, Th^{232}) have been quoted in terms of radioactivity (A_{Bq}) in Bq (disintegrations per second) per g of sample. This terminology is linked to the number of moles, n, of the isotope, by $A_{Bq} = nN_A(\ln 2)/t_{½}$, where N_A is the Avogadro constant and $t_{½}$ is the half-life in seconds of the isotope concerned.

Uranium belongs to the actinide series of the Periodic Table. It is not an essential element and its uptake and toxicity effects are dependent on the plant species, U speciation, and soil characteristics (Vanhoudt et al. 2011a). Plants accumulate U as the uranyl ion UO_2^{2+} (Ebbs et al. 1998). The bioavailability of U can affect the mineral nutrition, morphology, and physiology of plants by targeting the photosynthetic tissues (Aranjuelo et al. 2014; Saenen et al. 2014; Vanhoudt et al. 2011a, 2014) and can induce oxidative stress in plants, as shown in *Arabidopsis thaliana* (Brassicaceae) (Doustaly et al. 2014; Vanhoudt et al. 2008, 2011b). Cannon (1964) considered U concentrations in plant ash above 2 μg g^{-1} to be anomalous; this amount can be estimated to correspond to dry-weight concentrations above 0.1–0.2 μg g^{-1}. Brooks (1972) regarded an ash weight U concentration of 0.6 μg g^{-1} in plants as typical; a dry weight equivalent would be *ca.* 0.05 μg g^{-1}. Bowen (1979) quotes a normal range of 0.005–

Table 9 Unusual accumulation of Radioactive elements (U) (values converted from ash weight basis)

Foliar ($\mu g\ g^{-1}$ DW)	Species (family)	Region of origin	Location of record	Life form	References
U					
1250–2500	*Uncinia leptostachya* (**Cyperaceae)**	New Zealand	Lower Buller Gorge Region, New Zealand	Shrub	Whitehead and Brooks (1969)
80–100	*Coprosma arborea* (**Rubiaceae)**	New Zealand	Lower Buller Gorge Region, New Zealand	Shrub	Whitehead and Brooks (1969)

0.06 $\mu g\ g^{-1}$ (dry weight); Vargas et al. (1997) give a range of <0.008–1.3 $\mu g\ g^{-1}$. A conservative criterion for hyperaccumulation for U, two orders of magnitude higher than these putatively typical concentrations, might therefore be approximately 100 $\mu g\ g^{-1}$.

Raju and Raju (2000) give concentrations in the wood of the tree *Pterocarpus santalinas* as 1.22 and 2.57 $\mu g\ g^{-1}$ for U and Th, respectively; these amounts are presumed to be ash weight concentrations, as the discussion quotes other work on this basis. The associated soil concentration of U was 8.3 $\mu g\ g^{-1}$. Higher levels of both U and Th have been recorded in plants growing on naturally enriched soils and in industrial areas (Kabata-Pendias and Pendias 2001; Sarkar 2002). From an abandoned Pb-Zn-Cu mining area with soils containing U and Th, each at 1–70 $\mu g\ g^{-1}$ shoot concentrations of 0.02–2.76 $\mu g\ g^{-1}$ U and 0.07–12.3 $\mu g\ g^{-1}$ Th were found in *Euphorbia macroclada* (Euphorbiaceae), *Verbascum cheiranthifolium* (Scrophulariaceae), and *Astragalus gummifer* (Fabaceae) (Sasmaz and Yaman 2008).

Because of concern about the effects of radiation on human and animal health, a great deal of work has focused on the uptake of U and Th by crop plants on soils with normal and elevated levels of these elements. In many cases, U concentrations in roots have been shown to be higher than in plant shoots. For example, lettuce plants grown on a U-contaminated soil in Portugal contained 0.95–6 $\mu g\ g^{-1}$ U dry weight in roots and 0.32–2.6 $\mu g\ g^{-1}$ in shoots (Neves et al. 2008). In some cases of exposure to low U levels, the root concentrations are not consistently higher, as reported in the work of Olszewski et al. (2016), in which *Urtica dioica* (Urticaceae) was found with 0.01–0.20 $\mu g\ g^{-1}$ U dry weight in roots and 0.004–0.20 $\mu g\ g^{-1}$ in shoots on soils having 0.3–2.4 $\mu g\ g^{-1}$ U. This paper includes a summary of data from many studies by other workers on crop plants, grasses, and vegetables. Experiments with a focus on phytoremediation were carried out by Shahandeh and Hossner (2002) on 34 plant species grown on soils spiked with uranyl nitrate (applied at 100–600 $\mu g\ g^{-1}$); the greatest uptake was observed in sunflower (*Helianthus annuus*, Asteraceae) and Indian mustard (*Brassica juncea*, Brassicaceae), with maximum concentrations of 102 $\mu g\ g^{-1}$ in shoots and 6200 $\mu g\ g^{-1}$ in roots. Such high concentrations could be considered hyperaccumulation, although it has been argued that results from artificially spiked soil are insufficient, on their own, for the recognition of hyperaccumulators (van der Ent et al. 2013).

The strong interaction of soluble U species with organic matter may explain the elevated levels found in coal and peat, which can sometimes contain up to 200 $\mu g\ g^{-1}$ (Bowen 1979). Dunn (1986) notes the remarkable concentration of U in twigs of black spruce (*Picea mariana*, Pinaceae) in Saskatchewan, Canada, for which the concentration range of up to 2270 $\mu g\ g^{-1}$ on an ash weight basis makes biogeochemical prospecting for U feasible. A detailed discussion of plant uptake of U and Th in relation to prospecting was given by Boyle (1982). High U concentrations were measured in soils and crops from a U mining area in Jiangxi province of Southeast China. Soils there contained U at over 3000 mBq g^{-1}, whereas the various plants had 15–118 mBq g^{-1} in shoots and 108–1167 mBq g^{-1} in roots. For U^{238} 1 mBq g^{-1} is equivalent to approximately 0.08 µg g^{-1} (Chen et al. 2005). In a highly uraniferous part of the Buller Gorge in New Zealand, it was found that, on an ash weight basis, *Uncinia leptostachya* (Cyperaceae) accumulated up to 25 000 µg g^{-1} U (~1250–2500 µg g^{-1} dry weight), three bryophytes contained 670–4360 µg g^{-1} U (~34–436 µg g^{-1} dry weight), and four shrubs (from a larger selection analysed) contained 150–1000 µg g^{-1} U (~8–100 µg g^{-1} dry weight) (Whitehead and Brooks 1969). The highest of these concentrations may represent hyperaccumulation (Table 9).

Thorium is classified as a member of the actinide family located below Ce in the Periodic Table. It is a toxic element and not essential to plants. A concentration range for Th in uncontaminated soils has been quoted as 0.4–6.0 µg g^{-1} (Shacklette and Boerngren 1984); Kabata-Pendias and Pendias (2001) give 2–12 µg g^{-1} as a typical range, with a mean of 6 µg g^{-1}. Plants absorb Th in tetravalent form (Th^{4+}). Brooks (1972) gives a typical concentration in plant ash as 20 µg g^{-1}, implying a dry weight concentration of 1–2 µg g^{-1}. As noted above, the heartwood of *Pterocarpus santalinus* (Fabaceae) accumulates up to 2.6 µg g^{-1} Th (possibly on an ash weight basis) in Andhra Pradesh, India (Raju and Raju 2000). A study of a site in Bulgaria where soils contained about 50 times background levels of both U and Th was reported by Mihaylova et al. (2013). They found concentrations of U in grass and dandelion leaves of 0.128 and 0.137 µg g^{-1}, respectively, on soil having 45–65 µg g^{-1}, and corresponding Th concentrations of 0.021 and 0.081 µg g^{-1} where the soil contained 70 µg g^{-1} Th. These plant concentrations were elevated by factors of only about 4 to 6x compared to those on background soils. As noted above, Th concentrations up to 12 µg g^{-1} were found in shoots of plant species from a mined area with up to 62 µg g^{-1} Th in soil (Sasmaz and Yaman 2008). If the data of Brooks (1972), cited above, are taken as indicative of typical foliar concentrations, then

Table 10 Different pathways of elemental accumulation and suggested hyperaccumulation threshold values

Element	Example species	Concentration (µg g^{-1} DW)	Suggested hyperaccumulation threshold (µg g^{-1} DW)	Chemical form of uptake	Analog/Chemical form*	Pathway
Al	*Symplocos* spp.	up to 40 000	1000	Al^{3+}	None	Requirement
Cs		up to 200	100	Cs$^+$	K$^+$	Inadvertent
REE	*Dicranopteris linearis*	up to 7000	100	REE^{3+}	Al^{3+} or Si^{3+}	Inadvertent
Re		up to 30	10	ReO$_4^-$	NO$_3$-	Inadvertent
F	*Gastrolobium* spp.	up to 3000	100?	F$^-$		Inadvertent
I		up to 300	100?	I$^-$/IO$_4^-$?	Cl$^-$?	?
Si	*Oryza sativa*	up to 10 000	1000	(SiO$_4$)$^{4-}$	PO$_4^{3-}$	Requirement
B	*Puccinellia frigida*	up to 5000	3000	BO$_3^{3-}$	?	?
V	*Astragalus* spp.	up to 90	300	VO$_4^{3-}$	MoO$_4^{2-}$	Inadvertent
W		up to 1500	100	WO$_4^{2-}$	MoO$_4^{2-}$	Inadvertent

100 µg g^{-1} Th might be a suitable criterion for hyperaccumulation of this metal, but it seems unlikely that any plants yet tested exceed this threshold.

4 Discussion

Metal uptake by plants has been widely discussed in terms of factors such as the translocation factor (TF), bioconcentration factor (BF or BCF), or various kinds of enrichment factor (EF) (Yoon et al. 2006; Cui et al. 2007; Li et al. 2007). The TF is defined as the ratio of shoot/root (dry weight) element concentrations. The BF or BCF concerns the element concentration quotient of root/soil. Some authors also make use of the accumulation factor (AF), referring to the concentration quotient for the whole plant versus that of the soil. Other workers have used the EF or BAC (biological accumulation coefficient) to refer to shoot/soil concentration quotients; this term has also been used in connection with plant behaviour on contaminated versus uncontaminated sites: the metal concentration quotient for contaminated/uncontaminated media can be applied to the metal concentration in either the soil or the plant. In addition, for plants growing in aqueous media, the BCF refers to the quotient of the plant dry-matter concentration (in µg g^{-1}) to the aqueous medium concentration (in µg mL^{-1}). Importantly, great care must be taken in using these factors in discussing hyperaccumulation or even just accumulation, and especially in connection with promoting the use of particular plant species for phytoextraction. The BCF (root/soil) factor, for example, is useful only in discussing phytostabilization, or where whole-plant harvesting, including the root system, is feasible.

Too much importance should not be placed on a translocation factor (TF) > 1 as a criterion for suitability in phytoremediation, nor is its use appropriate in defining hyperaccumulation. This is partly because of the difficulties in removing all superficial trace elements and soils attached to plant roots prior to analysis, even by extensive washing. Furthermore, a TF > 1 for a particular element in a particular species may have little significance for phytoextraction, nor may this be an indication of any remarkable physiological interest: for example, a plant having 60 µg g^{-1} Zn in the shoot and 30 µg g^{-1} Zn in the root is not showing any extraordinary accumulation behaviour. These concentrations can be influenced in part by the proportions of structural material in the different plant parts, which in turn can depend on growing conditions. The translocation of metal into shoots relative to the roots may also reflect the nature of the external metal supply (Talke et al. 2006). A high root to shoot biomass ratio of some hyperaccumulators can also contribute to relatively high shoot to root metal quotients (Krämer et al. 1997). For phytoremediation of a particular element, the most important factors are its dry-matter concentration in the harvestable biomass and the rate of this biomass production.

The shoot-to-soil ratio of metal concentrations (EF or BAC, as noted above) has several limitations. It has been suggested that plants with BAC > 1 have the potential for phytoremediation (Yoon et al. 2006); such factors locally reach 50–100 (Cluis 2004). However, some cases where BAC is <1 may still be of interest, because high metal concentrations in soil can result in a low BAC. For example, an ultramafic soil may have a Ni concentration of 3000 µg g^{-1} and an overlying hyperaccumulator plant may have 2000 µg g^{-1}. Conversely, a plant growing on a soil deficient in an essential trace element (e.g. Zn) might be very efficient in sequestration and have a high bioaccumulation factor, yet still have a low tissue metal concentration.

In a seminal review on the ecological significance of trace element hyperaccumulation, Boyd and Martens (1992) summarized five hypotheses that might explain why plants have evolved patterns of elemental uptake and sequestration that potentially have a metabolic cost. The adaptive explanations included metal disposal, drought tolerance, defence against herbivores and pathogens, and allelopathic interactions with other plants. A fifth hypothesis, termed ‘inadvertent uptake’, suggested that hyperaccumulation may confer no selective advantage, but merely occurs as a side effect of nutrient acquisition mechanisms or other aspects of primary metabolism. After nearly 30 years, only the defence hypothesis has accumulated significant supporting evidence (Boyd 2007; Hörger et al. 2013). Because inadvertent uptake is in some sense a null hypothesis, it is therefore difficult to test experimentally, but has also been supported in some cases (Mizuno et al. 2006; DeGroote et al. 2018).

There appear to be several reasons for abnormal plant uptake of the elements discussed in this review (Table 10). With the exception of boron, none of the elements considered here is essential. In all cases under discussion, the unusual uptake still allows the plant to undergo its normal life cycle. Sometimes the accumulation occurs largely in the root system and translocation to the upper parts is limited; in other cases, the abnormally accumulated element is sequestered in a harmless form in the above-ground parts of the plant. The unusual behaviour may be 'inadvertent' and non-specific, perhaps related simply to maintenance of an osmotic balance somewhere within the plant. It may also be an inadvertent consequence of the root metabolism of particular species, in which processes that facilitate the uptake of an essential element also mobilise another (non-essential or essential) element to an unusual degree. Other cases are the result of obvious chemical analogies, e.g. between phosphate and antimonate. There are also clear cases where certain plant species have evolved a set of unusual and parallel metabolic pathways to deal with the unusually high concentration of an element normally occurring in only trace concentrations, such as the involvement of fluorocarboxylic acid synthesis in some F accumulators. However, to our knowledge, there are no such chemical analogues for Hg, Sn, PGEs and Au among the essential elements normally taken up by plants.

Acknowledgements The French National Research Agency through the national 'Investissements d'avenir' program (ANR-10-LABX-21, LABEX RESSOURCES21) is acknowledged for funding support to A. van der Ent and F. Abubakari. F. Abubakari is the recipient of an Australian Government Research Training Program Scholarship and UQ Centennial Scholarship at The University of Queensland, Australia.

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Global Distribution and Ecology of Hyperaccumulator Plants

Roger D. Reeves, Antony van der Ent,
Guillaume Echevarria, Sandrine Isnard, and
Alan J. M. Baker

Abstract

A large body of analytical data is available on the inorganic composition of many thousands of plant species, for which typical concentration ranges have been tabulated for major, minor, and trace elements. These elements include those that have been shown essential for plant growth, as well as others that lack this status, at least universally. Metalliferous soils, having abnormally high concentrations of some of the elements that are generally present only at minor (e.g. 200–2000 μg g^{-1}) or trace (e.g. 0.1–200 μg g^{-1}) levels, have attracted increasing attention during the last 50 years. The effects vary widely, depending on the species, the relevant elements, and soil characteristics that collectively influence the availability of metals to plants. Some of these soils are toxic to all or most higher plants. Others have hosted the development of specialized plant communities consisting of a restricted and locally characteristic range of metal-tolerant species. These typically show a slightly elevated concentration of the elements with which the soil is enriched, but in places a species may exhibit extreme accumulation of one or more of these elements, to a concentration level that can be hundreds or even thousands of times greater than that usually found in plants on the most common soils. These plants, now widely referred to as hyperaccumulators, are a remarkable resource for many types of fundamental scientific investigation (plant systematics, ecophysiology, biochemistry, genetics and molecular biology) and for applications such as phytoremediation and agromining. Systematic analysis of herbarium specimens by X-ray Fluorescence, combined with auxiliary collection data, can provide insights into phylogenetic patterns of hyperaccumulation, and has the potential to complement and add insights to biogeographical and phylogenetic studies.

R. D. Reeves (✉)
Palmerston North, New Zealand
e-mail: rdjmreeves@xtra.co.nz

A. van der Ent · G. Echevarria · A. J. M. Baker
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD 4072, Australia

A. J. M. Baker
School of BioSciences, The University of Melbourne, Melbourne, VIC 3010, Australia

G. Echevarria
Laboratoire Sols et Environnement, Université de Lorraine, UMR 1120, Vandœuvre-lès-Nancy, France

S. Isnard
AMAP, University of Montpellier, IRD, CIRAD, CNRS, INRAE, Montpellier, France

S. Isnard
AMAP, IRD, Herbier de Nouvelle-Calédonie, Nouvelle-Calédonie, Nouméa, France

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_7

1 Introduction

It has been known since the 1850s and 1860s that certain plant species then found on the zinc (Zn)-rich 'calamine' soils near Aachen in Germany accumulated Zn to very high concentrations. Although the first record referred to *Viola calaminaria* (Violaceae) (Fig. 1), a later report presented data showing that *Thlaspi alpestre* var. *calaminare,* now classified as *Noccaea caerulescens* (Brassicaceae) (Fig. 2), contained at least 1 wt% Zn in the dry leaf tissue, or 10 wt% in the inorganic ash (Sachs 1865). During the last century, unusual accumulation of other metals and metalloids has been found, including for Pb in the 1920s; Se in the 1930s; Ni in the 1940s; Co, Cu and As in the 1960s; and Cd and Mn in the 1970s.

2 Hyperaccumulation

Normal concentration ranges in plants have been tabulated for major, minor, and trace elements in many reviews (e.g. Reeves and Baker 2000). The term 'hyperaccumulation', describing a highly abnormal level of metal accumulation, was first applied by Jaffré et al. (1976) in the title of their paper on Ni concentrations in the New Caledonian tree *Sebertia acuminata* (Sapotaceae), now classified as *Pycnandra acuminata*. In discussing Ni concentrations in species of *Homalium* (Salicaceae) and *Hybanthus* (Violaceae) from various parts of the world, Brooks et al. (1977a) used the term to indicate a defined concentration threshold (>1000 μg g^{-1}) for Ni. A similar concept was used earlier by Jaffré and Schmid (1974), who referred to certain Ni-rich plants from the

Fig. 1 The metallophyte *Viola calaminaria* (Violaceae) from Zn-Pb-Cd-rich calamine soils in Belgium, Germany and France

Fig. 2 The Zn-Cd-Ni hyperaccumulator *Noccaea caerulescens* (Brassicaceae) can accumulate in excess of 3% Zn (dry wt)

ultramafic soils of New Caledonia as 'hypernickelophores', i.e. 'extreme nickel-bearers'.

Choice of the 1000 µg g^{-1} criterion was not entirely arbitrary. In many reports on Ni-rich soils, plant Ni concentrations are generally 5–100 µg g^{-1}; levels of 100–1000 µg g^{-1} are quite rare. The local cases of accumulation to >1000 µg g^{-1} seem to represent a distinct form of plant response, implying some characteristic and unusual physiological behaviour. Greater precision in the definition of hyperaccumulation was provided by Reeves (1992) for Ni: "*a hyperaccumulator of Ni is a plant in which a Ni concentration of at least 1000 mg kg^{-1} has been recorded in the dry matter of any above-ground tissue in at least one specimen growing in its natural habitat*." The criteria defining hyperaccumulation should therefore not be based on analyses of whole plants or subterranean plant parts, mainly because of the difficulty of ensuring that the samples are free of soil contamination, and also because plants that immobilize metals in the root system, and fail to translocate them further (Baker 1981), are of less interest for many purposes than those that actively accumulate metals into all tissues.

Definitions of hyperaccumulation have been extended to elements other than Ni. Malaisse et al. (1978) used the 1000 µg g^{-1} criterion for Cu accumulation, and Brooks et al. (1980) applied this to Co. Reeves and Brooks (1983b) used the same criterion in discussing Pb, but for Mn and Zn, which are normally present at higher and more widely varying concentrations (~20–400 µg g^{-1}), a 10 000 µg g^{-1} threshold was suggested by Baker and Brooks (1989), following use of the term 'hypermanganèsophore' for plants having this level of Mn accumulation (Jaffré 1980).

Extensive recent discussions of appropriate criteria for defining hyperaccumulation of many elements are those of Baker and Whiting (2002) and van der Ent et al. (2013) who summarized the history of development of this topic. These papers also pay attention to the limitations of hydroponic experiments in relation to hyperaccumulation, because such experiments have often involved the use of unrealistic concentrations of free metal ions that are not relevant to the continuing life cycle of naturally occurring metallophyte populations living on metalliferous soils.

3 A Note on Nomenclature

As is often the case with botanical discussions, complexities arise from continual changes in botanical nomenclature and the differing views on the importance of various criteria in circumscribing species, genera, and even families. Many recent changes have resulted from studies of partial or complete plant genomes, bringing new information on the degree of interspecies and interpopulation relationships, and clarifying genotypic and phenotypic effects. In following the history of studies of hyperaccumulator species, there is a need to be aware of any nomenclatural changes that have occurred since the first hyperaccumulation reports. In some instances, the changes are not completely new, but involve simple intergeneric transfers or the resurrection of earlier generic names. In the context of the present account, the following are some of the more important nomenclatural changes:

Sebertia acuminata to *Pycnandra* (New Caledonia); *Austromyrtus* species to *Gossia* (Australia and New Caledonia); *Peltaria emarginata* to *Leptoplax* and then *Bornmuellera* (Greece); *Cochlearia aucheri* and *C. sempervivum* to *Pseudosempervivum* (Turkey); *Ariadne shaferi* to *Mazaea* and many species of *Pentacalia* to *Antillanthus* (Cuba); species of *Alyssum* sect. Odontarrhena to the resurrected genus *Odontarrhena* (Mediterranean Europe, Turkey and further east); species of *Thlaspi* to the resurrected genus *Noccaea* (many temperate regions worldwide).

The major problems arise where a great deal of research has been done and many publications have appeared under the names that were first used in the hyperaccumulation literature. Future work and literature searches should include synonyms. Further changes are inevitable. The following account includes, in particular, reference to species of *Odontarrhena* under their earlier *Alyssum* classification, and to some species of *Noccaea* previously discussed under *Thlaspi* as this genus was earlier broadly understood in major Floras.

4 Ecology and Conservational Status of Hyperaccumulator Plants

The soils produced from the weathering of surficial ore deposits or naturally enriched metalliferous country rocks (e.g. ultramafics, Cu-Co mineralization, calamine deposits) can be regarded as primary habitats for most hyperaccumulator plants. In certain cases, as in some of the ultramafic terranes of Cuba and New Caledonia, such soils are believed to have been continuously available for plant life and evolution for tens of millions of years (Reeves et al. 1996, 1999; Pelletier 2006; Cluzel et al. 2012). Other naturally occurring metalliferous soils are much younger, having been subjected to more recent geological processes such as erosion and re-deposition, hydrothermal alteration, or glaciation. Secondary habitats (on the scale of decades to a few thousand years) have resulted from the exploitation of mineral deposits via metalliferous mining and ore processing activities. A tertiary category of distribution results from the superficial deposition of dusts and particles derived from smelting operations and the beneficiation of processed ores, where effluents are discharged into river systems leading to metal enrichment of alluvial floodplains (Baker et al. 2010).

Present-day plant species that show metal tolerance through occurrence on metalliferous soils (i.e. metallophytes) may therefore have experienced any of this wide variety of soil histories. In relation to species that appear to be endemic to metalliferous soils, there has been extensive discussion of the concepts of palaeoendemism and neo-endemism (Stebbins 1942; Kruckeberg 1954; Antonovics et al. 1971; Brooks 1987). Palaeoendemic metallophytes are species formerly widespread that have survived in the metalliferous environment, restricted by competitive pressures and often having no or few closely related surviving species. Neoendemic metallophytes are species that have evolved in the metalliferous environment, leading to morphological characteristics now recognized as distinctive. The concept as applied generally to metallophytes can also be used in discussion of the particular case of hyperaccumulator species and their putative origins. Some Ni hyperaccumulators, for example, in genera consisting of only one or two species growing on ancient soils and without close relatives, may be termed palaeoendemics. Examples include *Shafera platyphylla* (Asteraceae) and *Phyllomelia coronata* (Rubiaceae) from Cuba and *Oncotheca balansae* (Oncothecaceae) from New Caledonia. These phylogenetically isolated hyperaccumulators contrast with the intense diversification in some genera, as shown by the large numbers of Ni hyperaccumulating endemics present in genera such as *Odontarrhena* (synonym *Alyssum*, Brassicaceae) in Mediterranean Europe, Turkey, and nearby parts of Asia; *Buxus* (Buxaceae) and *Leucocroton* (Euphorbiaceae) in Cuba; and *Phyllanthus* (Phyllanthaceae) in several tropical parts of the world (Reeves et al. 2017).

Mineral wastes have locally enabled the growth of endemic species that are both hypertolerant and hyperaccumulating to extend their distributions regionally, such that the current distributions of some hyperaccumulator plants are well beyond their primary habitats. Additionally, some species are known from both non-metalliferous and metalliferous locations, exhibiting hyperaccumulation solely from the latter. This situation described as 'facultative hyperaccumulation' has been discussed in detail by Pollard et al. (2014). Further, some species are reported to show extreme accumulation of certain elements (e.g. Zn, Mn, Se) from normal soils or those having only modest concentrations of the element concerned. Examples include: *Noccaea caerulescens* (Brassicaceae) that hyperaccumulates Zn from both metalliferous and non-metalliferous soils in France and elsewhere in Europe (Reeves et al. 2001); *Gossia* (formerly *Austromyrtus*) *bidwillii* (Myrtaceae) from eastern Australia (Bidwell et al. 2002; Fernando et al. 2009) and *Alyxia rubricaulis* (Apocynaceae) from New Caledonia (Jaffré 1977), that hyperaccumulate Mn from soils having only a slightly elevated Mn content; and species of *Astragalus* (Fabaceae) in the USA (Rosenfeld and Beath 1964) that hyperaccumulate Se from soils in which the elevated Se content is commonly up to 50 µg g^{-1}.

In temperate regions, the plant assemblages on metalliferous soils generally consist of a limited range of obligate and facultative metallophytes (Baker 1987) that may or may not include hyperaccumulators. On ultramafic soils, in particular in Mediterranean Europe, almost monospecific communities of a Ni hyperaccumulator, e.g. *Odontarrhena* spp. can be found in Greece, Turkey and Albania. By contrast, in the tropics, ultramafic soils regularly show a high density of woody species where hyperaccumulators and non-hyperaccumulators may grow side by side. Often the most ancient and undisturbed metalliferous environments support the richest assemblages of hyperaccumulator plants (e.g. Reeves et al. 1996, 1999).

In spite of the rapidly increasing number of hyperaccumulator plants being discovered (especially for Ni), the overall rarity of this resource must be stressed because it represents only a few tenths of 1% of the known flora. Furthermore, anthropogenic and environmental factors threaten the habitats of many hyperaccumulator plants. These include ongoing mineral exploration and mining activities, reworking of ancient mine spoils, land reclamation and improvement for agricultural production, urbanization and development of brownfield sites, natural fire events, and probably climate change (Whiting et al. 2004; Baker et al. 2010; Wulff et al. 2013; Ibanez et al. 2018). Urgent conservation and management steps are clearly needed in areas under threat, in order to ensure persistence of the valuable phytotechnological resource. Appropriate options are the maintenance of living materials in botanical gardens and seed in germplasm banks, and regeneration in situ using 'seed orchards' on mining lands. Exploitation of the hyperaccumulator resource base for agromining must be considered with due caution and with appropriate management practices in place. An unfortunate incident has been reported in southwestern Oregon, USA, where poor crop management led to the extension of the distribution of *O. chalcidica* (under the name *Alyssum murale*) well outside the operational area, to the extent that this species is now regarded as a noxious weed in Oregon and future use has been banned (Strawn 2013). Invasions such as this may also affect the status of other local endemics native to the area that have been selected for agromining. Issues of the CITES convention may also apply when attempts are made to introduce an 'alien' species to a new country.

5 Instances of Hyperaccumulation

The following discussion outlines instances of hyperaccumulation of selected trace elements (Ni, Zn, Cd, Pb, Co, Cr, Cu, Mn, Se, As and Tl) for which a substantial body of reliable plant analysis data exists. Further exploration of various types of metalliferous environments, both natural and man-modified, will certainly uncover more examples. The exact enumeration of metal hyperaccumulator species is made difficult by the lack of recent and complete Floras for many tropical regions, in particular. The exact identification of some specimens of interest is still in doubt. In addition, since the first hyperaccumulator species were identified, numerous name changes have occurred, some species have been grouped into synonymy, whereas others have been split into several taxa (species, subspecies, and varieties). Some of the earlier information was published in periodicals that are difficult to access, and much useful detail has been omitted because of the space limitations of most journals. All of these difficulties have justified the initiative to create a Global Hyperaccumulator Database (www.hyperaccumulators.org), an ongoing project to encompass as much of the knowledge as possible on identified hyperaccumulator species, including synonymies and other taxonomic changes. A recent summary listed 721 hyperaccumulator species (532 Ni, 53 Cu, 42 Co, one Cr, 42 Mn, 20 Zn, two REEs, 41 Se, two Tl, seven Cs, five As and eight Pb) with some species showing hyperaccumulation of more than one element (Reeves et al. 2017). These numbers will change as more discoveries are made, or if earlier claims are shown to be spurious. The 721 hyperaccumulator species are from 52 families and *ca.* 130 genera; the families most strongly represented are the Brassicaceae (83 species) and the Phyllanthaceae (59 species). The countries with the greatest numbers of published hyperaccumulator plant species (including some sub-specific taxa) are Cuba with 128 (Reeves et al. 1999), New Caledonia with 99 (Jaffré et al. 2013; Gei et al. 2020), Turkey with 59 (Reeves and Adıgüzel 2008) and Brazil with at least 30 (Reeves et al. 2007).

5.1 Nickel

Unprecedented Ni concentrations (up to about 10 000 µg g^{-1} or 1 wt%) were discovered in the Italian ultramafic *Alyssum*, now regarded as *Odontarrhena bertolonii* (Brassicaceae) (Minguzzi and Vergnano 1948). In the 1960s, two additional Ni-accumulating *Alyssum* species, now *Odontarrhena muralis* from Armenia (Doksopulo 1961) and *O. serpyllifolia s.l.* from Portugal (Menezes de Sequeira 1969), were reported to behave similarly. These observations were followed by studies in Zimbabwe (Wild 1970) and two independent discoveries of high Ni concentrations (3000–9800 µg g^{-1}) in *Hybanthus floribundus* from Western Australia (Severne and Brooks 1972; Cole 1973).

Beginning in 1974, concerted attempts were made to discover the extent of Ni hyperaccumulation, both geographically and in terms of distribution in the plant kingdom. Detailed studies of the flora of ultramafic soils were carried out in New Caledonia (Jaffré and Schmid 1974; Jaffré et al. 1976, 1979a, b; Jaffré 1980).

Fig. 3 The Ni hyperaccumulator *Pycnandra acuminata* (Sapotaceae) from New Caledonia has a peculiar blue-green latex with up to 25% Ni (dry wt)

Particularly notable was the discovery that the latex of the New Caledonian tree *Pycnandra* (formerly *Sebertia*) *acuminata* contained about 10% Ni, yielding a dried solid with 20–25% Ni (Jaffré et al. 1976), in which citrate was a major organic constituent (Lee et al. 1977) (Figs. 3 and 4).

During the next 25 years, R.R. Brooks, R.D. Reeves, A.J.M. Baker, and co-workers in many other parts of the world collected and analyzed plant material from ultramafic areas in the search for further examples of Ni hyperaccumulation. Extensive use was made initially of leaf fragments from herbarium collections, but later this approach gave way to field studies. Brooks et al. (1977a) identified several species of *Homalium* and *Hybanthus* in New Caledonia as hyperaccumulators. A comprehensive survey of nearly all of the 170 then known species of *Alyssum* (Brooks and Radford 1978; Brooks et al. 1979) established the existence of 48 Ni hyperaccumulators, all in one section of the genus (now the genus *Odontarrhena*), distributed from Portugal across Mediterranean Europe to Turkey, Armenia, Iraq, Iran and Russia. Most are ultramafic-endemic species, and many have a very restricted geographical distribution. Several additions to the list of Ni hyperaccumulators in this genus have been made subsequently.

Further work by various groups has focused on other genera of the Mediterranean region, on species of ultramafic outcrops in the European Alps, southern Africa, Newfoundland (Canada), and the Pacific Northwest of the United States, and on plants of tropical ultramafic soils of Brazil, Cuba and other Caribbean islands,

Fig. 4 The Ni hyperaccumulator *Psychotria gabriellae* (Rubiaceae) from New Caledonia can accumulate in excess of 4% Ni (dry wt)

Queensland (Australia), Costa Rica, Sri Lanka, and Southeast Asia (especially certain islands of Indonesia and the Philippines). Hyperaccumulators discovered in temperate-zone areas include *Leptoplax* (formerly *Peltaria* and now *Bornmuellera*) *emarginata* from Greece (Reeves et al. 1980), species of *Bornmuellera* and *Cochlearia* (*Pseudosempervivum*) from Turkey and the Balkans (Reeves et al. 1983b; Reeves and Adıgüzel 2008), *Streptanthus polygaloides* from California (Reeves et al. 1981), and species of *Thlaspi* (*Noccaea*) from Europe (Reeves and Brooks 1983a), Turkey, and Japan (Reeves 1988; Reeves and Adıgüzel 2008), and California (Reeves et al. 1983b). Discoveries in tropical areas include several species from Palawan (Baker et al. 1992) and other parts of Southeast Asia (Wither and Brooks 1977), *Stackhousia tryonii* from Queensland (Batianoff et al. 1990), and numerous species from Brazil (Reeves et al. 2007).

The ultramafic soils of Cuba host the largest number of Ni hyperaccumulators reported from any one country. Following initial observations by Berazaín (1981), a survey of much of the Caribbean ultramafic flora revealed 128 such species in Cuba, as well as *Phyllanthus nummularioides* in the Dominican Republic (Reeves et al. 1996, 1999). *Psychotria grandis* is a Ni hyperaccumulator where it occurs on ultramafic soils in Puerto Rico (Reeves 2003; Campbell et al. 2013; McAlister et al. 2015). Other major sources of Ni hyperaccumulator plants, with more than 50 species identified in each country, are New Caledonia and Turkey. Substantial additions to the list are being made from ongoing work in New Caledonia (Jaffré et al. 2013; Gei et al. 2020), Brazil, Indonesia (Sulawesi and some of the smaller islands), Sabah (Malaysia) (van der Ent et al. 2015), and the Philippines (Fernando et al. 2013).

The most recent information brings the worldwide total of known Ni hyperaccumulator plant species to more than 500. Developments can be followed through earlier summaries, some of which deal with hyperaccumulators of other elements (Brooks 1987; Baker and Brooks 1989; Brooks 1998; Reeves et al. 1996, 1999; Reeves and Baker 2000; Reeves 2003, 2005); more recent results can be found in reports on Brazil by Reeves et al. (2007), and in Turkey by Reeves and Adıgüzel (2004, 2008). Ongoing investigations in Sabah (Malaysia) and New Caledonia continue to reveal numerous hyperaccumulator plants new to science (Gei et al. 2020).

Most Ni hyperaccumulators belong to two groups, geographically: (i) the Mediterranean region, extending from Portugal through Italy and the Balkans to Turkey and adjacent countries; and (ii) tropical and subtropical areas worldwide, particularly Cuba, New Caledonia and various islands of Indonesia and the Philippines. The plant family most strongly represented in the first group is Brassicaceae, whereas in tropical areas there is strong representation from Euphorbiaceae, Phyllanthaceae, Salicaceae, Buxaceae and Rubiaceae. Within Violaceae, species of *Hybanthus* (Severne and Brooks 1972; Brooks et al. 1974; Jaffré 1980; Paul et al. 2020) and *Rinorea* (Brooks and Wither 1977; Brooks et al. 1977b; Proctor et al. 1994) are notable as having potentially suitable biomass for agromining purposes. Hyperaccumulators in the Asteraceae appear in South Africa (*Berkheya* and *Senecio*; Morrey et al. 1989, 1992), in the Mediterranean-Turkey region (*Centaurea*; Reeves and Adıgüzel 2004), and in the neotropics (e.g. *Pentacalia* and *Senecio* in Cuba, Reeves et al. 1999; species in several genera in Brazil, Reeves et al. 2007). The Ni hyperaccumulator plants reported to date belong to about 40 different families, distributed widely throughout the plant kingdom; this syndrome is therefore presumed to have evolved independently many times (Boyd 2014; Cappa and Pilon-Smits 2014). It is certain that many more examples of Ni hyperaccumulation remain to be discovered. These will include species not yet discovered or described and known species that have never been analyzed. Further studies of plants growing on ultramafic areas in several islands of the Philippines and Indonesia, in Central America, mainland Asia, and possibly West Africa, are particularly likely to be fruitful.

The relatively large number of Ni hyperaccumulators discovered (compared with those of other elements) may be partly the result of the concerted attention to analytical work on ultramafic floras and partly to the ability to detect high Ni concentrations (>1000 μg g^{-1}) in leaf tissue by a simple test with dimethylglyoxime. Among various types of metalliferous soils, the Ni-enriched ultramafics are the most widespread on a global scale, and in places continuous ultramafic areas of tens or even hundreds of km^2 can be found (e.g. New Caledonia, Cuba, Turkey). Where such areas have been continuously available for plant colonization for millions of years, as appears to be the case in New Caledonia and eastern Cuba, a long-term opportunity has existed for the evolution of a characteristic flora with numerous endemic species, including some that have developed Ni accumulation as a particular response to growth on high-Ni soils (Isnard et al. 2016; Reeves et al. 1996).

Most of the known Ni hyperaccumulator species are endemic to ultramafic rocks, but some occur on a wider variety of soils and exhibit facultative hyperaccumulation, i.e. high Ni concentrations are found only in those specimens from Ni-rich soils. A tabulation of facultative hyperaccumulators, covering Ni and other elements, has been given by Pollard et al. (2014). In a few cases, ultramafic-endemic species may show a wide variation in Ni uptake, apparently being sensitive to parameters other than total soil Ni concentration, such as soil pH; this ‘erratic’ Ni hyperaccumulation occurs, for example, in the Queensland ultramafic endemic *Pimelea leptospermoides* (Thymelaeaceae) (Reeves et al. 2015).

When the focus is specifically on agromining potential, the interest logically moves towards those species that contain consistently >1 wt% Ni in their leaves (and ideally >1 wt% in total harvestable biomass). This property needs to be considered in conjunction with the rate of biomass production, and with other agronomic features considered elsewhere in this book by

Nkrumah et al. (Chapter "Agronomy of 'Metal Crops' Used in Agromining"). The observation that the Californian *Streptanthus polygaloides* (Brassicaceae) could accumulate Ni to 1.5% of the dry plant matter (Reeves et al. 1981) stimulated studies by Nicks and Chambers (1995, 1998) on the use of this plant for phytomining. These included investigations of various fertilization regimes and the optimization of harvest time. They estimated that a crop of nearly 5 t ha^{-1} could be obtained with unfertilized plants in a small-scale trial in the native environment and predicted that fertilization could double that yield. Work elsewhere has been carried out with species capable of producing a larger biomass. The discovery of Ni hyperaccumulation by the South African *Berkheya coddii* (Morrey et al. 1989, 1992; Howes 1991) has been followed by extensive work on its cultivation and extraction of the accumulated Ni (Robinson et al. 1997a; Brooks and Robinson 1998); yields in excess of 20 t ha^{-1} were calculated, again by extrapolation from studies involving small plots.

Several *Odontarrhena* hyperaccumulators have attracted attention for their phytoextraction potential. Although some work has been done on *O. bertolonii* (Robinson et al. 1997b), more investigations have centred on species that have higher biomass such as *O. corsica* and *O. muralis* (Li et al. 2003; Bani et al. 2015a, b). Other species of the Brassicaceae in the Mediterranean region, such as *Bornmuellera emarginata* and *B. tymphaea,* have also been studied (Chardot et al. 2005). These authors concluded that *B. emarginata* compared favourably with *O. chalcidica* and *Noccaea caerulescens* in its phytoextraction performance.

About 70 tropical hyperaccumulator taxa with >1 wt% Ni have been listed by Reeves (2003). These include the facultative hyperaccumulator *Rinorea bengalensis* (Violaceae) of Southeast Asia, a large number of Cuban species in the Phyllanthaceae, Buxaceae, and Rubiaceae, and several New Caledonian species. Many of these are shrubs or small trees, probably with good rates of biomass production, although in many cases no data are available on this aspect. Some of these species are rare, and in most cases agronomic studies are lacking or are only in early stages.

5.2 Zinc, Lead and Cadmium

Since the early discovery of Zn accumulation by certain *Viola* and *Noccaea* species (noted above), further work, particularly on *Noccaea* from German and Belgian calamine soils and from British mine wastes, has been reported frequently, as discussed with detailed references by Baker et al. (1994), Reeves and Baker (2000), and Reeves et al. (2001). This species, often referred to as *Thlaspi calaminare* or *T. alpestre* in earlier work, and later as *T. caerulescens*, is now classified as *N. caerulescens* after a taxonomic revision by Meyer (1973) and subsequent DNA work (Koch and Mummenhoff 2001; Al-Shehbaz 2014).

Following the observation of Rascio (1977) that *T. rotundifolium* subsp. *cepaeifolium* (now *N. rotundifolia*) from Zn-polluted soils near the border of Italy and Austria was also a hyperaccumulator of Zn, surveys of the genus *Thlaspi s.l.* (including those species now belonging to *Noccaea)* (Reeves and Brooks 1983a, b; Reeves 1988) revealed that many species of this genus are hyperaccumulators of Ni from ultramafic soils and often have Zn levels above 1000 μg g^{-1}, even from soils of background Zn content. Reeves and Baker (1984) showed that the ability of the Austrian species *N. goesingensis* to accumulate Ni and Zn was an innate or 'constitutional' property, i.e. not dependent on the geochemistry of the area from which the seed originated. Baker et al. (1994) showed that *N. caerulescens* grown in amended nutrient solutions had the ability to accumulate to high concentrations a wide variety of elements (Zn, Cd, Co, Mn and Ni throughout the plant; Al, Cr, Cu, Fe and Pb largely in the root system).

There are several other examples of Zn accumulation to a concentration of 10 000 μg g^{-1} set as the criterion for Zn hyperaccumulation by Baker and Brooks (1989), and later supported by van der Ent et al. (2013). The most notable is probably *Arabidopsis* (formerly *Cardaminopsis*) *halleri* (Brassicaceae) (Ernst 1968). Other occurrences, mainly from Zn-rich soils around mine sites or from the vicinity of smelters, are listed elsewhere (e.g. Reeves and Baker 2000).

Lead is usually present in vegetation at levels below <10 μg g^{-1}. Even where concentrations of 1–10 μg g^{-1} are measured in above-ground plant parts, it is likely that much of this metal comes from various forms of environmental and/or laboratory contamination. Plant root systems restrict severely the uptake of Pb and significant translocation to the upper parts is uncommon in plants in natural environments. There have been several reports of very high Pb concentrations in plants from areas of Zn-Pb mineralization, and from mine or smelter wastes; notably, these have not generally been subjected to rigorous scrutiny in relation to washing procedures and contamination possibilities. Increased uptake of Pb can be achieved in hydroponic experiments or by various treatments of soil with complexing agents (Raskin and Ensley 2000). However, such soil treatments designed to mobilize relatively insoluble elements such as Pb and Au into harvestable plants, as promoted by several groups, are now regarded as being both economically and environmentally unfavourable.

Elevated levels of Cd (10–200 μg g^{-1}, locally higher) can be found in soils containing waste materials from the mining of Zn ores but may also occur in soils treated with industrial wastes or Cd-rich phosphate fertilizers. Plant Cd is generally <3 μg g^{-1} but may reach 20 μg g^{-1} or more in the flora of Cd-rich soils. A plant concentration of >100 μg g^{-1} has been proposed as the threshold for hyperaccumulation of this element (van der Ent et al. 2013); such a level is exceptional, even on a Cd-contaminated site. However, on some Zn-Pb mine waste sites in the south of France and in Slovenia, *Noccaea* species such as *N. caerulescens* and *N. praecox* have been found to typically contain >100 μg g^{-1} Cd, and >1000 μg g^{-1} locally, with very large variations existing among sites and populations, and considerable intra-site variability (Robinson et al. 1998; Escarré et al. 2000; Lombi et al. 2000; Reeves et al. 2001; Schwartz et al. 2006). Similar observations have been made for *A. halleri* in Europe (Bert et al. 2002) and for *Sedum alfredii* (Crassulaceae) and *Viola baoshanensis* (Violaceae) in China (Liu et al. 2004; Deng et al. 2008). As stressed by van der Ent et al. (2013), further claims of hyperaccumulation of Cd (and other elements) should be restricted to the behaviour of self-sustaining natural populations. Extensive investigations of the behaviour of selected *N. caerulescens* populations have generally been carried out with a focus on phytoremediation rather than agromining (e.g. Chaney et al. 2005).

5.3 Cobalt and Copper

An earlier threshold of 1000 μg g^{-1} for plants to be considered as hyperaccumulators of Cu and Co (Baker and Brooks 1989) has been lowered to 300 μg g^{-1} (Krämer 2010; van der Ent et al. 2013) in the light of the apparent rarity of genuine accumulations of these elements in flora. Most reports of Co and Cu exceeding 1000 μg g^{-1} are derived from studies of the metalliferous soils of the Democratic Republic of the Congo, where the two metals occur together at elevated levels, although in widely varying proportions. Elsewhere, there are local early records of plants having >1000 μg g^{-1} Cu from Cu-mineralized areas (Blissett 1966; Dykeman and De Sousa 1966; Ernst 1966). These reports, and the plant species involved, need more detailed investigation, particularly in view of the potential for soil and dust contamination and the difficulty of its removal from many plant surfaces prior to analysis. The problem is exacerbated in the case of Cu mineral exposures by the common occurrence of more or less pure Cu compounds occurring as secondary mineralization products: a very small amount of such contamination remaining on the plant material can elevate the analytical result considerably (van der Ent et al. 2013; Lange et al. 2017). A similar problem arises in the case of plants sampled from the vicinity of smelters.

Normal concentrations of Co and Cu in plants are in the ranges of 0.03–2 μg g^{-1} and 5–25 μg g^{-1}, respectively. Even on Co-rich soils, such as those derived from ultramafic rocks, Co in plants rarely exceeds 20 μg g^{-1}. Plant Cu concentrations are also controlled within a remarkably narrow range, even in the presence of high soil Cu; plant Cu concentrations above 100 μg g^{-1} are rare. However, the black gum of the southeastern United States (*Nyssa sylvatica* var. *biflora* and var. *sylvatica*) (Nyssaceae) shows exceptional Co accumulation (as much as 845 μg g^{-1}) from normal soils (Beeson et al. 1955; Kubota et al. 1960; Brooks et al. 1977c). Duvigneaud (1959) found accumulation of Co to 354 μg g^{-1} by *Crotalaria cobalticola* (Fabaceae) on Co-rich soils in the Democratic Republic of the Congo; Brooks et al. (1980) reported even higher concentrations in this species. In the extensive survey of the Cuban ultramafic flora, using herbarium specimens and others collected directly from the field, notably elevated Co levels were measured in some of the Ni hyperaccumulators. Mention was made of Co attaining 1140 μg g^{-1} in *Phyllanthus williamioides* (Reeves et al. 1996) and of values in the range 100–800 μg g^{-1} in a number of other species (Reeves et al 1999). Details of the latter were not published, as the concentrations did not reach the threshold for Co hyperaccumulation that was being applied at that time. With the lowering of the threshold to 300 μg g^{-1} it can be noted that the following maximum Co concentrations were found (always accompanying Ni hyperaccumulation): *Buxus historica* (Buxaceae) 667 μg g^{-1}; *Euphorbia helenae* subsp. *grandifolia* (Euphorbiaceae) 357 μg g^{-1} and 392 μg g^{-1} in latex; *Phyllanthus myrtilloides* subsp. *erythrinus* (Phyllanthaceae) 378 μg g^{-1}; *Heterosavia maculata* var. *clementis* (Phyllanthaceae) 336 μg g^{-1}.

Extensive studies of the vegetation of many sites of mining and smelting activity throughout the Democratic Republic of Congo by F. Malaisse, R. R. Brooks, A.J.M. Baker, and co-workers identified 30 hyperaccumulator plants of Co and 32 of Cu, with 12 species being common to the two lists. The species involved have been summarized and updated in several papers and chapters (Brooks 1977; Malaisse et al. 1979; Brooks et al. 1978, 1980, 1987, 1995; Brooks and Malaisse 1985; Reeves and Baker 2000). Assessment of these data is difficult for several reasons: (i) numerous changes have been made to the classification and nomenclature of the species involved; (ii) uncertainties exist surrounding pretreatment of the samples prior to analysis, and in particular the efficacy of the washing regimes; (iii) few of the Co- and Cu-accumulating species appear to be absolutely restricted to metalliferous soils, although some have had local or regional uses as indicator plants; (iv) there are wide variations in the apparent metal concentrations occurring within many species, even from the same area; (v) a lack of reproducibility exists in cases where the plants from a given location have been re-examined later; and (vi) difficulties have been reported in attempting to reproduce the metal accumulating behaviour in plants in cultivation. A detailed re-assessment of several putative hyperaccumulators was presented by Faucon et al. (2007), who concluded that at least part of the previously reported elevated metal levels could be ascribed to inefficient washing of sample materials prior to analysis. However, in spite of the suspicion that the last of these possibilities is sometimes relevant, many records of Cu and Co hyperaccumulation represent some degree of abnormal uptake by the plant from the soil: Malaisse et al. (1994), for example, presented iron data that indicate little likelihood of soil contamination (e.g. *Anisopappus davyi* (Asteraceae) having 3504 μg g^{-1} Cu, 3 μg g^{-1} Co and 67 μg g^{-1} iron). A re-examination of putative Cu hyperaccumulation by *Millotia myosotidifolia* (Asteraceae) from a Cu mine site in South Australia (R.D. Reeves, unpublished data), has not supported the earlier finding of 4 wt% Cu in the plant ash or 2400 μg g^{-1} in the leaves (Blissett 1966), but instead showed Cu levels averaging 516 μg g^{-1}. This concentration is still abnormally high, and much higher than found in other species from the same site, apart from *Arctotheca calendula* (Asteraceae) that averaged 779 μg g^{-1} Cu. Extensive analyses of plants from some unusually Cu-rich ultramafic soils in Malaysia and Brazil have not shown any instance of Cu concentrations reaching 300 μg g^{-1} (van der Ent and Reeves 2015).

Even with the adoption of a 300 μg g^{-1} threshold in defining hyperaccumulation of Cu and Co, and with the addition of reports of Cu accumulation from Sri Lanka, China, and Indonesia, we conclude that Cu and Co hyperaccumulation in plants is very rare. From the point of view of agromining applications, it is scarcely relevant whether the threshold is set at 300 or 1000 μg g^{-1}, because the levels of 5000 to 10 000 μg g^{-1} of interest for agromining of these elements have never been observed. The high specificity of Ni hyperaccumulation, relative to uptake of Co by Ni accumulator plants on ultramafic soils, also implies that extracting Co as a by-product of Ni agromining will rarely be economically feasible.

5.4 Manganese

Jaffré (1977, 1979, 1980) found that 98 out of 445 species (22%) growing on ultramafic soils of New Caledonia had mean Mn concentrations above 1000 μg g^{-1}; six species had means exceeding 10 000 μg g^{-1}, and nine had at least one specimen above this level. The total Mn concentrations within these soils ranged from about 4000–6000 μg g^{-1} (Isnard et al. 2016), only a little above the range determined for many types of soils worldwide. High Mn concentration in leaves can be attributed to: (i) Mn bioavailability, which depends on soil pH, independently from total or exchangeable soil [Mn] (Fernando et al. 2008; Jaffré 1980); and (ii) the release of carboxylates by specialized cluster roots, such as in many Mn hyperaccumulators belonging to Proteaceae (Lambers et al. 2015).

Recognizing that normal levels of Mn in plant dry matter fall within the rather wide range of 20–500 μg g^{-1}, Baker and Brooks (1989) chose a level of 10 000 μg g^{-1} to define Mn hyperaccumulation. This criterion has been maintained in the review by van der Ent et al. (2013). After accounting for synonymies and changes of nomenclature for several species, data are now available for 42 species that have been found to meet this threshold in at least one specimen (Reeves et al. 2017). These include single species of *Alyxia* (Apocynaceae), *Beaupreopsis* and *Grevillea* (Proteaceae) (Jaffré 1977, 1979; Losfeld et al. 2015), all from New Caledonia; *Chengiopanax* and *Polyscias* (Araliaceae) from Japan and New Caledonia, respectively (Mizuno et al 2008; Losfeld et al. 2015); *Garcinia amplexicaulis* (Clusiaceae) from New Caledonia (Jaffré 1980); two species each of *Phytolacca* (Phytolaccaceae) from China (Xue et al. 2004); *Polygonum* (Polygonaceae) from China (Deng et al. 2010), three species of *Denhamia* (formerly in *Maytenus*—Celastraceae) from New Caledonia and Australia (Jaffré 1977); two of *Virotia* (formerly in *Macadamia*—Proteaceae) from New Caledonia (Jaffré 1979); and nine of *Gossia* (formerly in *Austromyrtus* and *Eugenia*—Myrtaceae) from eastern Australia (Jaffré 1980; Bidwell et al. 2002; Fernando et al. 2008, 2009; Losfeld et al. 2015). Because of the extreme levels of Mn, locally reaching 2–5 wt% in dry matter in some of these species, the plant ash may contain 10–25 wt % Mn, which should make agromining for Mn worthy of further study and field trials.

5.5 Chromium

Even on ultramafic soils having high Cr concentrations (500–5000 μg g^{-1}) it is normal to find Cr in plant material in the range of 1–30 μg g^{-1}. Occasional reports of much higher concentrations are believed to reflect contamination by windblown dusts or smelter fallout, or analytical problems. High Cr concentrations in plants from ultramafic soils (up to 1000 μg g^{-1}) have been used as an indicator of soil contamination (see e.g. Jaffré et al. 1979b; Brooks and Yang 1984). In the absence to date of evidence for consistently high Cr concentrations existing in any species, there seems to be little hope of finding a future for agromining of this element.

5.6 Selenium

Selenium is essential for animal and human health and exhibits a narrow range between the levels required to prevent deficiency diseases and those that produce symptoms of toxicity. The Se content of soils is typically below 2 μg g^{-1} but can reach several hundred μg g^{-1} in soils derived from certain Cretaceous shales. In plant dry matter, Se concentrations are generally below 1 μg g^{-1} and may even be <0.01 μg g^{-1} in areas of Se-poor soils. However, the accumulation of Se to high levels (locally >1000 μg g^{-1}) by legumes in the genus *Astragalus* (Fabaceae) from seleniferous soils in the western United States was found to be responsible for the poisoning of livestock (Byers et al. 1938). A detailed account of the discovery of Se-accumulating plants in the western USA can be found elsewhere (Rosenfeld and Beath 1964). Reeves and Baker (2000) tabulated values and references for 20 species that have shown maximum Se concentrations above 1000 μg g^{-1}. Because of the very low levels of Se that normally occur in plants, a case can be made (Reeves 2005; van der Ent et al. 2013) for taking 100 μg g^{-1} as the threshold for Se hyperaccumulators. The use of plants showing some degree of Se accumulation for economic extraction of elemental Se has not been proposed. However, there are potential applications in: (i) phytoremediation of soils that have become Se-contaminated through extensive use of Se-rich irrigation waters (Parker et al. 2003), (ii) harvesting crop plants suitable for stock feed from high-Se areas and transport of this material to areas of Se deficiency (Bañuelos and Mayland 2000), and (iii) Se biofortification for improving human health (Bañuelos et al. 2014).

5.7 Arsenic

Normal As concentrations in igneous rocks and soils are in the range of 1–10 μg g^{-1}. Higher soil As concentrations can be found in areas of polymetallic sulfide mineralization and of some pyritic black shales, in places contaminated through the smelting of chalcophile element or gold ores, in areas of geothermal activity, and where As compounds have been used as horticultural sprays (e.g. blueberry fields in Maine, USA) or timber preservation agents. Plant As concentrations are normally on the order of 1 μg g^{-1}, but higher values can be found in contaminated areas. Arsenic hyperaccumulation (based on a 1000 μg g^{-1} dry matter criterion) has been known for more than 50 years. Warren et al. (1964) found As in the ash of growing tips of *Pseudotsuga menziesii* to be 2500–10 000 μg g^{-1} over soils containing 1000–5000 μg g^{-1} As. The highest of these values almost certainly corresponds to >1000 μg g^{-1} As on a dry weight basis.

Studies by several groups on the behaviour of aquatic plants in the Waikato River in the North Island of New Zealand showed that three aquatic plants act as As hyperaccumulators. Natural geothermal activity, together with borefield drainage and wastewater from the Wairakei geothermal power plant that opened in 1953, combined to raise the As concentration in the river from ca. 0.01 mg L^{-1} to as much as 0.08–0.09 mg L^{-1} before dilution and sedimentation processes lower the concentrations downstream. The adventive aquatic weeds *Ceratophyllum demersum* (Ceratophyllaceae), *Egeria densa,* and *Lagarosiphon major* (Hydrocharitaceae) act as As hyperaccumulators (Lancaster et al. 1971; Aggett and Aspell 1980; Liddle 1982; Reeves and Liddle 1986), yielding As concentrations in the plant dry matter from ca. 100 μg g^{-1} to 1000–1500 μg g^{-1}. The bioaccumulation factor, taken as the plant/substrate concentration ratio, can be as high as 30 000, e.g. where the plants contain 1500 μg g^{-1} in water with 0.05 mg L^{-1} As.

More recent attention has been paid to As accumulation by fern species, particularly those growing in areas of As contamination from waste disposal related to timber preservation processes or mining. Ma et al. (2001) reported As at 3280–4980 μg g^{-1} in *Pteris vittata* (Pteridaceae) plants from soils containing 19 to 1603 μg g^{-1} As. Arsenic hyperaccumulation was also found by Vittoottiviseth et al. (2002) in the fern *Pityrogramma calomelanos* (Pteridaceae). A number of fern species may possess this capability of As accumulation as a constitutive property (Meharg 2002). However, applications of As hyperaccumulators seem likely to lie more in the area of remediation of As-contaminated waters and land, rather than in economic extraction of the As itself.

5.8 Thallium

Currently only a small number of Tl hyperaccumulator plants have been reported, mainly from France: *Biscutella laevigata* (Brassicaceae) with up to 15 200 μg g^{-1} Tl (Anderson et al. 1999), and *Iberis intermedia* (Brassicaceae) (now regarded as a synonym of *I. linifolia*) with up to 2810 μg g^{-1} Tl (LaCoste et al. 1999; Leblanc et al. 1999). Van der Ent et al. (2013) proposed a threshold value of 100 μg g^{-1} to define Tl hyperaccumulation. The substantial value of Tl might justify Tl agromining, but the locations at which this could take place appear to be rather limited (Zn-Pb mine tailings mainly).

6 X-ray Fluorescence for Discovery of Hyperaccumulator Plants: Case Studies

The discovery of hyperaccumulator plants has largely been based on analytical methods (e.g. AAS, ICP-AES) after acid digestion or dry ashing of dried leaf material obtained from herbarium collections or field sampling. New technical advances now permit massive screening of herbarium specimens using non-destructive and rapid portable X-ray Fluorescence Spectroscopy (XRF), an approach that has already led to the discovery of numerous hyperaccumulator species new to science. A full assessment of the advantages and limitations of this method is given in Chapter "Tools for the Discovery of Hyperaccumulator Plant Species in the Field and in the Herbarium" of this book.

6.1 Herbarium XRF Discoveries in Papua New Guinea

The flora of Papua New Guinea is one of the richest worldwide (estimated at 25 000 plant species). However, no plant species from this country were reported as hyperaccumulators until a herbarium XRF scanning of native Papua New Guinean specimens in Queensland Herbarium (Australia) was performed by Do et al. (2020). During the scanning campaign, 3164 plant specimens were measured and a total of 19 hyperaccumulators were identified: one Ni hyperaccumulator (>1000 μg g^{-1} Ni), eight Mn accumulators (>5000 μg g^{-1} Mn) and 10 Zn hyperaccumulators (>3000 μg g^{-1} Zn). These hyperaccumulators were previously unknown, thus adding to the global inventory of hyperaccumulator plants.

6.2 Herbarium XRF Discoveries in Central America

In Central America, the first successful endeavour to discover hyperaccumulators was performed using a portable XRF instrument (McCartha et al. 2019). This study aimed to confirm the status of three species from the genus *Psychotria* (*P. grandis, P. costivenia* and *P. viridis*) that were reported to be hyperaccumulators in its neighbouring region, Greater Antilles. Also, it evaluated whether four species (*P. clivorum, P. flava, P. lorenciana* and *P. papantlensis*) that are close relatives of the three known hyperaccumulators do indeed hyperaccumulate. Results show that *P. costivenia, P. grandis, P. lorenciana* and *P. papantlensis* were identified as valid hyperaccumulators, the two latter being obligate. The study also found that the geographic distribution of these Ni hyperaccumulators does not correspond to that of Ni-laterite soils or more widely to ultramafic outcrops. Such a finding is another benefit offered by herbarium XRF scanning that can be used in an initial stage of exploration. Recently, when sampling these species of *Psychotria* in the field in southeastern Mexico and Central America, two other interesting groups of Ni hyperaccumulators were identified. The first belongs also to the Rubiaceae and includes the monospecific genus *Blepharidium* (*B. guatemalense*) and the closely related *Arachnothryx longiflorum*. *Blepharidium guatemalense* exhibits the same extraordinary green Ni-rich phloem tissues as some of the Ni hyperaccumulators from Southeast Asia and the Pacific region. Its leaves can contain Ni concentrations above 2 wt%. The second group belongs to the Violaceae and includes two very closely related genera (*Orthion* and the mono-specific genus *Mayanaea*). Brooks et al. (1977a) found from herbarium sampling that a species of *Hybanthus* from Mexico, *H. malpighiifolius*, had unusually high Ni concentrations in leaves (638 μg g^{-1}) and therefore qualified as a 'strong' accumulator. Since then, several species of *Hybanthus* from the New World were grouped in the genus *Orthion* (Wahlert et al. 2014), in which nearly all species are Ni hyperaccumulators (facultative and obligate). Unsurprisingly, *Mayanaea* and *Orthion* belong to the same phylogenetic clade as the hyperaccumulating *Hybanthus* from Australia and New Caledonia (Wahlert et al. 2014). Among *Orthion* species, *O. subsessile* is an obligate hyperaccumulator that can have Ni concentrations in leaves that exceed 2 wt%. Further XRF herbarium scanning campaigns revealed that at least four of the six species of *Orthion* and *Mayanaea caudata* are Ni hyperaccumulators, and that these species are not endemic to ultramafic soils although some are obligate Ni hyperaccumulators (Navarrete-Gutiérrez, to be submitted). In total, 14 Ni hyperaccumulator taxa are now identified in Central America, most of which are not endemic to ultramafic areas (McCartha et al. 2019) because local soils have notably high Ni concentrations independent of the presence of ultramafic substrates.

6.3 Herbarium XRF Discoveries in New Caledonia

Pioneer geobotanical studies were carried out in the 1970s in New Caledonia, where many hyperaccumulator plants have been discovered (Jaffré et al. 1976; 1979a; 1979b; Kersten et al. 1979). Current knowledge of the flora and availability of plant specimens in herbaria have provided a unique opportunity to carry out a systematic assessment of the incidence of hyperaccumulation in the regional flora. XRF herbarium screening was undertaken at the Herbarium of New Caledonia (NOU) on ca. 11 200 herbarium specimens. The selection of herbarium specimens to scan was based on families that were already known to contain numerous hyperaccumulator species (Jaffré et al. 1976, 1979a, 2013). All available specimens were scanned in the Cunoniaceae, Phyllanthaceae, Salicaceae, Sapotaceae, Oncothecaceae, and Violaceae, as well as a systematic screening of one to four specimens (depending on availability) of species known to occur on ultramafic soils in New Caledonia (Isnard et al. 2016). This screening included 1484 species (1620 taxa) covering 35 orders, 96 families, 281 genera, and ~89% of the ultramafic-related dicotyledonous flora. The study led to the recording of numerous hyperaccumulator plant species: 99 taxa for Ni (65 known previously), 74 taxa for Mn (11 known previously), eight taxa for Co (two known previously), and four taxa for Zn (none previously recorded). This work demonstrated that XRF screening of herbarium specimens has the potential to discover vast numbers of new hyperaccumulator species, even in well-studied flora such that of New Caledonia. New hyperaccumulator species are also expected to be discovered in the field, as demonstrated by the recently described new species *Pycnandra caeruleilatex* (Swenson and Munzinger 2010) and *Pycnandra kouakouensis* (Swenson and Munzinger 2016), both of which have a bluish or greenish latex. These species were confirmed to be strong hyperaccumulators (Gei et al. 2020). This approach points to further opportunities to study the ecology and biogeography of hyperaccumulation.

6.4 Herbarium XRF Discoveries in Sabah, Malaysia

In Sabah, Malaysia, a recent herbarium XRF scanning campaign scanned a total of ~7300 plant species (van der Ent et al. 2019). This campaign recorded 91 hyperaccumulators: 28 Ni hyperaccumulators, 12 Co hyperaccumulators and 51 Mn hyperaccumulators. Among 51 Mn hyperaccumulators, 14 Mn hyperaccumulators were previously known from Sabah.

Interestingly, most Mn (hyper) accumulative plants encountered in this campaign did not occur in ultramafic soils. Cobalt hyperaccumulation is rare, even with a Co hyperaccumulation threshold of >300 μg g^{-1}. Nevertheless, this campaign discovered a species, *Ashtonia excelsa* that accumulates 1500 μg g^{-1} Co. The study further demonstrates the usefulness of the herbarium XRF scanning technique for identifying hyperaccumulators.

7 Knowledge Gaps: Priority Regions for Exploration and Discovery

Currently it is estimated that hyperaccumulation occurs in 0.2% of angiosperms and 1–2% of known ultramafic global flora (Baker et al. 2000; Baker and Brooks 1989; Cappa and Pilon-Smits 2014). It is certain that more unidentified hyperaccumulator species remain to be discovered. Systematic herbarium specimen XRF scanning, combined with auxiliary collection data, can provide insights into phylogenetic patterns of hyperaccumulation, and has the potential to complement and add insights to biogeographical and evolutionary studies.

There is also a need for further field exploration of ultramafic and other metalliferous areas that have not so far been subjected to extensive exploration and collection of herbarium material. Furthermore, because herbarium analysis most often consists of a single sample taken from a population, in the most striking cases of hyperaccumulation (where there is agromining potential), there is a need for more detailed field investigation of various occurrences of the species. This needs to be done (i) to obtain reliable statistical information on the distribution of metal concentration within each population, and their relation to local soil, (ii) to examine interpopulation variability, and (iii) to obtain information about the natural reproduction of the species and any significant interactions with other biota in the immediate environment of the specimens.

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Physiology and Molecular Biology of Trace Element Hyperaccumulation

Sylvain Merlot, Vanesa Sanchez Garcia de la Torre,
and Marc Hanikenne

Abstract

Metals (trace elements) are essential for plants but become toxic at high concentration. Remarkably, about 700 species worldwide are able to accumulate large quantities of metals in their leaves and are therefore called metal hyperaccumulators. In the context of sustainable development, there is renewed interest in understanding the mechanisms of metal hyperaccumulation that may become instrumental for improved metal phytoextraction from contaminated soils and for making metals available at lower environmental cost. In addition, studying the molecular mechanisms of hyperaccumulation in diverse plant species is necessary in order to understand the evolution of this extreme and complex adaptation trait in plants. Our current knowledge of metal hyperaccumulation is based mostly on the analysis of few species from the Brassicaceae family and suggests that the underlying mechanisms result from an exaggeration of the basic mechanisms involved in metal homeostasis. However, the development of Next Generation Sequencing technologies enables the study of new hyperaccumulator species and therefore the revealing of greater diversity in these mechanisms. The goal of this chapter is to provide background information on metal hyperaccumulation and give an instantaneous picture of what is currently known about the molecular mechanisms involved in this trait. We also attempt to outline for the reader the future scientific challenges that this field of research is facing.

1 Introduction

Investigating the mechanisms involved in metal hyperaccumulation allows observation of extreme adaptation of the metal homeostasis network in plants and identification of key players in metal distribution and tolerance within plant tissues. The study of metal hyperaccumulator species also allows understanding the genetic mechanisms involved in the evolution of an extreme adaptive trait (Shahzad et al. 2010; Hanikenne and Nouet 2011; Hanikenne et al. 2013). Several comprehensive reviews on metal hyperaccumulation have been published in the past years and we refer interested readers to those reviews (Verbruggen et al. 2009, 2013a; Krämer 2010; Hanikenne and Nouet 2011; Rascio and

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S. Merlot (✉) · V. S. Garcia de la Torre
Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC),
91198 Gif-sur-Yvette, France
e-mail: sylvain.merlot@i2bc.paris-saclay.fr

M. Hanikenne
InBioS-PhytoSystems, Functional Genomics and Plant Molecular Imaging, University of Liège,
4000 Liège, Belgium

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
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Navari-Izzo 2011; Ricachenevsky et al. 2015; Van der Pas and Ingle 2019).

Whereas hyperaccumulator species commonly accumulate one metal when growing in their natural environment, some species have the ability to tolerate and accumulate several metals when grown ex situ. The latter case is well documented for the hyperaccumulator species of the Brassicaceae family *Noccaea caerulescens*, in which serpentine-adapted accessions such as Puy de Wolf, Monte Prinzera, and Puente Basadre are able to accumulate Ni but also Zn and Cd (Peer et al. 2003; Assunção et al. 2003; Escarré et al. 2013; Gonneau et al. 2014; Callahan et al. 2016). This ability to tolerate and accumulate several metals likely reflects the relatively low specificity of some mechanisms involved in metal transport and chelation in plants.

Several metals that are concentrated in hyperaccumulator species (e.g. Zn, Ni, Mn) are essential nutrients but become toxic at high levels for most plants (i.e. non-accumulating species). Therefore, all plant species have developed mechanisms to regulate essential metal homeostasis according to their needs and metal availability in soils (Burkhead et al. 2009; Thomine and Vert 2013; Ricachenevsky et al. 2015; Shao et al. 2017; Clemens 2019; Kobayashi et al. 2019). Our current knowledge suggests that the molecular mechanisms involved in metal hyperaccumulation essentially derive from the mechanisms involved in metal homeostasis. In several examples, genes involved in metal homeostasis are differentially expressed in hyperaccumulators compared to related, non-accumulator species as a result of gene duplication and/or changes in gene promoter activity (Talke et al. 2006; van de Mortel et al. 2006; Krämer et al. 2007; Hanikenne et al. 2008; Shahzad et al. 2010; Suryawanshi et al. 2016). However, specific genes linked to hyperaccumulation may be discovered as future molecular analyses of hyperaccumulation are extended to additional non-model species from various plant families, thanks to the development of high-throughput DNA sequencing technologies (Verbruggen et al. 2013a; Halimaa et al. 2014b; Merlot et al. 2014; Meier et al. 2018). For most metals, the hyperaccumulation trait appeared independently in distant plant families. Therefore, some of the mechanisms involved in metal hyperaccumulation may be specific to a plant family or a species, whereas other mechanisms may be convergent among distant hyperaccumulators. For instance, several examples of convergent evolution have been identified between the Brassicaceae *Arabidopsis halleri* and *Noccaea caerulescens* (see below and Krämer et al. 2007; Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012), suggesting important functional constraints in the metal homeostasis network.

Because of its singularity, metal hyperaccumulation may appear as an exception with limited relevance. However, from a scientific point of view, metal hyperaccumulation in plants is fascinating, and understanding the mechanisms involved in this trait may provide tools needed in order to extract metals from soil with a lower impact on the environment in the near future. The goal in this chapter is to outline our current knowledge about molecular mechanisms of metal hyperaccumulation in plants and to highlight possible future developments in this field of research.

2 Molecular Physiology of Metal Hyperaccumulation

2.1 Main Steps of Metal Hyperaccumulation

When exposed to excess metals, most plant species adopt a so-called excluder strategy to prevent metal accumulation in photosynthetically active shoot tissues (Krämer 2010). This result can be achieved by limiting metal absorption by roots, increasing metal efflux from root tissues, and/or increasing metal storage in root cell walls and vacuoles. In contrast, achieving metal hyperaccumulation and associated (hyper)tolerance requires modifications at specific nodes of the metal homeostasis network to ensure that the metal flux in the plant is directed towards shoot tissues (Fig. 1; Clemens et al. 2002). At the physiological level, these alterations include some or all of the following steps:

1. An enhanced metal mobilization and uptake in roots;
2. An efficient radial metal transport towards the root vascular tissues. This includes a reduction of metal storage in root vacuoles;
3. An increased transport of metal from the root to the shoot, with efficient xylem loading. This step contributes to metal tolerance by enabling metal storage (and thus detoxification) in shoot tissues;
4. An efficient mechanism for xylem unloading and metal distribution in shoots together with high vacuolar storage capacity.

In addition, accumulating evidences suggest that modifications of the cell wall, which has a large metal-binding capacity (Krzesłowska 2011), is also contributing to knowledge of metal accumulation and tolerance (Meyer et al. 2015; Peng et al. 2017; Corso et al. 2018; Lešková et al. 2019). Further investigations will reveal in detail the role of the cell wall in roots and shoots in achieving metal hyperaccumulation and hypertolerance.

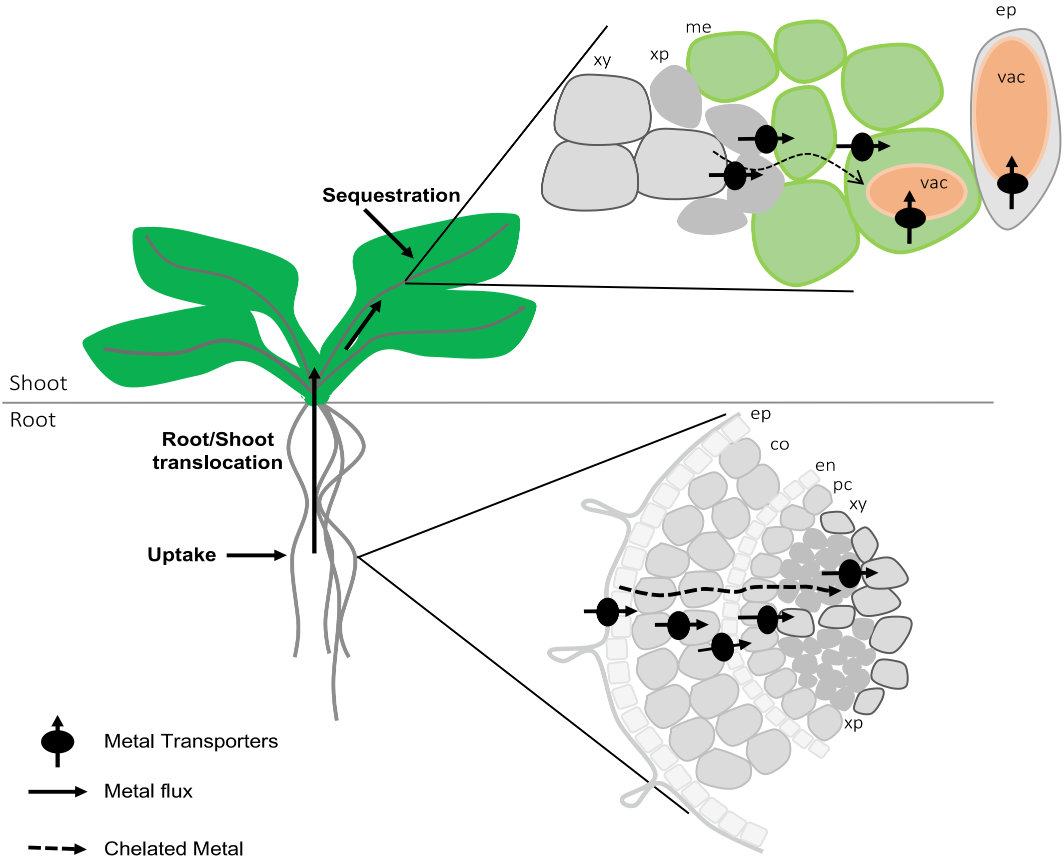

Fig. 1 Model of the physiology of metal hyperaccumulation and hypertolerance. Enhanced metal uptake and radial transport in roots, xylem loading/unloading and vacuolar storage in shoots all make major contributions to the traits. In roots, it is possible that the metal can travel in the apoplasm up to the endodermis cell layer before cellular uptake (not represented). Note that the tissue (epidermis or mesophyll) involved in metal storage varies depending on the species and the metal (see text). co: cortex; en: endodermis; ep: epidermis; me: mesophyll; Zinc-NA: Zinc-Nicotianamine chelatesmanganese accumulation and phosphorus-acquisition; pc: pericycle; vac: vacuole; xp: xylem parenchyma; xy: xylem. Figure modified from Hanikenne and Nouet (2011)

2.2 Metal Distribution in Shoots

Metal distribution in shoot tissues is specific to both the species and the metal considered. This topic has been extensively reviewed (Fernando et al. 2013; Leitenmaier and Küpper 2013; van der Ent et al. 2018; Kopittke et al. 2018). Briefly, in most cases, metals (Zn, Cd, Ni or Se) accumulate at the basis of the trichomes and in the vacuoles of epidermal cells. In contrast, mesophyll cells that are the main site of photosynthesis accumulate lower quantities of metals (Küpper et al. 1999, 2001; Lombi et al. 2002; Cosio et al. 2005). In the vacuoles of epidermal cells, metals can reach very high concentrations (e.g. several hundred mM, Küpper et al. 1999; Fernando et al. 2006a). There are, however, some exceptions. For instance, Zn and/or Cd are stored in the vacuoles of mesophyll cells of the hyperaccumulators *Arabidopsis halleri*, *Noccaea caerulescens* and *Sedum alfredii*, where Zn is mostly bound to malate (Küpper et al. 2000; Sarret et al. 2002, 2009; Tian et al. 2011; Lu et al. 2014; Isaure et al. 2015; van der Ent et al. 2019). A significant amount of Cd is also found in the vascular tissues of the main vein in leaves of *A. halleri* ssp. *gemmifera* (Fukuda et al. 2020). Lead, mostly bound to acetate, accumulates in the epidermis and collenchyma vascular cells of petioles and leaf blades in *A. halleri* ssp. *halleri* (Höreth et al. 2020). In the hyperaccumulator *Sedum plumbizincola*, Zn accumulates mostly in leaf epidermal cells, but also in large amounts in mesophyll cells of young leaves (Cao et al. 2014). In this species, Cd is mostly bound to cell walls in leaves (Peng et al. 2017). Accumulation of Mn in mesophyll cells is also observed in several Mn hyperaccumulators (Fernando et al. 2006a, b, 2013).

Because it requires access to rare and expensive infrastructures, metal imaging has been, and often still remains, a bottleneck in the characterization of metal hyperaccumulators, and more generally in investigations of metal homeostasis mechanisms in plants. The rapid development of metal imaging technologies, including sample preparation protocols that preserve metal distribution and speciation in tissues, is nonetheless progressively alleviating this bottleneck (van der Ent et al. 2018; Kopittke et al. 2018).

2.3 Identification of Molecular Actors Involved in Metal Hyperaccumulation

In the last 15 years, a number of complementary approaches have been used to identify the molecular actors underlying hyperaccumulation and hypertolerance. These approaches include:

1. Screens of cDNA libraries in yeast to isolate genes contributing to metal transport and tolerance (e.g. Bernard et al. 2004; Lasat et al. 2000; Papoyan and Kochian 2004; Pence et al. 2000);
2. Quantitative genetics analyses aiming to identify Quantitative Traits Loci (QTLs) co-segregating with the traits in progenies of crosses between an hyperaccumulator and a related non-accumulator species, or between accessions of a species with contrasting hyperaccumulation and/or tolerance phenotypes (Dräger et al. 2004; Deniau et al. 2006; Courbot et al. 2007; Filatov et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Baliardini et al. 2015; Karam et al. 2019);
3. Transcriptomic studies comparing gene expression levels in hyperaccumulator and related non-accumulator species or accessions. This approach has benefited more recently from the development of the RNA-Seq technology (Becher et al. 2004; Weber et al. 2004, 2006; Chiang et al. 2006; Talke et al. 2006; van de Mortel et al. 2006, 2008; Craciun et al. 2006; Filatov et al. 2006; Hammond et al. 2006; Gao et al. 2013; Halimaa et al. 2014b, 2019; Milner et al. 2014; Han et al. 2016; Peng et al. 2017; Corso et al. 2018; Garcia de la Torre et al. 2020 ; Schvartzman et al. 2018).

Candidate genes mostly involved in metal transport, metal chelator synthesis, or metal-induced oxidative stress response were indentified with these approaches. Note that the last category will not be discussed further in this chapter. Several candidate genes were characterized functionally (Pence et al. 2000; Persans et al. 2001; Dräger et al. 2004; Kim et al. 2004; Hanikenne et al. 2008; Gustin et al. 2009; Lin et al. 2009; Shahzad et al. 2010; Ueno et al. 2011; Milner et al. 2012, 2014; Deinlein et al. 2012; Merlot et al. 2014; Baliardini et al. 2015; Nouet et al. 2015; Charlier et al. 2015; Cornu et al. 2015; Ahmadi et al. 2018; Uraguchi et al. 2019; Spielmann et al. 2020). However, only a few candidates (i.e. *HMA4*, *HMA3*, *NAS2*, *ZIP6* and *CAX1*) were confirmed by reverse genetics in hyperaccumulator species as major players in metal hyperaccumulation and tolerance. Their functions are described in detail in the next sections.

A large part of our knowledge of metal hyperaccumulation comes from the study of two model Zn and Cd hyperaccumulating species of the Brassicaceae family, *A. halleri* and *N. caerulescens*, which are related to the sensitive and non-accumulating species *A. thaliana* (Yogeeswaran et al. 2005; Clauss and Koch 2006). These two species have been instrumental in successfully improving our understanding of the physiological, molecular, and genetic bases of metal hyperaccumulation and associated hypertolerance (Krämer et al. 2007; Milner and Kochian 2008; Pauwels et al. 2008; Roosens et al. 2008; Verbruggen et al. 2009, 2013b; Krämer 2010; Hanikenne and Nouet 2011; Honjo and Kudoh 2019). Those successes relied on the availability of the *A. thaliana* genome sequence (The Arabidopsis Genome Initiative 2000) and dedicated tools and resources combined with relatively high gene sequence conservation among Brassicaceae species: 94 and 88% identity with *A. thaliana* for *A. halleri* and *N. caerulescens*, respectively (Talke et al. 2006; van de Mortel et al. 2006). It is expected that our knowledge base will rapidly become broader with new species becoming accessible to molecular and genomic analyses (Gao et al. 2013; Verbruggen et al. 2013a; Merlot et al. 2014; Van der Pas and Ingle 2019).

3 Mechanisms of Zinc and Cadmium Hyperaccumulation

As mentioned above, most of our understanding of Zn and Cd hyperaccumulation was acquired using *A. halleri* and *N. caerulescens* (Fig. 2). More recent models include (i) the Crassulaceae *Sedum alfredii* and *Sedum plumbizincola* from Asia, which are among the few species reported to hyperaccumulate Cd outside the Brassicaceae (Yang et al. 2004, 2006; Deng et al. 2007; Wu et al. 2013; Cao et al. 2014; Peng et al. 2017; Li et al. 2018); and (ii) the Amaranthaceae *Gomphrena claussenii* from South America, which is strongly tolerant to Zn and Cd, and presents indicator levels of Zn and Cd accumulations (Villafort Carvalho et al. 2013, 2015; Pongrac et al. 2018).

Arabidopsis halleri and *N. caerulescens* display constitutive Zn hyperaccumulation and hypertolerance, although intraspecific variation for those traits has been reported (Bert et al. 2000, 2002; Reeves et al. 2001; Assunção et al. 2003; Molitor et al. 2005; Pauwels et al. 2006; Besnard et al. 2009; Stein et al. 2017). Similarly, hyperaccumulation of Cd shows substantial intraspecific variation (Escarré et al. 2000; Bert et al. 2002; Roosens et al. 2003; Verbruggen et al. 2013b; Meyer et al. 2015; Stein et al. 2017). Metal hyperaccumulation has evolved independently in the two species (Krämer 2010). However, both share a set of alterations of their metal homeostasis networks in comparison to the non-accumulator *A. thaliana*, which we detail below.

3.1 Uptake of Zinc and Cadmium

Prior to uptake, it is suggested that metals are actively mobilized from the soil, by acidification and/or chelate secretion (Clemens et al. 2002). It was, however, suggested that *A. halleri* roots secrete elevated levels of nicotiniamine (NA), a metal chelator able to form NA-Zn complexes (Curie et al. 2009; Clemens et al. 2013) that may reduce root Zn uptake and increase tolerance to this metal (Tsednee et al. 2014). Another report

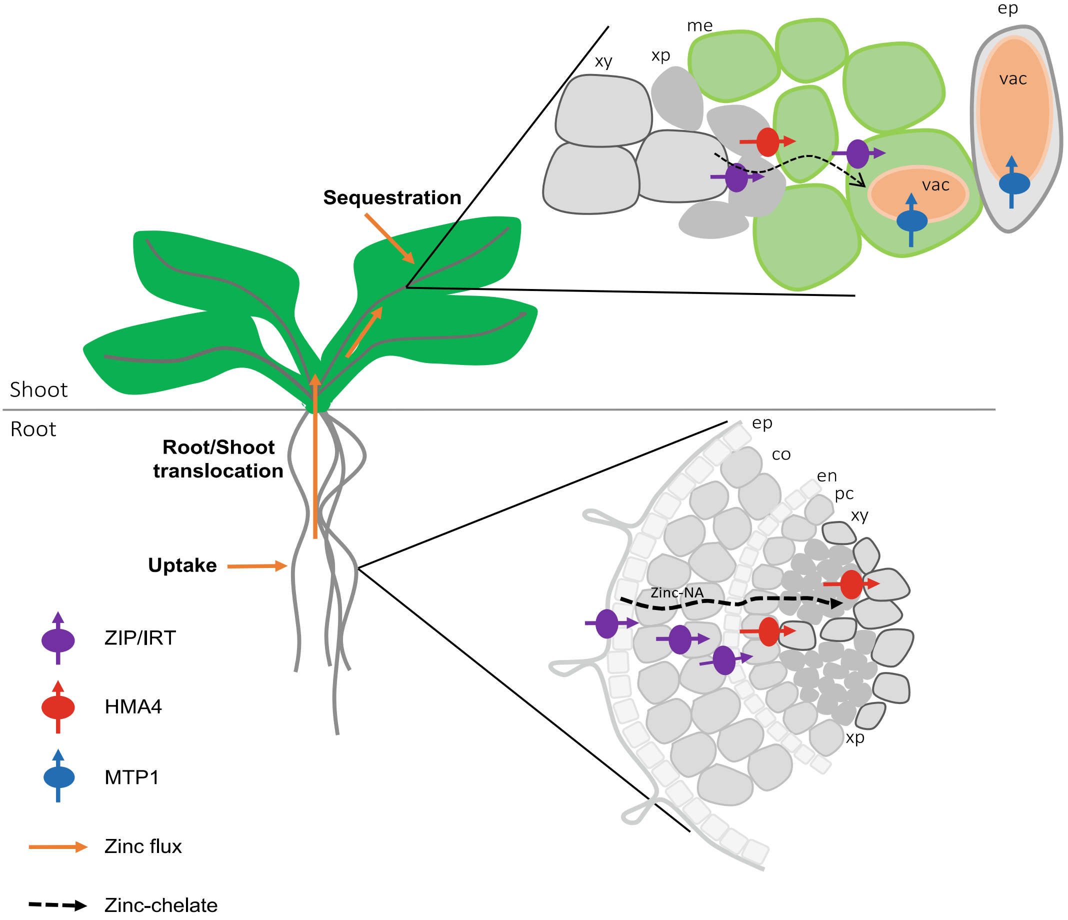

Fig. 2 Model for Zn hyperaccumulation and hypertolerance in the Brassicaceae *A. halleri* and *N. caerulescens*. Enhanced functions of ZIP transporters in cellular uptake, of the P-type ATPase HMA4 in xylem loading/unloading and of MTP1 in vacuolar storage all make major contributions to the traits. The exact functions and localizations of individual ZIPs are unknown. In roots, the metal chelator nicotianamine (NA) possibly favours Zn radial transport towards the xylem by symplastic inter-cellular mobility of Zn by either allowing Zn movement through plasmodesmata and/or preventing vacuolar storage. Vacuolar storage in shoots occurs in the epidermis in *N. caerulescens* and in the mesophyll in *A. halleri*. Additional metal homeostasis genes that are highly expressed in both hyperaccumulators are discussed in the text. Note that similar mechanisms have been identified as more active in a metal hyperaccumulating population of the Crassulaceae *S. alfredii* compared to a non-accumulating population or in or *S. plumbizincola*. co: cortex; en: endodermis; ep: epidermis; me: mesophyll; Zinc-NA: Zinc-Nicotianamine chelates; pc: pericycle; vac: vacuole; xp: xylem parenchyma; xy: xylem. Figure modified from Hanikenne and Nouet (2011)

indicated higher organic acid levels and Zn mobilization in the dissolved organic matter in the rhizosphere of hyperaccumulator, compared to non-accumulator accessions of *S. alfredii* (Li et al. 2012).

Several divalent metal transporters of the ZIP (Zrt-Irt-like Protein) family are highly expressed in roots and/or shoots of both *A. halleri* and *N. caerulescens* (Talke et al. 2006; Krämer et al. 2007; Lin et al. 2009, 2016; Wu et al. 2009). This presumably results in enhanced rates of metal uptake in roots or mobilization from root storage sites. By contributing to Zn radial transport towards the xylem in roots, it may also contribute to metal partitioning between root and shoot tissues. Several ZIP genes are induced by Zn deficiency under the control of the bZIP19 and bZIP23 transcription factors in *A. thaliana*

(Assunção et al. 2010). Their high expression in *A. halleri* and *N. caerulescens* roots could be the direct consequence of the high activity of HMA4 (Heavy Metal ATPase 4, see below), which depletes Zn in roots (Talke et al. 2006; Hanikenne et al. 2008; Gustin et al. 2009). Several ZIP genes are also highly expressed in *S. plumbizincola* and in *S. alfredii* hyperaccumulator individuals (Peng et al. 2017; Yang et al. 2018). Recently, intraspecific comparison in both *A. halleri* and *N. caerulescens* has revealed that the expression of the ZIP transporter IRT1 (Iron-Regulated Transporter 1) correlates with variation of Zn and/or Cd accumulation among populations (Corso et al. 2018; Schvartzman et al. 2018; Halimaa et al. 2019). IRT1 encodes the main Fe-uptake transporter at the root epidermis in *A. thaliana* (Vert et al. 2002; Thomine and Vert 2013), but because of a low selectivity, it is also responsible for the uptake of additional divalent metal cations such as Zn, Cd, or Ni (Korshunova et al. 1999; Vert et al. 2002; Nishida et al. 2011; Barberon et al. 2011, see also Ni section below). Constitutive differences in gene expression and protein levels of IRT1 were linked to differential Zn and Cd shoot accumulation among Polish and Italian metalicollous populations of *A. halleri* (Corso et al. 2018; Schvartzman et al. 2018). Variation of *IRT1* expression level, together with altered functionality of the protein, is also observed among two calamine populations of *N. caerulescens* with distinct Cd accumulation capacity (Halimaa et al. 2019). These later reports suggest that Zn and Cd differentially interfere with Fe homeostasis and induce a Fe deficiency response in these *A. halleri* and *N. caerulescens* populations. The authors also suggest that maintaining Fe homeostasis is key in Zn and Cd hyperaccumulators and that alternative strategies evolved to do so among populations.

Additional studies are required to determine the individual function of ZIP transporters in hyperaccumulation. The respective contribution of Zn and/or Fe transport mechanisms to Zn and Cd uptake also needs to be assessed in detail (Meyer and Verbruggen 2012).

3.2 Root-to-Shoot Transfer of Zinc and Cadmium

Increased rate of root-to-shoot metal transfer is key to achieving metal hyperaccumulation in shoots. It requires enhanced radial transport to xylem, decreased vacuolar storage in root cells, and efficient xylem loading. Several *NAS* (nicotianamine synthase) genes are highly expressed in *A. halleri* and *N. caerulescens* (Weber et al. 2004; van de Mortel et al. 2006; Deinlein et al. 2012). *NAS* transcript levels are also higher in roots of a hyperaccumulator accession compared to a non-hyperaccumulator accession in *S. alfredii* (Liang et al. 2014). Elevated levels of NA have been measured in roots of *A. halleri* compared to *A. thaliana* (Weber et al. 2004; Deinlein et al. 2012). It was further shown using *A. halleri* RNAi lines that high expression of the *NAS2* gene provides increased NA levels for Zn symplastic mobility towards the xylem, and for controlling the rate of Zn xylem loading in roots (Deinlein et al. 2012; Cornu et al. 2015). This process is important in order to enable Zn hyperaccumulation in plants exposed to a wide range of Zn availability in the soil (Uraguchi et al. 2019). The concentration of the amino-acid histidine (His) weakly correlates with Zn content in *N. caerulescens* and His was shown to enhance Zn xylem loading, thus contributing to reduce Zn storage in roots (Callahan et al. 2007; Kozhevnikova et al. 2014).

In *A. halleri*, Zn and Cd loading into the xylem is driven by the HMA4 protein (Talke et al. 2006; Courbot et al. 2007; Hanikenne et al. 2008), which is a plasma membrane P-Type ATPase pump that uses the energy released from the hydrolysis of ATP to transport the metal against the electro-chemical gradient (Hussain et al. 2004; Wong and Cobbett 2009; Pedersen et al. 2012; Hanikenne and Baurain 2014). The *HMA4* gene co-segregates with QTLs for Zn and Cd tolerance and accumulation (Courbot et al. 2007; Willems et al. 2007, 2010; Frérot et al. 2010; Meyer et al. 2016). High expression of *HMA4* is required for both hyperaccumulation and hypertolerance in *A. halleri* (Talke et al.

2006; Hanikenne et al. 2008). Increased gene dosage of *HMA4* was selected during the evolutionary history of *A. halleri*, and evolved through tandem triplication and activation in *cis* of the promoters of all three copies (Hanikenne et al. 2008, 2013). The *A. halleri HMA4* locus was shaped by positive selection, resulting in a selective sweep and ectopic gene conversion (Hanikenne et al. 2013). The three *HMA4* copies are mainly active in vascular tissues of *A. halleri*, which allows acting in xylem metal loading in roots and possibly in metal distribution in leaves. It may also ensure metal exclusion from metal-sensitive tissues (e.g. root tip, cambium). By controlling highly active Zn xylem loading, HMA4 also acts as a physiological regulator: it depletes the root Zn pool, which triggers a Zn-deficiency response resulting in high expression of several *ZIP* genes (Hanikenne et al. 2008). In agreement, modeling of the Zn supply-dependent spatio-temporal evolution of Zn concentration in root symplast and apoplast of *A. thaliana* predicted that slight changes in *HMA4* transcript levels have a major impact on the radial distribution of Zn in roots and on the root to shoot Zn gradient (Claus et al. 2013). This result was recently confirmed in *A. halleri* using ^{65}Zn imaging (Kajala et al. 2019). It was further shown that a certain extent of functional differentiation exists among the three *AhHMA4* copies when expressed in *A. thaliana*, stemming from differences in expression levels rather than in expression profiles. Interestingly, *AhHMA4* copy 3 was subjected to the strongest, possibly most recent, positive selection during the evolutionary history of *A. halleri*, thus linking sequence diversity patterns and function in vivo (Hanikenne et al. 2013; Nouet et al. 2015).

HMA4 is also highly expressed in *N. caerulescens* as well as in Zn and Cd hyperaccumulator accessions of *S. alfredii* and *S. plumbizincola*, wherein it very likely plays similar roles to the *A. halleri HMA4* (Bernard et al. 2004; Papoyan and Kochian 2004; van de Mortel et al. 2006; O' Lochlainn et al. 2011; Craciun et al. 2012; Zhang et al. 2016; Peng et al. 2017). Moreover, the gene coding for the ZIP transporter ZNT1 of *N. caerulescens* is highly expressed in cortex, endodermis, and pericycle root cells. When expressed in *A. thaliana*, it contributes to Zn and Cd tolerance and accumulation. The *NcZNT1* gene therefore may be involved in Zn and Cd influx into cells responsible for xylem loading, providing metals for transport by HMA4 (Milner et al. 2014; Lin et al. 2016). The ortholog of *ZNT1* in *A. halleri*, *ZIP4*, is also highly expressed and may contribute to a similar function (Talke et al. 2006). Note that NRAMP1 (Natural Resistance-Associated Macrophage Protein 1) may also play a similar role for Cd in *N. caerulescens* (Milner et al. 2014). Moreover, the vacuolar metal efflux transporters NRAMP3 and NRAMP4 are highly expressed in *N. caerulescens* and *A. halleri* roots, and were proposed to limit vacuolar storage and increase metal mobility (Weber et al. 2004; Oomen et al. 2009). *NRAMP3* is also highly expressed in *S. plumbizincola* (Peng et al. 2017).

Once in the xylem sap, metals are transported to the shoot via the evapo-transpiration stream. Within this compartment, Zn is mainly bound to organic acids such as malate and citrate (Monsant et al. 2011; Lu et al. 2013; Cornu et al. 2015).

3.3 Storage of Zinc and Cadmium in Leaves

It is suggested that HMA4 and ZIP transporters also play an important role in Zn unloading and distribution in shoot tissues (Krämer et al. 2007; Hanikenne and Nouet 2011). However, their exact contribution, as well as the contribution of metal ligands or other transporters, to these processes remains to be detailed. Zinc storage in vacuoles is most likely ensured by the MTP1 (Metal Tolerance Protein 1) protein in *A. halleri*; MTP1 is a vacuolar transporter implicated in Zn tolerance (Krämer 2005). The MTP1 gene is constitutively highly expressed in both root and shoot of *A. halleri* and is present in four to five copies that are located on three distinct linkage groups in the genome (Dräger et al. 2004; Talke et al. 2006; Willems et al. 2007; Shahzad et al. 2010; Fasani et al. 2017). The two most highly expressed copies each co-segregate with QTLs for Zn tolerance (Dräger et al. 2004; Willems et al. 2007; Shahzad et al. 2010).

MTP1 is also highly expressed in *Noccaea goesingensis*, another Zn and Ni hyperaccumulator, in *N. caerulescens* and in Zn-hyperaccumulating populations of *S. alfredii* (Milner and Kochian 2008; Gustin et al. 2009; Zhang et al. 2011). It likely plays a similar role in these species. No detailed information is currently available on the molecular mechanisms of Cd storage in *A. halleri* shoot vacuoles (Meyer and Verbruggen 2012). Indeed, MTP1 is not associated with high Cd tolerance or accumulation in *A. halleri* (Courbot et al. 2007; Willems et al. 2010). However, expression of MTP1-related proteins from the Ni hyperaccumulator *N. goesingense* were shown to confer Cd tolerance when expressed in yeast, suggesting an activity of Cd transport (Persans et al. 2001). The Heavy Metal ATPase HMA3, which localizes at the vacuole (Morel et al. 2009), may also contribute to this process in *N. caerulescens* (Ueno et al. 2011). Owing to RNAi lines, HMA3 was shown to be essential for Cd tolerance in shoots of *S. plumbizincola* (Liu et al. 2017).

Recent work suggests that specific mechanisms take place in the leaves of metal hyperaccumulators to protect the photosynthetic apparatus from excess of Zn and Cd (Bayçu et al. 2017; Szopiński et al. 2019). Indeed, *HMA1* (Heavy Metal ATPase 1), encoding a chloroplastic metal transporter (Hanikenne and Baurain 2014), is highly expressed in shoots of *S. plumbizincola* (Zhao et al. 2019). HMA1 is required for Cd exclusion from the chloroplast: *hma1* mutant lines display increased Cd accumulation in chloroplasts, increased Cd sensitivity, and strongly altered photosystem II activity (Zhao et al. 2019). In *A. halleri*, mapping in a F2 progeny of an intraspecific cross between Italian metallicolous and non-metallicolous individuals identified one major Zn tolerance QTL for photosynthetic yield. The *NRAMP3* gene is associated with this QTL and was highly expressed in Zn-tolerant F2 plants (Karam et al. 2019). In *A. thaliana*, NRAMP3 and its paralog NRAMP4, contribute to excess Zn and Cd tolerance by mediating appropriate Fe and Mn supply to chloroplasts from vacuole stores, thus maintaining the photosynthetic function (Molins et al. 2013). NRAMP3 may play a similar role in *A. halleri*.

3.4 Additional Candidate Genes for Zinc and Cadmium Accumulation and Tolerance

A few additional candidate genes for a role in Zn or Cd tolerance have been functionally characterized. In *A. halleri*, the PDF1.1 (Plant Defensin 1.1) protein was identified through a cDNA screen in yeast as being a contributor to Zn tolerance. It also confers Zn tolerance when ectopically overexpressed in *A. thaliana* and is more highly expressed in shoots of *A. halleri* compared to *A. thaliana* (Mirouze et al. 2006). PDFs were initially known as secreted antifungal proteins and are characterized by a cysteine-stabilized α-helix β-sheet structure (De Coninck et al. 2013; van der Weerden and Anderson 2013). However, AhPDF1.1 localizes in intracellular compartments (Oomen et al. 2011). Family wide comparison of *A. halleri* and *A. thaliana PDF1* genes revealed that the molecular function of the *A. thaliana* and *A. halleri* proteins in Zn tolerance and antifungal activity is conserved, and that functional differences in the two species may result from differential expression levels and regulation (Shahzad et al. 2013; Nguyen et al. 2014).

The fine-scale mapping of a QTL in *A. halleri* allowed the identification of *CAX1* (cation/hydrogen exchanger 1) as a candidate gene for Cd tolerance (Courbot et al. 2007; Baliardini et al. 2015). CAX1 is localized in the vacuolar membrane and plays a key role in Ca homeostasis (Conn et al. 2011). *CAX1* is more expressed in roots of *A. halleri* compared to *A. thaliana*, and high expression of *CAX1* co-segregated with Cd tolerance in a back-cross 1 population of an *A. halleri*/*A. lyrata* cross. The *CAX1* QTL is conditional on Ca supply in the medium and is detected at low Ca supply only. Analyses of *A. thaliana cax1* mutant and *A. halleri* RNAi lines suggest that, at low Ca supply, *CAX1* is required to tolerate Cd-induced oxidative stress (Baliardini et al. 2015, 2016; Ahmadi et al. 2018). Recently, the high expression in shoots of multiple genes involved in the flavonoid pathway was linked to the capacity of a *A. halleri* population to tolerate and accumulate high Cd concentrations, suggesting that the capacity of accommodating Cd-induced oxidative damages is an important feature of Cd hyperaccumulation (Corso et al. 2018). Alternatively, flavonoids may bind Cd and be involved in Cd transport and sequestration (Kasprzak et al. 2015).

4 Mechanisms of Nickel Hyperaccumulation

Currently, more than 500 Ni hyperaccumulator species have been identified worldwide. These species are scattered in more than 50 plant families, mostly dicotyledons (Krämer 2010; van der Ent et al. 2013; Cappa and Pilon-Smits 2014; Reeves et al. 2018). Despite this large diversity and the interest in understanding the underlying mechanisms as instrumental to improve Ni phytoextraction, only a limited number of studies have focused on the molecular mechanisms of Ni hyperaccumulation. Ni is an essential microelement for plants because it is required for urease activity (Polacco et al. 2013). Plants have, therefore, evolved mechanisms for the regulation of Ni homeostasis and Ni hyperaccumulation that likely derive from these mechanisms (Fig. 3). In *A. thaliana*, the regulation of Ni homeostasis is strongly linked to the regulation of Fe homeostasis, but some responses to Ni excess are independent of Ni-induced iron deficiency responses (Schaaf et al. 2006; Morrissey et al. 2009; Nishida et al. 2011; Lešková et al. 2019). Interestingly, in Ni hyperaccumulators of the *Odontarrhena* (*Alyssum*) genus, it was shown that Mn treatment reduces Ni accumulation, suggesting that in some species Ni hyperaccumulation may also use mechanisms primary involved in Mn homeostasis (Leigh Broadhurst et al. 2009; Ghaderian et al. 2015).

4.1 Uptake of Nickel

Efficient uptake of Ni by the roots of hyperaccumulators requires divalent metal importers (*e.g*. ZIP, NRAMP) or transporters able to carry conjugated forms of Ni (e.g. Yellow Stripe-Like (YSL) family). However, identity of the transporters involved in Ni uptake in hyperaccumulators is still not clearly established. In *A. thaliana*, the metal transporter IRT1 required for the uptake of Fe from soil was shown to be involved in Ni uptake (Vert et al. 2002; Nishida et al. 2011, 2012). Interestingly, the high expression of *IRT1* orthologs in the roots of *N. caerulescens* and *Senecio coronatus* (Asteraceae) is correlated with the Ni hyperaccumulation capacity of tested accessions (Halimaa et al. 2014b; Meier et al. 2018). In addition, de novo sequencing of *NcIRT1* in Monte Prinzera revealed sequence polymorphism in the large cytoplasmic loop of IRT1 that may play a role in transport specificity and/or regulation (Halimaa et al. 2014a). However, in other Ni hyperaccumulator accessions of *N. caerulescens* (*i.e.* Puy de Wolf and Bergenbach), we were unable to detect correlation between *NcIRT1* expression and Ni hyperaccumulation (V.S. Garcia de la Torre, S. Merlot, unpublished data). These data suggest that metal transporters orthologous to IRT1 are likely involved in the efficient uptake of Ni in hyperaccumulator species. Other types of metal transporters may also participate in this important step. Indeed, several species of the ZIP and the NRAMP families have been linked to Ni transport or accumulation, but further studies will be required to support their implication in the efficient uptake of Ni in hyperaccumulators (Mizuno et al. 2005, 2007; Wei et al. 2009; Halimaa et al. 2014b; Meier et al. 2018).

4.2 Root-to-Shoot Transfer of Nickel

The long-distance transport of Ni from roots to shoots requires several steps that involve metal transporters and chelators that are able to bind Ni in different pH environments. In

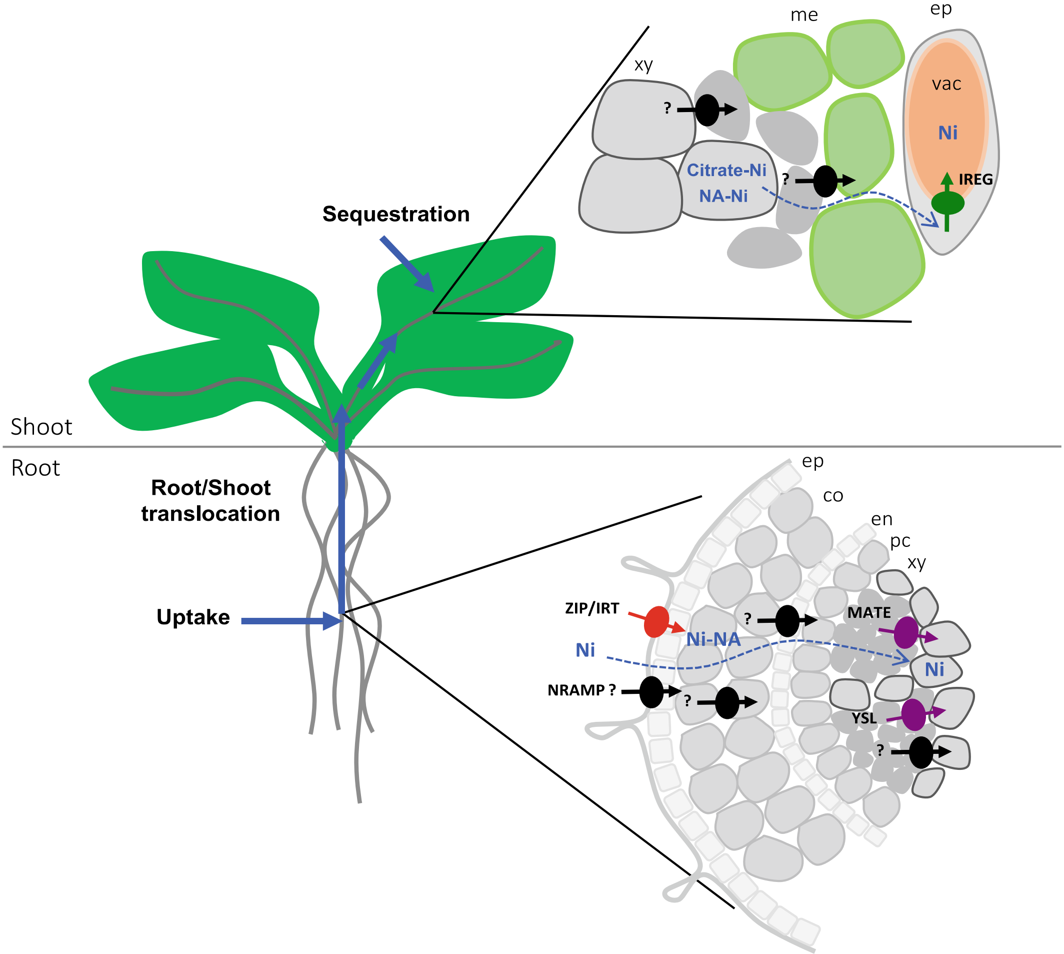

Fig. 3 Proposed mechanisms of Ni transport in Hyperaccumulators. Efficient Ni uptake is mediated by metal transporters of the ZIP/IRT family and possibly other transporters such as NRAMPs located at the plasma membrane of root epidermal cells (ep). Ni is then transported through the cortex (co) and the endodermis (en) by a combination of Ni export and import transporter activities. During this step, Ni is chelated [e.g. by nicotianamine (Ni-NA)] to reduce its reactivity in the cytoplasm. Binding to His might prevent vacuolar sequestration to favor radial transport. In the pericycle (pc), Ni is loaded in the xylem (xy) together with chelator molecules (e.g. NA, citrate…) by YSL and MATE transporters and transported to the shoot. The mechanisms involved in xylem unloading and transport to the epidermal cell are not well known but may be similar as the one involved in Ni transport in roots. In epidermal cell, Ni is transported and stored in the vacuole (vac) by IREG transporters. Figure modified from Hanikenne and Nouet (2011)

hyperaccumulators, a large proportion of Ni is found as complexes with carboxylic acids including citrate and malate (for reviews see Callahan et al. 2006; Sarret et al. 2013). These organic acid complexes are stable in acidic compartments such as vacuoles and xylem. In particular, citrate-Ni was identified in the xylem sap of the Ni hyperaccumulator *Odontarrhena serpyllifolia* (Alves et al. 2011). Interestingly, an ortholog of the *A. thaliana* citrate transporter FRD3 of the Multidrug and Toxic compound Extrusion family (MATE) is more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (van de Mortel et al. 2006). AtFRD3 and its orthologue in rice, OsFRDL1, are involved in the translocation of Fe from root to shoot (Rogers and Guerinot 2002; Yokosho et al. 2009). Therefore, high expression of MATE transporters in the root pericycle of hyperaccumulators would increase the loading of xylem with citrate and therefore favour the translocation of citrate-Ni complex from root to shoot. However, no direct evidence supports the implication of MATE transporters in Ni hyperaccumulation. It is interesting to note that *FRD3* is also highly expressed in *A. halleri*, which hyperaccumulates Zn. FRD3 transporters may therefore have a general function in metal hyperaccumulation, favouring long-distance transport of metal from root to shoot (Talke et al. 2006; Charlier et al. 2015).

NA also has a strong affinity for Ni over a wide range of pH and is proposed to bind Ni in more neutral compartments such as the cytoplasm or phloem (Callahan et al. 2006; Rellan-Alvarez et al. 2008; Alvarez-Fernandez et al. 2014). Accordingly, over-expression of NA synthase in transgenic *A. thaliana* increases Ni tolerance but is not sufficient to improve Ni accumulation (Pianelli et al. 2005). A NA-Ni complex was identified in the xylem sap of *N. caerulescens* (Mari et al. 2006), in the latex of the Ni hyperaccumulator *Pycnandra acuminata* (Schaumlöffel et al. 2003), and in extracts of several hyperaccumulator species (Callahan et al. 2012). Transporters of the YSL family have been shown to transport NA-metal complexes (Curie et al. 2009; Conte and Walker 2012). Several genes coding for YSL transporters are more expressed in the hyperaccumulator *N. caerulescens* than in the related non-accumulator *A. thaliana* (Gendre et al. 2007). Among these transporters, NcYSL3 that is able to transport the NA-Ni complex, is expressed in the vasculature of roots and leaves, suggesting a role in long-distance Ni transport.

Finally, the amino acid histidine (His), whose concentration in some hyperaccumulators of the *Odontarrhena* and *Noccaea* genera correlates with Ni accumulation, is proposed to play a role in the radial transport of Ni (Krämer et al. 1996; Richau et al. 2009). His has a strong affinity for Ni and an His-Ni complex has been identified in samples from *Odontarrhena* and *Noccaea* hyperaccumulators (Krämer et al. 1996; Persans et al. 1999; Callahan et al. 2006; McNear et al. 2010). Genes acting at different steps of His biosynthesis have been shown to be more expressed in Ni hyperaccumulators from several plant families than in related non-accumulator species (Ingle et al. 2005; Garcia de la Torre et al. 2018). The over-expression of the first enzyme of the His biosynthetic pathway, ATP-phosphoribosyltransferase, in *A. thaliana* increases Ni tolerance but not Ni content, suggesting that other mechanisms are necessary for accumulation of this metal (Wycisk et al. 2004; Ingle et al. 2005).

Treatment of plants with His-Ni increases xylem loading and inhibits Ni uptake from root vacuoles (Richau et al. 2009). It was therefore proposed that the high concentration of His in roots of Ni hyperaccumulators prevents vacuolar storage, favouring radial transport and xylem loading (Kerkeb and Krämer 2003; Richau et al. 2009). However, the mechanism responsible for the inhibition of Ni vacuolar sequestration by His is still unknown. In addition, it is currently not known if this strategy is widely conserved in Ni hyperaccumulators and if this is relevant in the natural environment (e.g. serpentine soil), where nitrogen is limiting (Alves et al. 2011; Centofanti et al. 2013).

4.3 Storage of Nickel in Leaves

In most of the hyperaccumulators that have been studied to date, Ni is stored in the vacuole of leaf epidermal cells (for review Sarret et al. 2013). However, the cell wall of leaf cells can also represent a reservoir for nickel (Krämer et al. 2000; van der Ent et al. 2019). Several lines of evidence indicate that Ferroportin (FPN)/Iron Regulated (IREG) transporters play an essential role in the sequestration of Ni in vacuoles. In *A. thaliana*, *AtIREG2* is expressed in roots in response to Fe starvation and the AtIREG2 protein localizes on the vacuole. The analysis of the *ireg2* mutant indicated that AtIREG2 is involved in the storage of Ni excess and Co in the vacuole of root cells (Schaaf et al. 2006; Morrissey et al. 2009). In addition, a second closely related IREG transporter in *A. thaliana*, named FPN1/IREG1, is localized at the plasma membrane and is proposed to play a role in the loading of metals in the xylem in roots (Morrissey et al. 2009). Interestingly, the ortholog of *AtIREG2* in *A. lyrata* is genetically linked to serpentine adaptation (Turner et al. 2010). Recent comparative transcriptomic analysis using RNA-Seq technology revealed that a high expression of genes coding for orthologs of AtIREGs in both roots and shoots is correlated with the Ni hyperaccumulation trait in several plant families (Halimaa et al. 2014b; Meier et al. 2018; Garcia de la Torre et al. 2020). For example, the PgIREG1 transporter from the Ni hyperaccumulator *Psychotria gabriellae* (Rubiaceae) localizes in the vacuolar membrane and is able to transport Ni when expressed in yeast. Therefore, PgIREG1 seems to be a functional homolog of AtIREG2. Interestingly, *PgIREG1* is more expressed in leaves of *P. gabriellae* than in the closely related non-accumulator *P. semperflorens* when both species are growing in their natural environment on ultramafic soil (Merlot et al. 2014). The overexpression of *AtIREG2* and *PgIREG1* in transgenic *Arabidopsis* plants significantly increases Ni tolerance but does not increase Ni accumulation, indicating that the high expression of these transporters is insufficient to trigger Ni hyperaccumulation (Schaaf et al. 2006; Merlot et al. 2014). Together, these results suggest that FPN/IREG transporters play a conserved role in the sequestration of Ni in the vacuoles of hyperaccumulators. We cannot exclude the possibility that FPN/IREG transporters located at the plasma membrane might play a role in the radial transport of Ni and the exclusion of Ni to the cell wall of epidermal cells. Other families of divalent metal exporters such as MTP transporters could mediate the transport of Ni in vacuoles but their role in hyperaccumulation needs to be further supported (Persans et al. 2001).

5 Hyperaccumulation of Other Trace Elements

Species that are able to hyperaccumulate Mn have been identified in more than 20 genera mostly in the Myrtaceae (e.g. *Gossia*) and Proteaceae (e.g. *Virotia*) families (Fernando et al. 2013; Losfeld et al. 2015; Reeves et al. 2018). Similar to other metals, Mn hyperaccumulation likely evolved from basic mechanisms involved in Mn homeostasis, although these mechanisms are poorly investigated in Mn hyperaccumulators (Pittman 2005; Fernando et al. 2013; Socha and Guerinot 2014; Shao et al. 2017; Li et al. 2019). In hyperaccumulators, Mn was found to accumulate in the vacuole of non-photosynthetic epidermal cells, but also more surprisingly in photosynthetic palisade mesophyll cells in the hyperaccumulator *Virotia neurophylla* (Fernando et al. 2012). These differences in the localization of Mn suggest that some mechanisms involved in Mn accumulation and detoxification might be divergent among hyperaccumulators. The high concentration of Mn measured in the leaves of several species is proposed to be the consequence of their strategy to acquire P from soil. For example, Proteaceae species excrete carboxylates in their rhizosphere that not only solubilize P but also micronutrients including Mn (Lambers et al. 2015). Several metal transporter families, such as NRAMP, ZIP, YSL and MTP have been shown to transport Mn in plants but their role in hyperaccumulation is not clearly established (Fernando et al. 2013; Socha and Guerinot 2014; Shao et al. 2017; Li et al. 2019). Most NRAMP transporters are able to transport Mn in the cytoplasm either from the exterior of the cell or from the vacuole, and therefore could participate in several steps of Mn hyperaccumulation. In particular, the NRAMP1 transporter from *A. thaliana* was shown to be the main transporter involved in Mn uptake in roots (Cailliatte et al. 2010). The ShMTP8 transporter (previously known as ShMTP1) was isolated from the Mn-tolerant species *Stylosanthes hamata* (Fabaceae). This MTP transporter confers Mn resistance when expressed in yeast and was proposed to mediate accumulation of Mn in the vacuole of plant cells (Delhaize et al. 2003). Further molecular studies on Mn hyperaccumulators will be required in order to identify the mechanisms that are key for Mn hyperaccumulation.

In addition to the above-mentioned metals, mechanisms involved in the hyperaccumulation of the metalloid element Se in plants are extensively studied because of their relevance to improve Se phytoremediation and biofortification. We only briefly address Se hyperaccumulation here and refer interested readers to recent thorough reviews of our current knowledge on Se homeostasis and hyperaccumulation (Barillas et al. 2011; White 2016; Schiavon and Pilon-Smits 2017; Lima et al. 2018; Reynolds and Pilon-Smits 2018). Selenium hyperaccumulation has been described in more than 40 taxa scattered among seven families (Reeves et al. 2018). More than half of Se hyperaccumulators have been described in the genus *Astragalus* (Fabaceae), but other well-described Se hyperaccumulators have been found in the genera *Stanleya* (Brassicaceae), *Oonopsis* and *Xylorhiza* (Asteraceae). The distribution of Se hyperaccumulators among plant families suggests that Se hyperaccumulation likely evolved independently at least six times (Cappa and Pilon-Smits 2014). Selenium is available to plants mostly as selenate (SeO_4^{2-}), a structural homologue of sulfate, or as selenite (SeO_3^{2-}), depending on the nature of the soil (Elrashidi et al. 1987). In cultivated soils, selenate uptake by root cells is catalyzed by high-affinity sulfate transporters of the SULTR family (Shibagaki et al. 2002; El Kassis et al. 2007; Barberon et al. 2008). A recent comparative RNA-Seq study revealed that several members of the SULTR family are more expressed in the hyperaccumulator *Stanleya pinnata* than in the non-accumulator *S. elata* (Wang et al. 2018), thus confirming and extending previous results observed in the *Astragalus* genus (Freeman et al. 2010; Cabannes et al. 2011; Schiavon et al. 2015). The high and constitutive expression of these transporters are proposed to significantly increase SeO_4^{2-} uptake and translocation to aerial parts of plants. The hyperaccumulation of Se is also linked to the capacity to preferentially take up selenate over sulfate. Sequence analysis of SULTR1 transporters from *Astragalus* identified a Gly to Ala polymorphism linked to the hyperaccumulation trait (Cabannes et al. 2011). However, it is not yet known if this difference explains the preferential uptake of selenate over sulfate. In rice growing in anerobic soils (e.g. paddy fields), selenite forms are transported in root cells by the phosphate transporters OsPT2 (Zhang et al. 2014) and by aquaporins (Zhao et al. 2010). Long-distance transport of selenate to the shoot is also proposed to be mediated by SULTR transporters (Takahashi et al. 2000). In shoots, the main fraction of selenate is metabolized into organo-selenium compounds (SeCys and SeMet) in chloroplast (Barillas et al. 2011; White 2016; Zhu et al. 2009). In hyperaccumulator species, genes involved in the synthesis of organo-selenium compounds and in the methylation of SeCys to produce the non-toxic form MeSeCys were shown to be constitutively more expressed than in non-accumulator species, supporting the metabolism of Se as also key for its hyperaccumulation (Pickering et al. 2003; Freeman et al. 2010; Schiavon et al. 2015; Wang et al. 2018).

6 Perspectives and Conclusions

6.1 Interaction of Hyperaccumulators with Biotic Environment

The so-called ‘elemental defense’ hypothesis proposes that metal hyperaccumulation provides defense against pathogens and/or herbivores by direct toxicity (Boyd and Martens 1992; Hörger et al. 2013; Cabot et al. 2019). Indeed, it has been shown that Ni and Zn accumulation can protect different *Brassicaceae* species from bacterial and fungal infection (Boyd et al. 1994; Ghaderian et al. 2000; Fones et al. 2010). However, it is also well documented that in environmental conditions, specific populations of bacteria are associated with the root system of metal hyperaccumulators (Aboudrar et al. 2012; Cabello-Conejo et al. 2014; Lucisine et al. 2014; Muehe et al. 2015). Endophytic bacteria have also been identified in shoots and roots of metal hyperaccumulators (Idris et al. 2004; Mengoni et al. 2009a). However, since the majority of these bacteria are not cultivable, current studies mostly describe endophytic populations using metagenomics approaches (Luo et al. 2011; Sessitsch et al. 2012; Chen et al. 2014; Visioli et al. 2014; Cao et al. 2020; Wang et al. 2020). Interestingly, it was shown that the inoculation of *N. caerulescens* and *O. serpyllifolia s.l.* with cultivable endophytic bacteria increases Ni translocation to shoots (Ma et al. 2011b; Visioli et al. 2015), and the inoculation of *S. alfredii* with *Pseudomonas fluorescens* enhances lateral root growth, photosynthesis, carbon fixation, and Cd accumulation in shoots (Wu et al. 2020a, b).

Little is known about the interactions between metal hyperaccumulators and associated bacteria. Metal hyperaccumulators represent an extreme niche for metal-tolerant bacteria (Mengoni et al. 2009b). On the other hand, such bacteria can improve plant growth and confer protection against abiotic stress by the production of hormones (auxins, cytokinins, etc.), or protect the host plant against other pathogens by the production of antagonistic substances or by competition for space and nutrients (Ma et al. 2011b; Reinhold-Hurek and Hurek 2011; Wu et al. 2020a). Metal-tolerant bacteria can also produce organic acids and metal chelators that can favour metal solubility, transport, and tolerance (Idris et al. 2006; Ma et al. 2011a; Visioli et al. 2015). These observations suggest intimate interactions between hyperaccumulators and associated bacteria; however, the mechanisms and genes involved in these interactions are mostly unknown. The development of next-generation sequencing technologies for metagenomic and meta-transcriptomic (or dual-transcriptomic) analyses, combined with functional and signalling networks, will uncover those genes expressed by plants and associated bacteria involved in their symbiotic interaction (Camilios-Neto et al. 2014; Pankievicz et al. 2016). Understanding these mechanisms will be instrumental for improving metal phytoextraction and plant biofortification, or for producing secondary metabolites such as metal chelators of bacterial origin that can be used in metal-based therapies (Franz 2013).

6.2 Evolution of Hyperaccumulation Mechanisms

After this brief review of our current knowledge of the molecular mechanisms of Zn, Cd and Ni hyperaccumulation, it is apparent that several candidate genes involved in these processes are involved in the control of metal homeostasis in non-accumulator plants. These genes display an enhanced function in hyperaccumulators, through gene copy number amplification and/or altered regulation, which profoundly modifies the metal flux in the plants towards accumulation in shoots. Many examples were also presented on the high level of convergent evolution between *A. halleri*, *N. caerulescens,* and *S. alfredii*. This convergent evolution likely reflects (and sheds light on) the functional constraints of the metal homeostasis network (Krämer et al. 2007; Verbruggen et al. 2009; Krämer 2010; Hanikenne and Nouet 2011; Preite et al. 2019). The key function of HMA4 in several hyperaccumulator species represents a potent example of this convergent evolution for Zn and Cd accumulation (Hanikenne et al. 2008; O'Lochlainn et al. 2011; Craciun et al. 2012). The high expression of FPN/IREG transporters in leaves of Ni hyperaccumulators from different families represents another example of convergence for the evolution of Ni hyperaccumulation (Halimaa et al. 2014b; Meier et al. 2018; Garcia de la Torre et al. 2018). Recently, polymorphism and high expression of IRT1 orthologs was found to be directly associated with both Cd and Ni hyperaccumulation in distant plant species (Halimaa et al. 2014a, b; Corso et al. 2018; Meier et al. 2018; Schvartzman et al. 2018). IRT1 represents the major high-affinity Fe uptake system in Dicotyledons, but has a low specificity for divalent metal ions in contrast to the more specific Fe-chelates system used by Monocotyledons (Korshunova et al. 1999; Vert et al. 2002; Nishida et al. 2011; Thomine and Vert

2013; Kobayashi et al. 2019). These results therefore support the hypothesis that the different pathways used for Fe uptake explain why the majority of metal hyperaccumulators have been identified in Dicotyledons.

Metal hyperaccumulation and associated (hyper)tolerance are complex traits that required the fine-tuning of multiple mechanisms during the course of plant evolution. Only a small number of large-effect QTL have been detected to date, suggesting that additional modifier genes involved in metal tolerance and hyperaccumulation remain to be detected. Moreover, if several key players have now been identified, how the tolerance and hyperaccumulation traits evolved remains an open 'chicken and egg' question. Hence, Bayesian inference suggested that speciation between *A. halleri* and *A. lyrata* closely coincided with *HMA4* duplication (Roux et al. 2011). Complex signature of selection detected at the *HMA4* locus of *A. halleri* further supports the key role of the gene in the evolution of the hyperaccumulation trait (Hanikenne et al. 2013). Recent adaptations to anthropogenic metal-polluted sites possibly occurred independently within distinct phylogeographic units of the *A. halleri* European distribution (Pauwels et al. 2012). Hypertolerance of metallicolous populations thus potentially evolved using a variety of genetic mechanisms (Meyer et al. 2009, 2010; Pauwels et al. 2012; Babst-Kostecka et al. 2018). Moreover, the study by Meyer et al. (Meyer et al. 2016) suggests that, if HMA4 contributes to Zn tolerance in both metallicolous and non-metallicolous populations of *A. halleri* (Hanikenne et al. 2013), the function of MTP1 in Zn tolerance may have evolved later in metallicolous populations that have colonized polluted soils recently. Indeed, the co-segregation of *MTP1* with Zn tolerance is only observed in a back-cross 1 population of a cross between a French metallicolous individual (i.e. living on metal-polluted soil) of *A. halleri* and *A. lyrata* and is lost when a non-metallicolous Slovakian (i.e. living on non-polluted soil) *A. halleri* individual is used as parent (Meyer et al. 2016). In contrast, the co-segregation of *HMA4* with Zn tolerance is independent of the edaphic origin of the *A. halleri* populations used in the analysis (Willems et al. 2007; Meyer et al. 2016). This result partially contradicts the hypothesis that MTP1 is required for metal detoxification accommodating the high HMA4-dependent metal flux into *A. halleri* shoots, which was proposed based on the observation that expression of *AhHMA4* in non-accumulator plants resulted in increased sensitivity to excess Zn (Hanikenne et al. 2008; Barabasz et al. 2010). However, *MTP1* is highly expressed in four metalicollous populations from distinct genetic units (Dräger et al. 2004; Talke et al. 2006; Schvartzman et al. 2018), which either suggests convergent evolution in several metalicollous populations with parallel acquisition of high expression of multiple *MTP1* copies or that the lack of *MTP1* may be a specific feature of the non-metallicolous Slovakian population previously described (Meyer et al. 2016).

More recently, the QTL (Karam et al. 2019) and transcriptomic (Halimaa et al. 2014b; Milner et al. 2014; Corso et al. 2018; Schvartzman et al. 2018) approaches used so far to compare hyperaccumulator and non-accumulator related species were extended to comparisons of contrasting populations within species, taking advantage of important intraspecific variation of hypertolerance and hyperaccumulation traits observed among populations of distinct geographic regions or established on distinct edaphic types (Escarré et al. 2000; Pauwels et al. 2006; Gonneau et al. 2014; Stein et al. 2017). Exploiting this natural variation, application of Genome-Wide Association Studies (GWAS) in large cohorts of wild plants (or accessions) of a species will further allow identifying novel alleles linked to the variation of a phenotype. As complementary techniques, Transcriptome-Wide Association Studies (TWAS) and expression Quantitative Trait Loci (eQTL) could also be used to identify regulatory sequence variants and to prioritize candidate genes at the identified loci. Furthermore, these techniques not yet applied to address metal tolerance and hyperaccumulation in plants will facilitate the modelling of functional and/or regulatory networks underlying the complex traits.

Recent studies have used omics approaches to characterize genome- or transcriptome-wide genetic variation within species and to uncover mechanisms of evolution of metal hyperaccumulation (Yang et al. 2017; Paape et al. 2018; Sailer et al. 2018; Halimaa et al. 2019; Preite et al. 2019; Honjo and Kudoh 2019). These studies started to reveal the pattern of polymorphisms in genomes and to shed light on how selection acts on those genomes. This variation has been exploited to select or breed lines of *N. caerulescens* with increased tolerance and/or accumulation (Nowak et al. 2018; Sterckeman et al. 2019).

The study of metal hyperaccumulation in distant plant families combined with the comparison of distinct accessions with contrasting accumulation capabilities within a species will likely shed light on the evolution of the hyperaccumulation and hypertolerance traits. This approach may indeed reveal the commonalities and differences in mechanisms underlying these traits, highlighting evolutionary divergence and convergence. It may also reveal evolutionary 'intermediates' (i.e. genotypes that do not display the full extent of hyperaccumulation or tolerance), which may allow ordering the evolutionary events that took place during the adaptation of the metal homeostasis network.

6.3 How Can Phytoextraction Technologies Benefit from Molecular Knowledge?

To date, most of our knowledge on metal hyperaccumulation arises from studies of a few model hyperaccumulator species (i.e. *A. halleri* and *N. caerulescens*) of the Brassicaceae family. In the future, it will be necessary to pursue molecular studies and improve genetic manipulation of these species in order to identify and demonstrate the role of key mechanisms involved in metal hyperaccumulation. Furthermore, these species have a low biomass and a relatively restricted distribution worldwide. Therefore, one of the coming challenges for the development of agromining/phytoextraction will be to transfer knowledge of the mechanisms involved in metal hyperaccumulation to species having a high potential for phytoextraction. As mentioned above, the development of Next Generation Sequencing technologies opens the possibility for studying 'non-model' species at the genomic and transcriptomic levels.

As for other crop plants, this molecular knowledge will be instrumental to develop markers for the selection of genotypes with the best potential for metal phytoextraction. The level of expression of key genes involved in metal hyperaccumulation can be used to predict metal accumulation capacities. These marker genes can also be used to study the interaction between metal accumulation and agricultural practices (e.g. fertilization), and for improving biomass production while maintaining efficient metal accumulation.

Finally, genome-editing technologies such as CRISPR-CAS9 are currently being implemented in plants to specifically modify the sequence of target genes (Chen et al. 2019). This technology offers several advantages compared to traditional transformation technologies used to produce Genetically Modified Organisms (GMO), and therefore could be better accepted by civil society and political stakeholders to engineer crop plants for metal phytoextraction. Recently, this technology was used to inactivate the *SdHMA1* gene coding for a chloroplast Cd exporter in the hyperaccumulator *S. plumbizincicola* (Zhao et al. 2019). Further development of this technology may allow for specifically introducing point mutations in the sequence of genes involved in metal accumulation to increase their activity, improve their specificity, or modify their selectivity towards metals of interest (Rogers et al. 2000; Menguer et al. 2013; Pottier et al. 2015).

Acknowledgements We thank colleagues in our laboratories for critical reading of the manuscript. The research of SM and VSGT was supported by the French National Research Agency (ANR-13-ADAP-0004) and by CNRS (Defi Enviromics and Defi X-Life). Funding to MH is from the 'Fonds de la Recherche Scientifique–FNRS' (PDR-T.0206.13, MIS-F.4511.16, CDR J.0009.17, PDR-T0120.18) and the University of Liège (SFRD-12/03, ARC GreenMagic). MH is a Senior Research Associate of the FNRS.

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Tools for the Discovery of Hyperaccumulator Plant Species in the Field and in the Herbarium

Imam Purwadi, Vidiro Gei, Guillaume Echevarria,
Peter D. Erskine, Jolanta Mesjasz-Przybyłowicz,
Wojciech J. Przybyłowicz, and Antony van der Ent

Abstract

Globally, the discovery of metal and metalloid hyperaccumulator plants has been hindered by systematic screening of plant species and is highly biased towards Ni hyperaccumulators due to the existence of a paper test that is only specific to Ni (based on dimethylglyoxime reagent). Consequently, more than 500 of the approximately 700 known hyperaccumulators species accumulate Ni. New technological advances now permit massive screening of herbarium specimens using non-destructive multi-element sensitive portable X-Ray Fluorescence Spectroscopy (pXRF) instead of via destructive techniques such as Atomic Absorption Spectrophotometry (AAS) or Inductively-coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). This approach has already led to the discovery of numerous hyperaccumulator species for various elements new to science. This chapter introduces established and novel tools that can be used for discovering hyperaccumulator plant species in the field and in the herbarium.

1 Introduction

Endeavours to identify hyperaccumulator plants typically start with studying geological maps to provide information on the location of metalliferous soils. There is a high probability that hyperaccumulator plant species grow on such soils (Reeves et al. 2018). The large proportion of identified Ni hyperaccumulator species in comparison to hyperaccumulators that concentrate other elements is primarily because ultramafic soils constitute by far the most widespread metalliferous anomaly worldwide (Echevarria 2018). In addition, many hyperaccumulator plant species have been encountered in mining areas, and therefore, searches for hyperaccumulator plant species could centre on mining areas (e.g. Reeves and Brooks 1983; Yang et al. 2002; Liu et al. 2014). The effectiveness and efficiency for identifying hyperaccumulator plant species can be attributed to the technique used for quantifying the elemental concentrations in plant material

I. Purwadi · V. Gei · P. D. Erskine ·
A. van der Ent (✉)
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD 4072, Australia
e-mail: a.vanderent@uq.edu.au

G. Echevarria
Laboratoire Sols et Environnement, Université de Lorraine-INRAE, Vandoeuvre-lès-Nancy, France

J. Mesjasz-Przybyłowicz · W. J. Przybyłowicz
Department of Botany and Zoology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

W. J. Przybyłowicz
Faculty of Physics & Applied Computer Science, AGH University of Science and Technology, al. Mickiewicza 30, 30-059 Kraków, Poland

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_9

samples. For example, an easily deployed field-testing method for Ni using dimethylglyoxime (DMG)-treated paper is available and has helped in the initial discovery of numerous Ni hyperaccumulator plants during field screening (see Fig. 1). Meanwhile, confirmation of hyperaccumulator status has traditionally been achieved using Atomic Absorption Spectrophotometry (AAS) (e.g. Kelly et al. 1975; Jaffré et al. 1979; Kersten et al. 1979) and Inductively-coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (e.g. Reeves et al. 1996, 2007; Fernando et al. 2009; van der Ent and Reeves 2015) following acid digestion of samples of dried leaf material. Although the analysis with AAS or ICP-AES takes only minutes, the sample preparation is time- and resource-consuming (e.g. 100 samples require 2–3 days to digest). Importantly, recent technological advances in more sensitive and compact X-ray fluorescence (XRF) instruments enable non-destructive, multi-elemental screening of a vast number of samples, including herbarium specimens, in a relatively short period of time (van der Ent et al. 2019a).

This chapter presents various established and novel tools and methods that can be used for discovering hyperaccumulator plant species in the field and in the herbarium.

2 Field Spot Tests for Hyperaccumulator Plants

The most popular and widely used spot test in the field is paper impregnated with dimethylglyoxime (DMG). This colorimetric reagent reacts with Ni to form a bright purple complex precipitate. The reagent has practically no interfering elements (except for Cd and Cu) and is sensitive down to about 20 µg g^{-1} Ni (Gramlich et al. 2011). It is prepared by dissolving 1% DMG in either ethanol or acetone (as it is highly

Fig. 1 Field spot test based on the Ni-specific colorimetric reagent dimethylglyoxime demonstrating the reaction with Ni from the fresh leaf of the Ni hyperaccumulator species *Psychotria gabriellae* (Rubiaceae) in the field in New Caledonia. The test is fast and sensitive and allowed for the identification of numerous Ni hyperaccumulators globally

insoluble in water), soaking the solution in normal filter paper rounds, and letting the solvent evaporate to create paper impregnated with this chemical. Its use for field testing for Ni hyperaccumulator plants has a long history, and remains very useful today (Vergnano Gambi 1967). Another colorimetric reagent, aluminon, which forms a pink to crimson complex with Al, also has a long history of discovering Al hyperaccumulator plants (Chenery 1948; Jansen et al. 2000, 2002) and has been extensively used to test many thousands of herbarium specimens for Al accumulation. Small (a few cm^2) fragments of the herbarium specimen are excised and heated in boiling water with aluminon reagent solution to obtain a semi-quantitative indication of the Al concentration. This test has been cross-validated with acid digestion and ICP-MS analysis and shows that a concentration of 1000 $\mu g\ g^{-1}$ Al in plant tissue can be determined, although Fe can interfere and also give coloured complexes (Jansen et al. 2003; Schmitt et al. 2017). It may also be used in the field by squashing fresh plant leaves with some of the reagent in aqueous solution. This approach was tested by our group on *Symplocos* spp. in Malaysian Borneo. More recently, the colorimetric agent Zincon (2-hydroxy-5-sulfonyl azobenzylidene hydrazinobenzoic acid) was tested for analysis of Zn in plant leaves after digestion with plant material using 2% sulphosalicylic acid (Macnair and Smirnoff 1999). Zincon reacts with Cu and Zn to produce a blue complex that can be measured spectrophotometrically. The results of this test match to >90% those obtained by using nitric acid digestion and AAS. We have prepared Zincon impregnated test paper for spot testing of Zn accumulation in plants in the field, and the results were satisfactory.

3 Portable XRF Analysis for Leaves in the Field and Herbarium Screening

In the portable XRF (pXRF) instrument, primary X-ray photons generated in the miniature X-ray tube excite secondary X-ray photons of the atoms present in the measured sample. The spectrum of excited fluorescent X-rays is then analyzed to determine the presence of different elements and to calculate their relative concentrations in the sample. The latest generation of such XRF instruments (equipped with an Ag or Rh anode operating at 6–50 kV, with currents up to 200 μA) allow detection limits in the range of ~100–300 $\mu g\ g^{-1}$ for most transition elements (i.e. Co, Ni and Zn) (van der Ent et al. 2019a). Readers can find more information on the principles of X-ray spectroscopy and portable X-ray fluorescence spectrometry in Jenkins (1974, 1988, 1999), Bertin (1978), Jenkins et al. (1995), and Potts and West (2008).

Use of the portable XRF instrument to obtain elemental data for herbarium specimens was first proposed by van der Ent et al. (2019a) and has now been used in several areas around the world. The instrument has demonstrated its usefulness in systematic screening of various plant families with the aim of discovering new hyperaccumulator plant species (van der Ent et al. 2019a, b). Previously, no hyperaccumulator plant species were reported from Papua New Guinea and Central America, but recently many new examples have been identified using this technology (McCartha et al. 2019; Do et al. 2020). The use of XRF instruments offers several key benefits, but also has some limitations that are discussed below.

Portable XRF spectroscopy is a time- and cost-effective method to measure elemental concentrations in many plant specimens. It has the ability to measure a range of different elements simultaneously within seconds in dry samples. Elements such as Ni, Mn, Co or Zn in herbarium specimens can be measured at a rate of ~300 specimens per day (translating to ~6000 specimens per month), thus enabling mass screening of tens of thousands of herbarium samples in a relatively short time span (see Fig. 2 for setup of XRF scanning of herbarium specimens) (van der Ent et al. 2019a). Additionally, XRF screening may be combined with the digitisation process of herbarium specimens, an effort already underway in many global herbaria. From such initial mass screenings, species may be selected for further

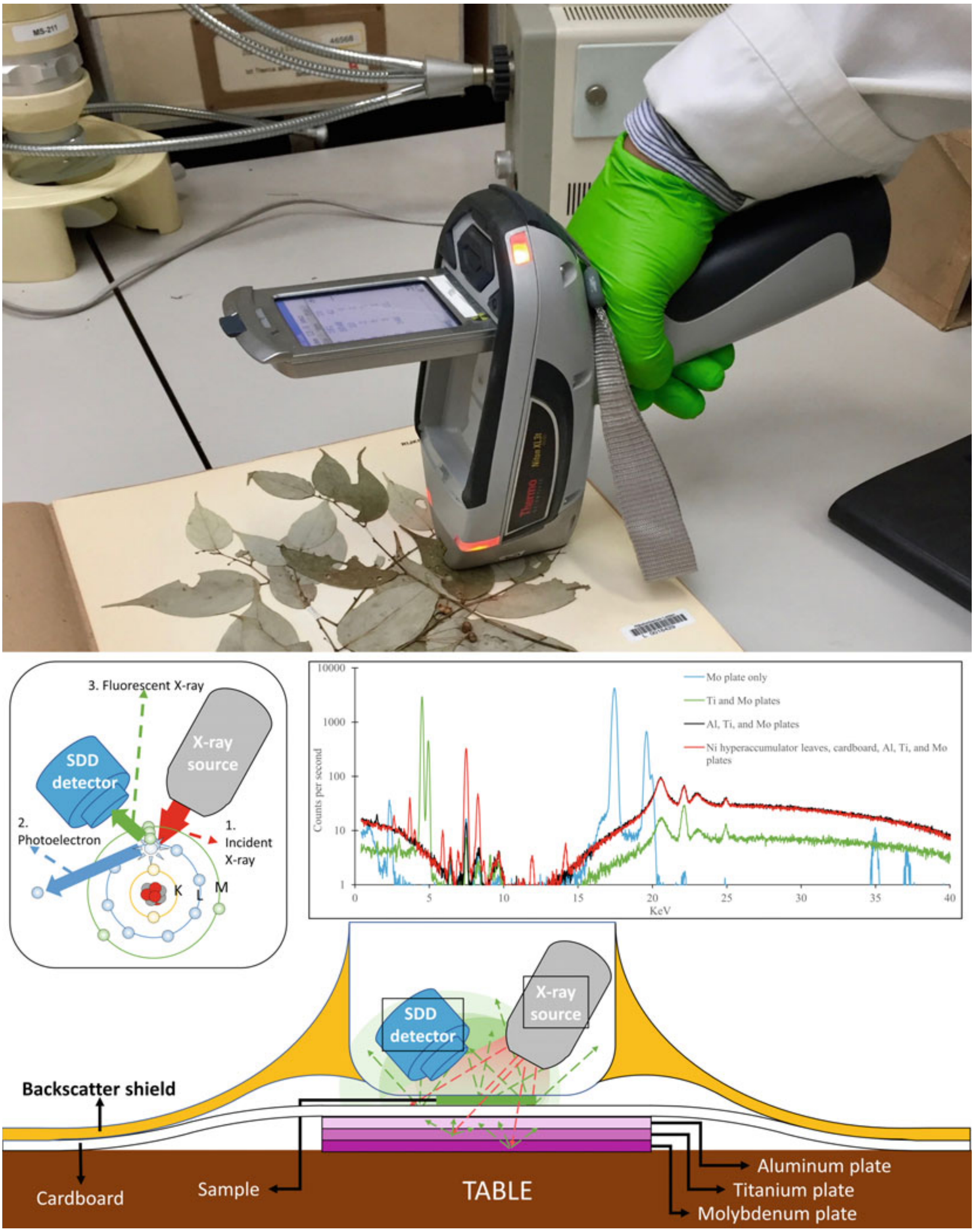

Fig. 2 **Above**: photo of a pXRF measurement of a herbarium specimen (shown without the use of a backscatter shield.) **Below**: The herbarium specimens sits on top of the table on a Al-Ti-Mo plate stack to block transmitted X-rays and to provide a clean background. Insert on the left side illustrates the operation principle: the X-ray source produces primary X-ray radiation onto the sample which results in fluorescent X-rays being emitted from the sample that are recorded by the SDD detector. Insert above the diagram shows XRF spectra with either Mo plate, Ti-Mo plates, Al-Ti-Mo plates and herbarium sample with Al-Ti-Mo plates

investigation to determine elemental distributions at the tissue and cellular level, after dedicated field collection of samples.

Even though pXRF devices will not be accessible to all researchers due to their high capital costs, availability of these instruments is becoming more widespread and it is the most powerful tool known for surveying hyperaccumulator plants in the field. It has proved indispensable for identifying Cu- and Co-accumulating plants in the field in Zambia (van der Ent et al. 2017). The devices are small and relatively light (<1.5 kg), the rechargeable battery lasts for 6–8 h, and semi-quantitative results for fresh plant leaves can be obtained within seconds. Owing to its multi-elemental capability, the pXRF is particularly useful for screening purposes. However, the obtained concentrations of elements should be regarded as approximate, since requirements for fully quantitative analysis are often not met (e.g. excess water content and insufficient sample thickness). Nevertheless, values that exceed 1000 µg g^{-1} for Ni, Co, Zn, As and Se or 10 000 µg g^{-1} for Mn, should be regarded as highly interesting for follow-up analysis.

4 Technical Considerations for Using Portable XRF

Recently, portable XRF instruments have been validated for the measurement of elements in plants in the laboratory via powdering and pelletization of sample material before analysis (McLaren et al. 2012; Reidinger et al. 2012; Guerra et al. 2014). Hyperaccumulator plant organs such as leaves clearly show a heterogenous distribution of different elements, with preferential accumulation of certain elements in epidermal cells or mesophyll, or in trichomes, etc. However, homogenizing herbarium specimens is out of the question, and homogenizing leaves in the field is not practical. Therefore, the heterogeneity of sample material will result in accuracy problems (Török et al. 1986). In order to minimize the heterogeneity effect, taking the average of several measurements from different parts, if possible, of herbarium specimens and of leaves in the field, is desirable to assess within-specimen variation.

The intensity of fluorescence of a sample is dependent on the full matrix composition and sample thickness. The intensity increases as the sample gets thicker until reaching its critical thickness (Sitko 2009). The critical thickness is that from which 99% of the fluorescence signal originates, and the sample that reaches its critical thickness is called a ‘thick sample’. Thin samples are those for which all matrix effects (both absorption and enhancement) can be neglected; the thickness of a thin sample corresponds to the one from which 1% fluorescence originates. Intermediate samples are those that have a thickness between thin and thick samples. As an illustration, assume 250 µm-thick dry and fresh (hydrated) *Berkheya coddii* leaves of cellulose composition measured with a pXRF instrument, where angles of the incident primary and fluorescence radiation are both 45°. Given the mass attenuation coefficient data provided by Hubbell and Seltzer (1996) and the density of dry and fresh/hydrated *B. coddii* leaves as 0.378 g cm^{-3} and 0.781 g cm^{-3}, respectively (mean values of 69 measurements of dry and fresh (hydrated) *B. coddii* leaves values of the X-ray penetration depths were calculated. This shows that the leaf should be treated as a thick sample for light elements ($Z < 14$ for dry leaves and $Z < 16$ for fresh/hydrated leaves) (see Fig. 3). These differences are appreciable and signify that corrections of the XRF results cannot be generalized. The red line in Fig. 3 is also known as critical thickness or escape depth that indicates the travel distance of the K-α fluorescence radiation within the samples; 99% of K-α fluorescence radiation originates from the surface to the escape depth. The escape depth of Al, for example, is 125 µm for fresh leaves and 258 µm for dry leaves, thus making dry and fresh (hydrated) leaves intermediate and thick samples, respectively. As the distribution of Al in the leaf might be uneven, the reading of Al concentration can be overestimated where Al is deposited in the upper epidermis and underestimated where Al is deposited in the lower epidermis. It is important

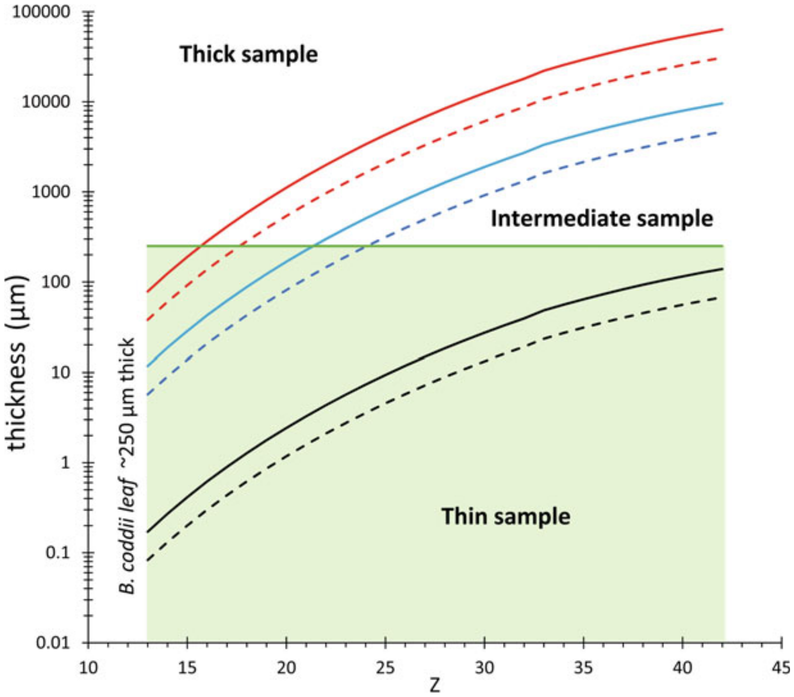

Fig. 3 Curves of leaf thickness for thin, intermediate, and thick samples plotted against the atomic number Z. Black lines indicate the boundary between thin and intermediate samples, and red lines indicate the boundary between intermediate and thick samples. Solid lines are the boundary for dry *B. coddii* leaves, while dashed lines are the boundary for fresh (hydrated) *B. coddii* leaves. The density of dry and fresh (hydrated) *B. coddii leaves* are 0.378 g cm^{-3} and 0.781 g cm^{-3} respectively, assuming the thickness of *B. coddii* leaves is 250 µm. Cellulose is assumed to be the matrix of *B. coddii* leaves and the mass attenuation coefficient of cellulose was calculated based on data provided by Hubbell and Seltzer (1996). Red, blue, and black lines represent the depths from which 99%, 50% and 1%, respectively, of the K-alpha fluorescence radiation originate. The lines are based on a computational simulation of measuring dry and fresh (hydrated) *B. coddii* leaves with, an incident primary radiation of 25 keV energy, and both incident and take-off angles of X-Ray radiation φ_1 and φ_2 equal to 45°

to note that leaves having a greater density will have shorter escape depth than those shown in Fig. 3, whereas those with lower density will have a longer escape depth.

Measuring high-Z elements is challenging because the excitation of X-rays of elements with energies of an absorption edge higher than the energy of the K-line of the anode (20.2 keV for Rh) relies on the Bremsstrahlung background tail, which is highly inefficient (Harada and Sakurai 1999). The portable XRF instrument is equipped with SDD detectors that have a resolution of about 150 eV. In many cases, the L-lines of the elements of interest can be used instead, but certain elements present a problem, notably Cd. The Lα1 line of Cd (at 3.133 keV) is spectroscopically indistinguishable from the Kα1 of K (at 3.313 keV), an element typically abundant in plant material samples at concentrations in the order of 10 000s µg g^{-1}. Another example is the overlap of the Lα1 line of Pb (at 10.551 keV) and the Kα1 of As (at 10.543 keV), meaning that it is nearly impossible to correctly measure Pb in samples having high As concentrations. Another common peak overlap issue involves the interference of the Fe Kβ1 line (at 7.058 keV) with the Co Kα1 line (6.9303 keV), which makes it difficult to quantify Co if Fe is present at high concentrations. Rare Earth Elements (REEs) present specific challenges. The energy required to excite the K-lines of the REEs (38.92 keV absorption edge for La) can only barely be reached (highly inefficient from excitation from the Bremsstrahlung background) by X-ray sources in handheld devices. Therefore, one has to mainly rely on exciting the L-lines of REEs (ranging from 4.65 keV for La to 7.41 keV for Yb), which are only a few 10s of eV apart, hence a wavelength-disperse XRF spectrometer is more suitable for solving the overlapping L-lines of REEs (Sitko et al. 2005).

Another consideration when undertaking pXRF measurements is that some herbarium specimens were treated with $HgCl_2$ for long-term insect protection. Therefore, readings for Hg can be extremely high for such samples (i.e. >500 μg g^{-1}). Furthermore, surficial contamination with soil particles must be considered as it could mask the true concentrations of elements under investigation. Soil dust adhering to leaves can preclude meaningful measurements, but may be gauged from unusually high concomitant Cr, Fe and Ti concentrations (Cary and Kubota 1990). The risk of particulate contamination can be addressed by observing selected 'suspect' specimens (i.e. specimens with >2000 g g^{-1} concomitant concentrations of Fe, Cr, Ti) under a scanning electron microscope (SEM). Soil particles, by virtue of their typical composition of higher-Z elements, are clearly visible in backscatter mode on the SEM when adhered to plant leaf surfaces. Contamination with soil particulates typically presents itself as minute, high-signal intensity speckles (dominated by soil abundant elements such as Si, Fe and Cr). Co-localization of the ostensibly 'hyperaccumulated' element with such speckles thus strongly suggests that contamination is responsible for any high bulk concentrations.

For elements with atomic number <17, not all XRF signal emerges from the sample as their escape depth is less than the thickness of the sample. The emerging fluorescent radiation is further absorbed by the air between the sample and the detector. Aluminium K-α fluorescence radiation, for example, will undergo absorption by 42.1% and 66.5% when the distance between the sample and the detector are either 1 cm or 2 cm, respectively (Veigele 1970). To deal with this problem, helium gas can be used to flush the chamber of the portable XRF instrument. By doing so, the transmission of the Al K-α fluorescence radiation improves to 99.9% where the distance between the sample and the detector is <2 cm (Henke et al. 1993). Using a He flush, the sensitivity of the portable XRF instrument improves for (very) light elements. For example, using this approach, the instrument is capable of measuring 0.74% Na and 0.32% Mg of certified reference material with a net count rate RSD (relative standard deviation) of ~6% and 4%, respectively (Adams et al. 2020). However, bringing a helium gas tank to the field is not practical. Thus, using He flush is only applicable for measurements of herbarium specimens, but the cost of using helium gas should be considered.

5 Safety Precautions for Using Portable XRF

It is important to adhere to basic X-ray safety principles when using the pXRF instrument whether measuring fresh leaves in the field or herbarium specimens. As paper and cellulose (e.g. herbarium cardboard sheet with specimen and leaves measured in situ) are highly transmissive for high-energy X-rays, it is recommended to place solid metal plates beneath the herbarium specimen during the XRF measurement to block transmitted X-rays. The configuration of the metal layers should consist of three metal layers that are stacked from top to bottom, having the following composition: light matrix (99.999% pure Al plate), medium matrix (99.999% pure Ti plate), and heavier metal (99.999% pure Mo plate) (see Fig. 2). Besides blocking X-rays, this stack configuration absorbs Mo fluorescence by the Ti plate and Ti fluorescence by the Al plate, and finally Al fluorescnece is absorbed by the cardboard sheet, thereby producing a clean background spectrum. Furthermore, it is critical to properly shield the X-ray source in order to avoid exposure to ionising radiation. X-rays will invariably escape from the area between the instrument snout and the specimen, and therefore a 'backscatter shield' (a stand attached to the face of the instrument with a flexible Pb or W-infused plastic collar) should be used. Such an attachment has the benefit that the instrument will stand up on itself, which simplifies the measurements of herbarium specimens (the XRF device can simply be placed carefully on top of the specimen without further support). In the field, the measurement should be taken by placing the metals/plant leaf stack on the ground, and never while handholding the device nor while holding it against one's body.

6 Calibration of Portable XRF

There are two types of calibrations required to convert the raw data of the pXRF instrument measurement to elemental concentrations, namely empirical calibration and so-called Fundamental Parameters (ThermoFisher 2015; Bruker 2018). Empirical calibration involves modelling the quantitative correlation between measured net intensities and concentrations of a set of standards whose matrix is the same as that of the unknown sample (Rousseau et al. 1996). In the case of herbarium specimens, a series of standards could comprise of dried leaves in which elemental concentrations were quantified using ICP-AES analysis to obtain correction factors. However, the empirical calibration may contain errors because the standards are not homogeneous and uniform in thickness. Homogeneity is important in order to obtain good agreement between the fluorescent intensity and concentration of an element, and thickness uniformity is a must for avoiding thickness dependency (see Fig. 3). Also, the standards consist of several elements that could enhance and absorb the fluorescent radiation of an element. A well-known example is the strong absorption of Fe intensity by Cr, and the strong enhancement of Fe intensity by Ni; a large number of standards as well as more complex mathematical treatments are required to correct for such matrix effects (Kalnicky and Singhvi 2001; Sitko 2009).

Most portable XRF instruments are pre-calibrated based on Fundamental Parameters (Bosco 2013). From a physics point of view, the fluorescent radiation of elements in a sample can be calculated using the Fundamental Parameters as long as all variables required by the Fundamental Parameter equations are known. Also, the Fundamental Parameter method requires all elements within the sample to be measured and their fluorescence spectra recorded, but very light elements such as H, C, N and O can not be recorded by the instrument (Markowicz 2008). Therefore, the Fundamental Parameter method is not suitable for herbarium specimens and leaves because their thickness is intermediate, and such specimens are mainly made of very light elements. Instead of the Fundamental Parameters, Compton normalization is designed for dealing with a sample consisting of very light elements (Nielson 1977).

7 Bulk Elemental Analysis of Hyperaccumulator Plant Samples and Acid Digestion

Even though portable XRF instrumentation has revolutionized the discovery phase of research on hyperaccumulator plants, bulk elemental analysis remains a critical method for quantifying elemental concentrations. Most popular and accessible options are AAS and ICP-AES. Table-top XRF is less popular and can be used on dry homogenized sample matreial in the form of pressed pellets. ICP-AES remains the method of choice in quantifying elemental concentrations (covering most of the Periodic Table) in plant samples with a level of sensitivity only rivalled by Inductively-coupled Plasma-Mass Spectrometry (ICP-MS). Of course, independent validation and calibration of (portable) XRF instruments typically also relies on ICP-AES. In a usual workflow, when a new species is identified as a hyperaccumulator from herbarium XRF screening, this is followed by bulk analysis using AAS, ICP-AES, or ICP-MS to precisely and accurately determine the elemental concentrations in plant tissue samples.

ICP-AES relies on converting the measured light intensities of spectral emission lines (in the range of 160–900 nm) within a hot (6000–

8000 °C) argon plasma. A set of calibration standards is made by diluting certified reference material, with the measured intensities of the calibration standard being strongly correlated ($r > 0.999$). This method contrasts with XRF analysis, which involves the use of advanced physics for correcting matrix-based effects. The strengths of ICP-AES analysis are its ability to measure many different elements simultaneously (up to 25 typically) with a dynamic range of at least six orders of magnitude (capable of measuring of ~50 ppb to several %). Unlike typical XRF analysis, ICP-AES and ICP-MS are destructive methods that rely on complete digestion of the plant material in liquid (acid solution) before analysis. Consequently, sample preparation is time consuming and the acid digestion process itself is fraught with potential issues. In general, the plant material is are digested with a strong oxidizer, typically nitric acid (HNO_3), in order to fully breakdown organic matter and release all elements in ionic form into the liquid medium. However, before the acid digestion process, plant tissues in general are required to be dried in an oven to reach constant weight, then homogenised by grinding, and finally digested. Other acids have also been used, such as perchloric acid ($HClO_4$), but this has serious associated safety risks. Some protocols also include the use of hydrogen peroxide (H_2O_2) to aid in the removal of organic matter (Huang and Schulte 1985). The acid digestion itself takes place either in a microwave in closed (or sometimes open) vessels, or on a hot block. Silicates cannot be dissolved by nitric acid alone and hence any element entrapped in silicates within plant material will not be released in solution for analysis. Only the use of hydrofluoric acid (HF) enables full dissolving of silicates, but its extreme toxicity precludes routine use.

Some specific issues pertain to the remarkably high concentrations of certain elements in tissues of hyperaccumulator plants. For example, Mn oxides are nearly insoluble, even at very low pH, and digestion with nitric acid can result in blackish-brown precipitates in the supernatant of Mn hyperaccumulator plant samples. This problem can be rectified by using a reducing acid such as hydrochloric acid (HCl) to get the Mn oxides into solution.

Advances in acid digestion and ICP-AES technology have greatly increased throughput and sensitivity. Whereas previously 1000 mg dry weight of plant material samples diluted to 50 mL was used (Soltanpour and Havlin 1980), nowadays 1–10 mg of plant tissue diluted to 5 mL can be used (Hansen et al. 2009). Smaller amounts of samples and liquids mean that smaller vials can be used, resulting in increased throughput of the digestion step.

8 Sensing Metals in Hyperaccumulator Leaves Using VNIR Spectroscopy

The wavelength range of Photosynthetically Active Radiation (PAR: 400–700 nm) covers the range in which chlorophylls absorb the radiation during photosynthesis (Kume et al. 2018). Almost all incident solar radiation at blue (~450 nm) and red (~650 nm) regions are absorbed (e.g. Johnson, 2016; Lodish et al. 2000), whereas at green (~550 nm) regions some of the incident solar radiation is reflected to avoid damage to leaves as green light constitutes the greatest intensity of solar radiation (Terashima et al. 2009; Kume 2017) (see Fig. 4). Reflectance in the wavelength longer than the PAR range is independent of leaf pigments (Knipling 1970); the reflectance of leaves in the near infrared region can be affected by the internal structure of plant leaves (Mestre 1935). In the longer wavelengths, the reflectance is controlled by water content (Allen et al. 1969), and four absorption bands due to the vibrational process of water molecules (H_2O: the combination of H–O–H and the asymmetric OH stretches) can be observed at ~975, 1200, 1400 and 1900 nm (Danson et al. 1992).

Application of the VNIR reflectance spectroscopy technique in plant analysis is not new, and has been used to indicate stresses in plants (Carter 1993). Such stresses occurs due to many factors, one of which is metal-toxicity induced stress (Atkinson and Urwin 2012). As plants are

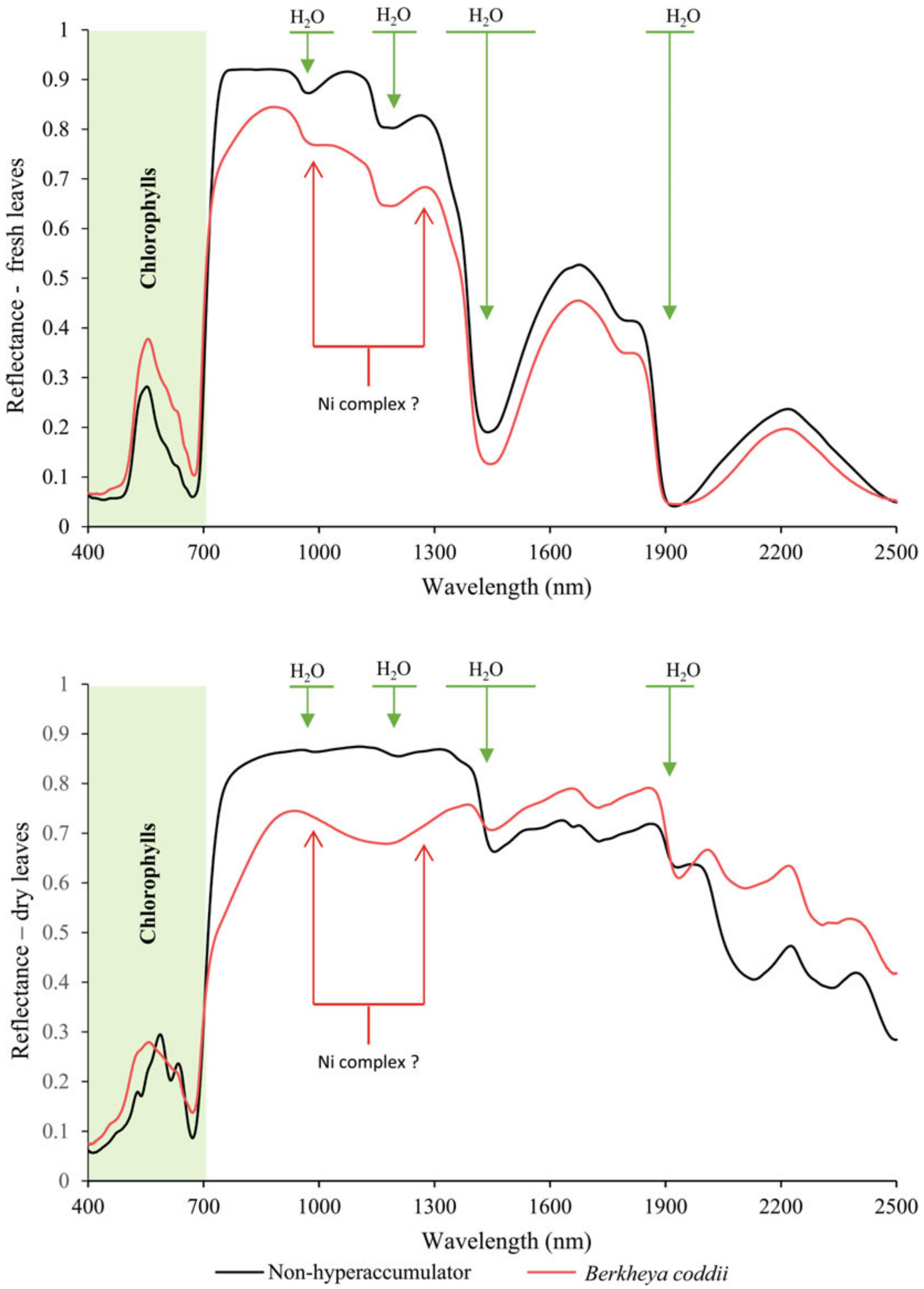

Fig. 4 Reflectance spectra of *B. coddii* leaf. The reflectance of fresh *B. coddii* leaf behaves differently in the region between two water absorption wavelength bands centred at ~975 and ~1200 nm compared to that of fresh non-hyperaccumulator plant leaf (above). As the water content in the leaves decreases, the reflectance dips of water absorption bands become reduced, and the absorption in the region between them becomes prominent. This spectral variation is suspected to be caused due to the presence of the Ni metal complex

undergoing stress, the reflectance of plant leaves shows a decrease or increase in overall reflectance values (Horler et al. 1980). Hyperaccumulator plants attain exceptionally high metal concentrations and transition metals are spectrally active in absorbing VNIR radiation (Nicholls 1974), causing spectral variations. *Berkheya coddii* (Asteraceae), for example, is a Ni hyperaccumulator plant that can accumulate Ni up to 76 100 µg g^{-1} (Mesjasz-Przybyłowicz et al. 2004). Because Ni^{2+} complexes can absorb radiation with a wavelength of ~1 µm (Goldcamp et al. 2003), spectral variations at ~1 µm in the reflectance of *B. coddii* leaves are expected to occur. Figure 4 shows the reflectance of a *B. coddii* leaf, and as expected, the absorption feature at ~1 µm is observed. There is a high probability that this absorption is due to the presence of Ni^{2+}. VNIR reflectance spectroscopy has the advantage that the VNIR sensor and platform could be used to discover hyperaccumulator plants from a airborne platform or even from a satellite.

9 Conclusions and Outlook

Metal hyperaccumulation plants are rare as only 721 known hyperaccumulators species were reported up to 2017, including 532 Ni hyperaccumulators (Reeves et al. 2017). The use of portable XRF instruments enables mass screening of herbarium collections that will likely lead to the discovery of substantial numbers of new hyperaccumulator plant species. Data obtained from systematic screening of entire phylogenetic lineages can also lead to better insights in the evolution of the hyperaccumulation plants. Many metals and metalloids could be tested using colorimetric methods spot tests, but these are not a widespread approach. Despite recent advances in technical methods for determining elements in plant materials, there is still a need for screening vast numbers of samples in the field or at field stations to identify samples for (costly and time-consuming) ICP-AES or ICP-MS analysis. Although portable XRF can fulfil this role, its use is very costly (the purchase price is >US$35 000 for an instrument), and radiation safety requirements (permits are needed in most jurisdictions) limit widespread use. Remote sensing techniques may be another approach for discovering hyperaccumulator plants that could be applied from remote sensing platforms. However, methodological studies are required in order to assess its fidelity and accuracy.

Acknowledgements Imam Purwadi is the recipient of an Australian Government Research Training Program Scholarship and an UQ Centennial Scholarship at The University of Queensland, Australia. V. Gei was the recipient of an Australia Awards PhD Scholarship from the Australian Federal Government. W.J. Przybyłowicz and J. Mesjasz-Przybyłowicz are recipients of South African National Research Foundation incentive grants No 114693 and 114694, respectively.

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Methods for Visualizing Elemental Distribution in Hyperaccumulator Plants

Antony van der Ent, Imam Purwadi,
Hugh H. Harris, Peter M. Kopittke,
Wojciech J. Przybyłowicz,
and Jolanta Mesjasz-Przybyłowicz

Abstract

Crucial to many investigations on the ecophysiology of hyperaccumulator plants is visualization of the spatial distribution of metal(loid)s in their tissues. A wide variety of methods can be used to obtain information on the distribution of elements in plant tissues, and X-ray elemental mapping techniques are especially widely used. Microanalytical investigations place strict demands on sample collection, preparation, and analytical conditions, in order to avoid elemental redistribution, chemical modification, or ultrastructural alterations. This chapter summarizes a range of techniques that can be used to visualise the elemental distribution in hyperaccumulator plants, including synchrotron and laboratory-based X-ray fluorescence microscopy (micro-XRF), proton-induced X-ray emission (PIXE), scanning/transmission electron microscopy with energy-dispersive X-ray spectroscopy (SEM/TEM-EDS), laser ablation-inductively-coupled plasma—mass spectrometry (LA-ICP-MS), nanoscale secondary ion mass spectroscopy (NanoSIMS), autoradiography, histochemical methods, and confocal microscopy using fluorophores.

1 Introduction

Understanding the ecophysiology of hyperaccumulator plants is no less important than finding them. Ecophysiology is a discipline that attempts to quantify the interaction between the external and internal environment of an organism (Ferry-Graham and Gibb 2008) and is key to understanding plant productivity and adaptation (Ainsworth et al. 2016). Advances in understanding the ecophysiology of hyperaccumulator plants are crucial for agromining practice, allowing for manipulations to stimulate metal uptake. Revealing the coordination chemistry and spatial distribution of metal(loid)s in plant tissues is a means to advance our knowledge of

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A. van der Ent (✉) · I. Purwadi
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD, Australia
e-mail: a.vanderent@uq.edu.au

H. H. Harris
Department of Chemistry, The University of Adelaide, Adelaide, SA, Australia

P. M. Kopittke
School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD, Australia

W. J. Przybyłowicz
Faculty of Physics & Applied Computer Science, AGH University of Science and Technology, al. Mickiewicza 30, 30-059 Kraków, Poland

W. J. Przybyłowicz · J. Mesjasz-Przybyłowicz
Department of Botany and Zoology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_10

the biomolecular mechanisms responsible for hyperaccumulation (van der Ent et al. 2018).

This chapter summarizes a range of techniques that can be used to visualize elemental distribution in hyperaccumulator plants, including scanning/transmission electron microscopy with energy-dispersive X-ray spectroscopy (SEM/TEM-EDS), synchrotron-based and laboratory-based X-ray fluorescence microscopy (micro-XRF), proton-induced X-ray emission (PIXE), laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), nanoscale secondary ion mass spectroscopy (NanoSIMS), autoradiography, and confocal microscopy using fluorophores. Reviews of in situ analysis of metal(loid)s in plants have been given e.g. by Lombi et al. (2011) and Wu and Becker (2012). Overviews of micro-PIXE applications in plant sciences are available (Przybylowicz et al. 1997; Mesjasz-Przybyłowicz and Przybyłowicz 2002), as well as a specific review of micro-PIXE applied to metal hyperaccumulation (Mesjasz-Przybyłowicz and Przybyłowicz 2011). Recently, other reviews have provided an explanatory approach of the main synchrotron techniques and their applications in plant sciences (e.g. Punshon et al. 2009, 2013; Lombi and Susini 2009; Sarret et al. 2013; Pushie et al. 2014; Castillo-Michel et al. 2017). This chapter builds upon more extensive reviews, such as those of van der Ent et al. (2018), who examined the use of X-ray-based approaches in hyperaccumulator plants, and Kopittke et al. (2018, 2020) who examined synchrotron-based approaches in plants.

2 Sample Preparation for Micro-analytical Investigations

The preparation of plant tissue samples for physiological investigations is critical in order to achieve preservation of the intrinsic elemental distribution. Proper preservation of plant tissues for micro-analytical investigations principally involves using cryo-techniques – various forms of cryo-fixation followed by analysis of frozen hydrated or freeze-dried plant tissue material (van der Ent et al. 2018). There are three main approaches to cryo-fixation:

1. ***Rapid plunge cryo-fixation***: plunging of small samples into a liquid cryogen (usually propane, ethane, or isopentane) cooled by liquid nitrogen. Liquid nitrogen should not be used as a primary cryogen due to the Leidenfrost phenomenon (i.e. formation of an insulating gas layer around the warm object that results in a slow freezing speed).
2. ***Metal mirror fixation***: quickly pressing small excised samples against a solid block of Cu (sometimes covered by Au) cooled by liquid nitrogen. This method is possible for use in the field.
3. ***High pressure freezing***: requires specialized equipment, with several restrictions regarding the method of preparation and specimen size. This method is mainly recommended for cryo-ultramicrotomy.

The recommended starting point for investigations is the collection of samples from plants growing in their natural habitat. Collecting plant tissue samples in the field is challenging, but two strategies have been successfully used: (i) collecting whole plants with soil in pots from metalliferous outcrops and transport to a suitably equipped laboratory (e.g. Koosaletse-Mswela et al. 2015), or (ii) freezing tissue samples in the field using metal-mirror fixation and transport to laboratory in a cryoshipper at liquid nitrogen temperature (Fig. 1) (Mesjasz-Przybyłowicz et al. 2016; van der Ent et al. 2017, 2020a, b). It is important that cryo-fixation is done as soon as possible (i.e. within seconds after excision) in order to minimize artefacts associated with sample deterioration. Air drying, or chemical fixation followed by resin embedding (including freeze-substitution), should be avoided for micro-analytical investigations because of the very substantial risk of elemental re-distribution (e.g. Turnau et al. 2001; Budka et al. 2005).

Once cryo-fixed, the samples can be analyzed in either a frozen-hydrated state (e.g. Tylko et al. 2007a, b; Wang et al. 2013) or in a freeze-dried state, employing a very slow, temperature-controlled freeze-drying regime (e.g. Mesjasz-

Fig. 1 Fieldwork in Malaysia: freezing samples in the field using metal-mirror fixation and transport to laboratory in a cryoshipper at liquid nitrogen temperature

Przybyłowicz et al. 2007; van der Ent et al. 2018). Frozen samples may be sectioned for analysis of specific tissues and cells. This approach is advisable because the penetration depth of the X-rays is generally greater than the thickness of a cell layer, and where there is more than one overlapping layer (e.g. upper epidermis, palisade mesophyll, spongy mesophyll, vascular bundle, and lower epidermis), it becomes impossible to ascertain from which layer the fluorescence signal is originating (Donner et al. 2012). A cryo-microtome can easily produce sections ~30 μm thick that typically correspond to a single cell layer. When using a cryo-ultramicrotome, semi-thin sections of the order of 1–2.5 μm in thickness, and thinner, can be obtained. Cryo-sections need to be kept at cryogenic temperature (−130 °C or colder) until micro-analytical examination, or they can be freeze-dried.

3 Elemental Localization Within Plant Tissues

A range of approaches can be employed for assessing elemental distribution within plant tissues. Below, a number of techniques are discussed including those that illuminate the sample with photons (laboratory- or synchrotron-based micro-XRF) and irradiate the sample with electrons (SEM/TEM-EDS) or ions (PIXE and SIMS). The elemental detection is based upon either the emitted fluorescent X-rays (laboratory micro-XRF, synchrotron micro-XRF, SEM/TEM-EDS, and PIXE) or by mass spectrometry (SIMS and LA-ICP-MS). In addition, we also describe the use of light microscopy and histochemistry, as well as autoradiography. The various techniques differ in their sensitivity (i.e. limits of elemental detection), lateral resolution, and sample preparation requirements. Although discussed in detail below, laboratory micro-XRF, synchrotron micro-XRF, SEM/TEM-EDS, PIXE, SIMS, and LA-ICP-MS potentially allow the analysis of (frozen) hydrated samples, thereby reducing the likelihood of experimental artefacts. Synchrotron (micro-XRF) and nuclear microprobe (micro-PIXE) facilities are often not easily accessible for many users, in contrast to SEM/TEM-EDS that are comparatively readily accessible in most large research institutions.

3.1 SEM/TEM-EDS

In SEM/TEM-EDS, incident electrons excite core electrons of the elements of interest within the plant tissue sample, with the subsequent measurement of emitted fluorescent X-rays (Warley 1997; Zierold 2002). Among the techniques considered here, SEM/TEM-EDS has relatively poor detection limits, in the order of 1000–5000 μg g^{-1} for most transition elements. 'Traditional' SEM analyses are conducted in a vacuum, requiring samples that are dehydrated, although samples can be analyzed in the frozen state where a cryogenic stage is available. The sample surface must be conductive. Conductivity is achieved by deposition of a very thin conductive layer onto the sample surface. The X-ray signal from a deposited layer should not interfere with the measured X-ray spectrum from the sample, hence only a carbon coating is used. More recently, the development of Environmental SEM has facilitated the analysis of hydrated/fresh samples. Although it is possible to obtain high resolution when imaging with SEM, elemental information is limited to a resolution of ca. 1–5 μm due to the interaction of the incident electrons with the sample, resulting in a tear drop-shaped penetration of the electrons into the sample (Warley 1997). Figure 2 shows SEM-EDS elemental maps of a petiole cross-section. TEM/EDS (Zierold 1988) is seldom used as it requires time-consuming preparation of ultrathin cryo-sections, although this is possible (e.g. Frey et al. 2000).

3.2 Laboratory Micro-XRF

Laboratory micro-XRF instruments use a focused beam of high-energy X-rays generated from a tube as the excitation source. These types of instruments provide high spatial resolution with spot sizes as small as 5 μm (compared with 20 nm–1 mm for synchrotron-based systems) and detection limits down to 50 μg g^{-1} for most transition elements (compared with <1 μg g^{-1} for synchrotron-based systems). The availability of laboratory micro-XRF facilities is particularly attractive for assessing fresh/hydrated plant specimens subjected to metal-dosing treatments, as well as for undertaking time-resolved studies. The lower X-ray flux in the laboratory micro-XRF system translates to typical per-pixel dwell times of 20–100 ms to achieve the desired sensitivity for major and trace elements. This result compares with 0.5–5 ms per pixel in synchrotron micro-XRF systems. This is offset by essentially ‘unlimited’ beamtime access to laboratory micro-XRF facilities at local institutions (within financial constraints), compared to highly restricted availability of beamtime for synchrotrons.

To date, however, limited attempts have been made to translate this to laboratory micro-XRF (Fittschen et al. 2017; Rodrigues et al. 2018). Off-the-shelf systems include the Bruker TORNADO, the EDAX Orbis PC system, and the ‘Maia Mapper’ that CSIRO has developed for the Advanced Resource Characterisation Facility (ARCF) in Perth (Australia). The University of Queensland (Australia) micro-XRF facility is a custom-built system manufactured by IXRF Systems, which consists of two 50 kV 1000 μA sources fitted with polycapillary focusing optics: a microfocus Mo-target tube producing 17.4 keV X-rays focused to 25 μm, and a Rh-target tube producing 20.2 keV X-rays focused to 5 μm. The system is fitted with two silicon drift detectors (SDD) of 150 mm^2 active area each. The fast motion stage allows for scanned areas up to 300 × 300 mm. Per pixel dwell times are typically 50–100 ms. It also has a cryo-stage (50 × 50 mm active area held at −50 °C) for analysis of samples in a frozen-hydrated state. Figure 3 shows the analytical measurement conditions for a laboratory micro-XRF instrument at The University of Queensland; Fig. 4 displays elemental maps of a Ni hyperaccumulator leaf (*Pycnandra acuminata*).

Sample preparation for the laboratory-based micro-XRF instrument is simple and does not require electrically conductive coatings (Rodrigues et al. 2018). The limits of detection of micro-XRF are much lower than those of SEM-EDS because of the greater efficiency of X-ray excitation compared to electron excitation. Operating the analysis chamber under vacuum or at helium atmosphere (to reduce absorption of low-energy X-rays in the air path) allows for the analysis of light elements down to sodium. In both, laboratory and synchrotron micro-XRF, the possibility of radiation-induced damage (especially in fresh hydrated samples) is an important consideration that may limit information sought from the analysis (van der Ent et al. 2018). In a recent study, radiation dose limits for micro-XRF analysis were assessed, and in hydrated plant

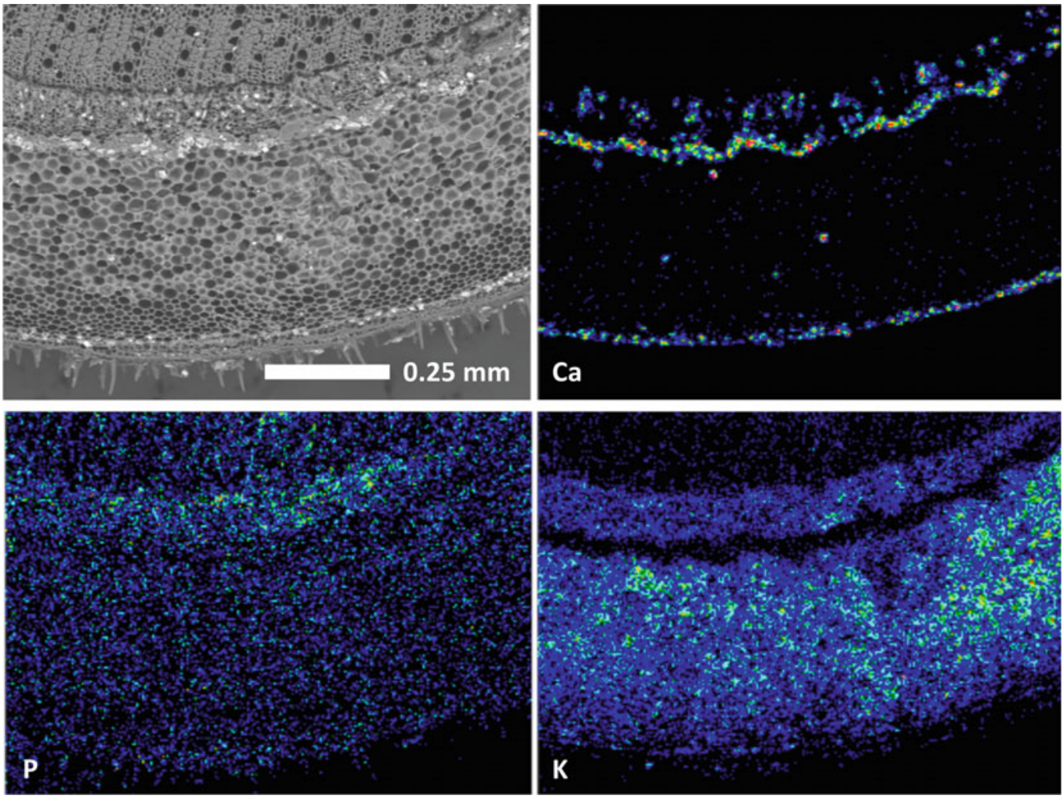

Fig. 2 Examples of elemental maps obtained with SEM-EDS analysis. The top left panel shows the typical electron microscopy image, with the other panels showing the elemental maps of Ca, P and K respectively. The sample is a freeze-dried petiole cross-section of the Ni hyperaccumulator *Rinorea bengalensis* (Violaceae)

tissues dose-limits are 4.1 kGy, above which damage occurs (Jones et al. 2020). Laboratory micro-XRF systems typically use a concave-focused, polychromatic X-ray source emitting Bremsstrahlung background. This represents an important difference compared with a monochromatic, highly parallel X-ray source in the synchrotron micro-XRF. Because normally there is no energy tunability in laboratory micro-XRF, use of X-ray absorption spectroscopy to determine chemical speciation of selected elements in plant specimens is not possible.

3.3 Synchrotron Micro-XRF

Synchrotron light is emitted by relativistic (very near to the speed of light) electrons when affected by a magnetic field resulting in photons that range from infrared and ultraviolet to X-ray wavelengths (Watson and Perlman 1978). The electromagnetic radiation emitted from emission devices is delivered to ‘end stations’ within individual beamlines. At ‘end stations’ the X-rays illuminate samples in a spectrometer, and fluorescence spectra may be recorded by different types of detectors (Miller and Dumas 2006; Sarret et al. 2013; Vijayan et al. 2015). Techniques that are possible using synchrotron XRF microscopy beamlines include scanning X-ray fluorescence microscopy (micro-XRF), X-ray fluorescence micro-computed tomography (XRF-μCT), X-ray absorption spectroscopy (XAS) and imaging, and differential phase-contrast imaging of low-Z ultrastructure (DPC), amongst others. A practical summary for potential synchrotron users was prepared by Donner et al. (2013). There are more than 50 synchrotrons in the world (https://lightsources.org/lightsources-of-the-world/). One of these is the

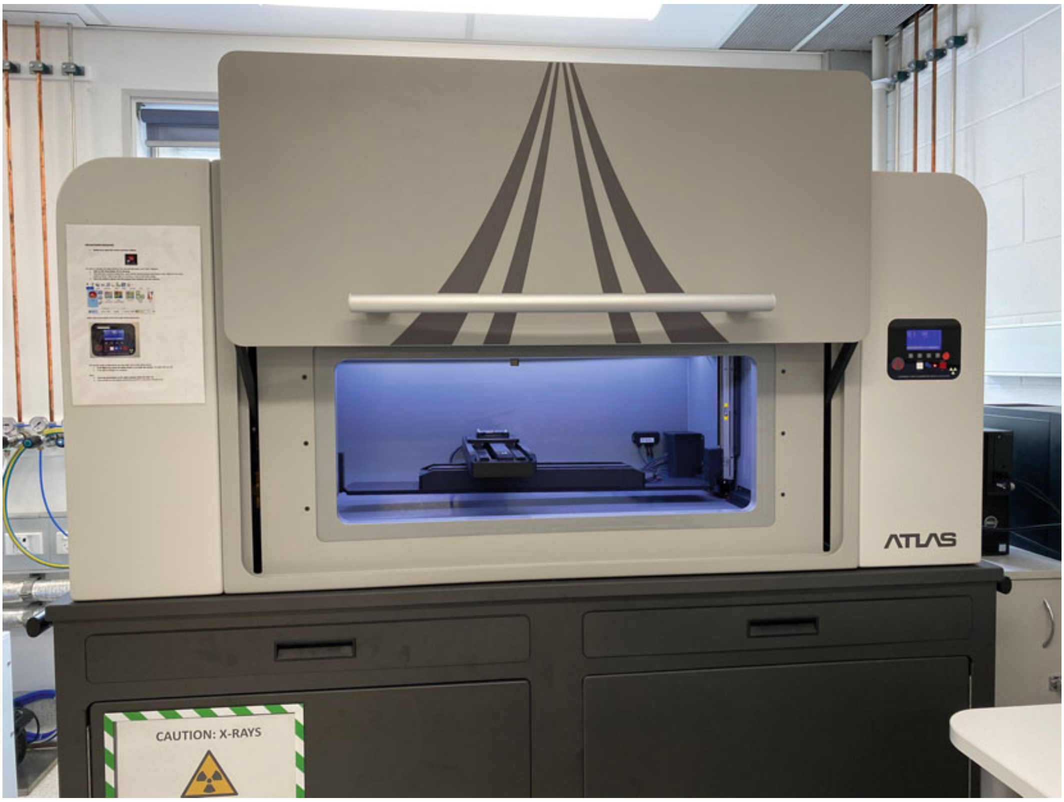

Fig. 3 Laboratory micro-XRF instrument at The University of Queensland, Australia

Australian Synchrotron in Melbourne. The XFM beamline at the Australian Synchrotron achieves a spatial resolution of ~1 μm, with an energy range of 4–25 keV operating on an in-vacuum undulator source (Paterson et al. 2011). The micro-XRF beamlines have the ability to generate fine-scale elemental maps with spatial resolutions and quantification within plant tissues, cells, and organelles using a micro-focused X-ray beam (McRae et al. 2009). Fluorescence emitted from the irradiated spots is detected as a function of location on the specimen, when the specimen is moved relative to the stationary X-ray beam. Synchrotron micro-XRF is a highly sensitive technique, with detection sensitivities as low as the femtogram (10^{-15}) to attogram (10^{-18}) level, hydrated samples can be analyzed in vivo, and it has the capacity to perform simultaneous, multi-elemental analysis (Sarret et al. 2013). Figure 5 shows the analytical setup at the XFM beamline of the Australian Synchrotron with the Maia detector system; Fig. 6 displays the elemental maps of a Ni hyperaccumulator leaf (*Pycnandra acuminata*).

New generation fast-detector systems such as Maia, which comprises of an array of 384 detectors, can process photon rates exceeding 10^7 s^{-1}, with pixel transit times as small as 50 μs, thereby producing megapixel elemental maps (Ryan et al. 2010, 2014; Kirkham et al. 2010). The use of these new-generation detector systems permits new insights into both hyperaccumulators and other plant species. It is now possible to image living plants repeatedly following exposure to elevated levels of trace metals (such as Ni or Mn) in order to examine progressive changes in metal distribution in plant tissues. For example, using the non-accumulating plant species cowpea (*Vigna unguiculata*), leaves were scanned repeatedly for up to 48 h after exposure to 30 μM Mn. In this study, images were obtained six times over 48 h, with the

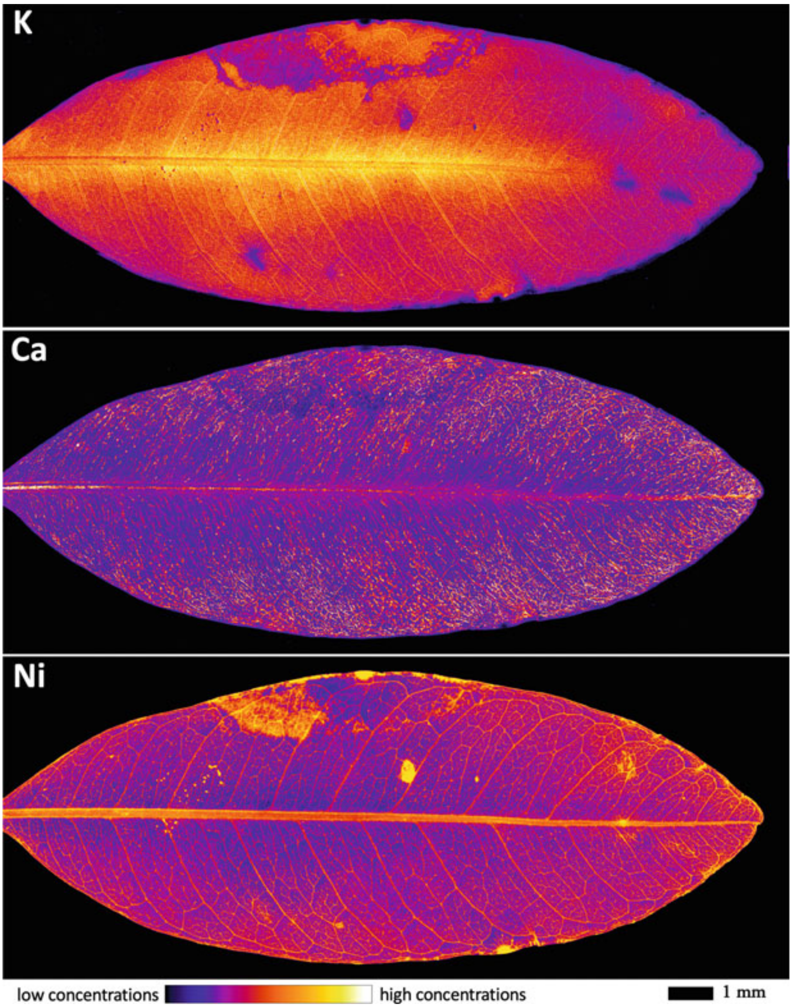

Fig. 4 Example of elemental maps obtained with laboratory micro-XRF analysis. The sample is a freeze-dried leaf portion of a Ni hyperaccumulator leaf *Pycnandra acuminata* from New Caledonia

images composed of 4 μm × 4 μm pixels collected over an area of 25 mm × 5 mm (Fig. 7). An essential component of such studies, however, is the need to determine that such repeated scanning does not cause damage to the living leaf tissues and hence experimental artefacts.

3.4 Micro-PIXE

In micro-PIXE, characteristic X-rays of elements present in analysed samples are excited with focused ion beams, typically protons with energies of 2–3 MeV (Johansson et al. 1995; Mandò and Przybyłowicz 2016). This technique allows quantitative studies of the concentration and distribution of elements from Na to U with detection sensitivity down to 1–10 μg g^{-1} for transition elements, and lateral resolution on the order of 1 μm. Such a broad elemental range enables quantification of a large group of elements such as S, P, K, Ca, Mn, Fe, Cu, and Zn (Mesjasz-Przybyłowicz and Przybyłowicz 2002, 2011). Simultaneous use of at least one additional analytical technique is a common practice, and the facility is referred to as a nuclear microprobe or a proton microprobe, because a proton beam is most often used. All research

Fig. 5 The analytical endstation of the XFM beamline at the Australian Synchrotron

related to hyperaccumulator plants using micro-PIXE has been carried out using protons and beam spot sizes of a few microns. Typically, areas up to 2.5 mm × 2.5 mm can be analysed in a single scan; mapping of larger structures requires specimen movement and later mosaicking of maps. Specimens of any thickness can be analysed. Fundamentally, PIXE is a standardless method that uses a fundamental parameter model approach. Once the experimental system has been calibrated, no additional standards matching the composition of biological samples are necessary. Quantification of analyses is obtained by direct measurements of accumulated charge deposited by protons or a value proportional to it. Simultaneously used proton backscattering spectrometry (BS) allows for the determination of specimen thickness (areal density) and the concentrations of main components of a biological material i.e. C, O, N, and indirectly, H. For samples fully penetrated by the proton beam, the thickness can be also determined using the scanning transmission ion microscopy (STIM) method (Pallon et al. 2009). Numerous studies of hyperaccumulators have been conducted mainly at the three laboratories – iThemba LABS in Cape Town, South Africa (Fig. 8), MIC in Ljubljana, Slovenia, and ANSTO in Melbourne, Australia. The first two laboratories offer facilities suitable for the analysis of frozen-hydrated samples and have reported results of elemental mapping of hyperaccumulator plants (Tylko et al. 2007a, b; Wang et al. 2013; Vavpetič et al. 2013; Hu et al. 2015). However, the majority of studies were conducted on freeze-dried samples (Mesjasz-Przybyłowicz and Przybyłowicz 2011).

The first micro-PIXE study on a hyperaccumulator was undertaken in 1981 (Morrison et al. 1981). More systematic elemental mapping of hyperaccumulator plants started from 1994 (Mesjasz-Przybyłowicz et al. 1994). Overviews

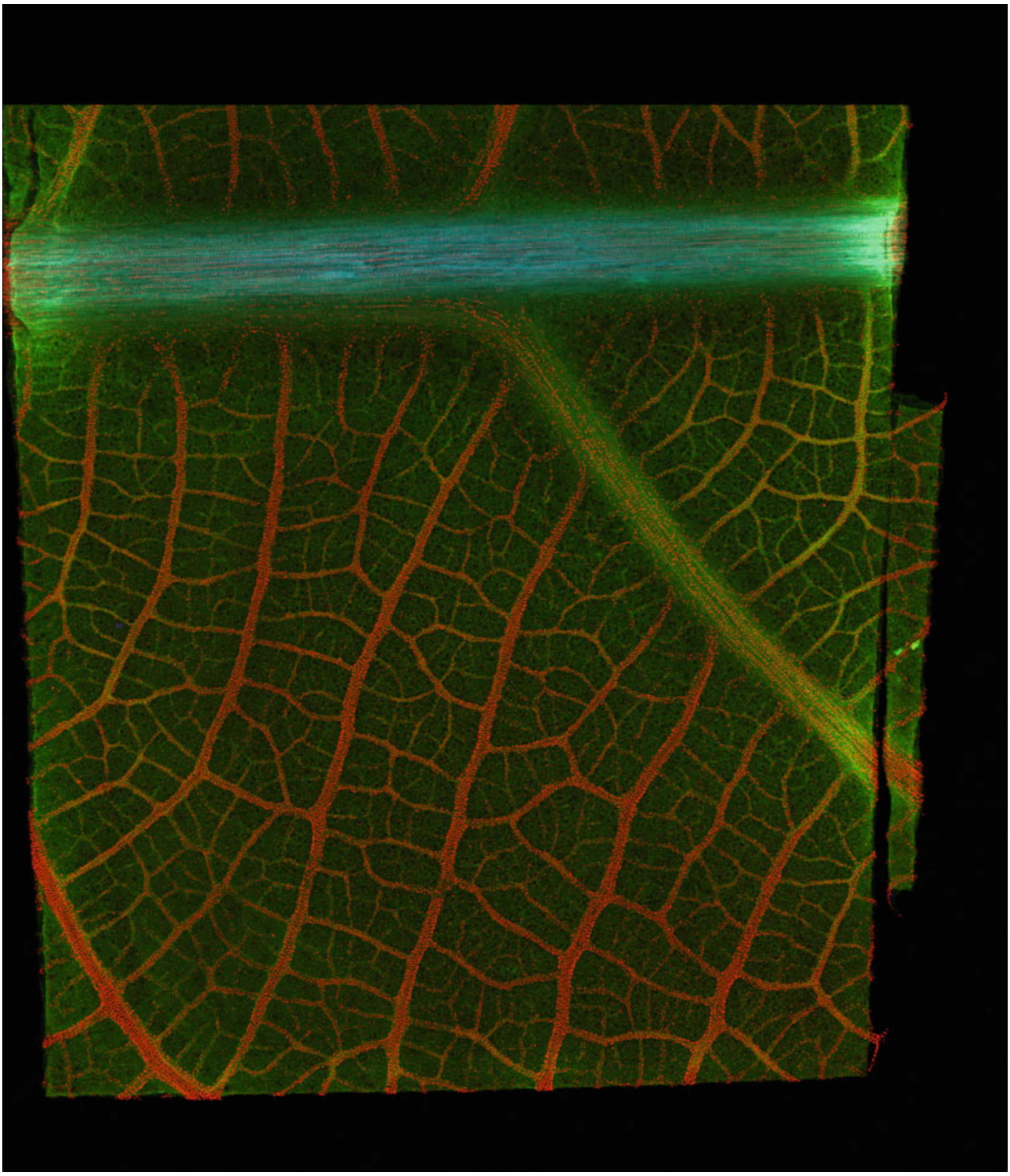

Fig. 6 Example of elemental map obtained with synchrotron micro-XRF analysis. The sample is a leaf portion of a Ni hyperaccumulator leaf *Rinorea bengalensis* from Malaysia. This is an RGB colour composite with red showing calcium, green showing nickel and blue showing cobalt

of numerous micro-PIXE studies related to hyperaccumulation of Ni, Co, As, Mn, Zn, and Cd can be found e.g. in Przybylowicz et al. (1997) and Mesjasz-Przybyłowicz and Przybyłowicz (2011). Studies in which micro-PIXE was employed extend from elemental mapping of organs, tissues, and plant cells, to the role of mycorrhizas in hyperaccumulating plants and relationships between these plants and herbivorous insects feeding on them (Mesjasz-Przybyłowicz and Przybyłowicz 2011, 2020). Figure 9 shows an example of micro-PIXE

Fig. 7 Live plants (cowpea, *Vigna unguiculata*) to be analyzed with the Maia detector system at the XFM beamline of the Australian Synchrotron. The leaves were scanned (4 µm pixels, area of 25 mm × 5 mm) repeatedly for up to 48 h after exposure to 30 µM Mn

quantitative mapping of a hyperaccumulator. In this case, the leaf cross-section of *Senecio coronatus* (Asteraceae) was analysed in a frozen hydrated state. After mapping of the whole leaf cross-section (left), an additional scan was performed for a selected smaller region (right). Significant enrichment of Ni in the vacuoles of epidermal cells is clearly visible, whereas Ca shows significant depletion in the vacuoles, enrichment in the cell walls, and very high concentrations in the outermost cell wall. Maps demonstrate that micro-PIXE is able to reveal elemental distributions at the sub-cellular level (van der Ent et al. 2018).

3.5 NanoSIMS

SIMS uses ions as the incident beam that collides with the sample surface and causes atoms and molecules from the upper layers of the sample to be ejected into the vacuum of the instrument (De Rue et al. 2006; Hoppe et al. 2013). Ionized particles, referred to as secondary ions, are extracted to a mass spectrometer for analysis (which also enables isotopic identification). Given that the sputtering depth is 5–20 nm (Hoppe et al. 2013), NanoSIMS is a surface-sensitive technique, allowing for a spatial resolution as low as <50 nm. The primary beam is typically O$^-$ or Cs$^+$, with a negatively charged primary beam (O$^-$) tending to favour the production of positively charged secondary ions; a positively charged primary beam (Cs$^+$) tends to favour the production of negatively charged secondary ions. Thus, for metals (such as Ni) an O$^-$ beam is generally preferable, whereas for metalloids (such as As) a Cs$^+$ beam is generally preferable. For some elements, such as Zn, Cd, and Mn, a poor secondary ion yield makes

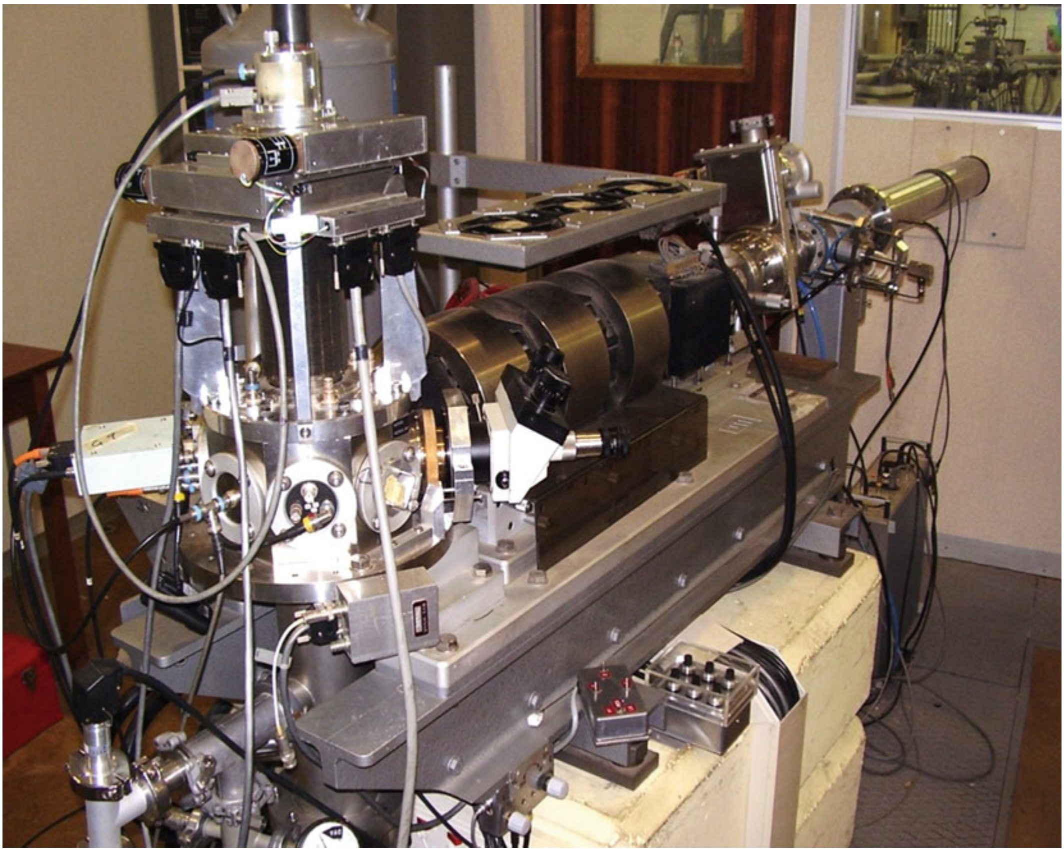

Fig. 8 The experimental setup of the nuclear microprobe at the Materials Research Department of iThemba LABS, South Africa

analyses more difficult (Zhao et al. 2014). NanoSIMS analyses are conducted within an ultra-high vacuum. Notably, the technique has a number of potential practical limitations when applied to biological specimens. It is very sensitive to topography; therefore flat, microtomed specimens are required. Specimens analysed at room temperature must be chemically fixed, dehydrated, and embedded in resin. SIMS is also subject to strong matrix effects. Importantly, it is very difficult to quantify SIMS data (Moore et al. 2011). However, there are some very interesting examples of studies on hyperaccumulators using NanoSIMS. In the hyperaccumulator *Odontarrhena lesbiaca*, Smart et al. (2007) used NanoSIMS to examine leaf sections, with Ni found to accumulate in the peripheral regions of large unicellular trichomes and in the epidermal cell layer. In a similar manner, Smart et al. (2010) examined the distribution of Ni and other elements in highly vacuolated leaf tissue of *O. lesbiaca*.

3.6 LA-ICP-MS

LA-ICP-MS uses a focused laser beam to ablate the sample surface, with ablated particles then transported to the mass spectrometer. Elemental and isotopic analyses are both possible, as for NanoSIMS. However, there are significant differences between the two techniques. The spatial resolution of LA-ICP-MS is 2 to 3 orders of magnitude worse than for NanoSIMS, being 1–50 µm, but the technique is comparatively matrix insensitive, and the signal to concentration ratio

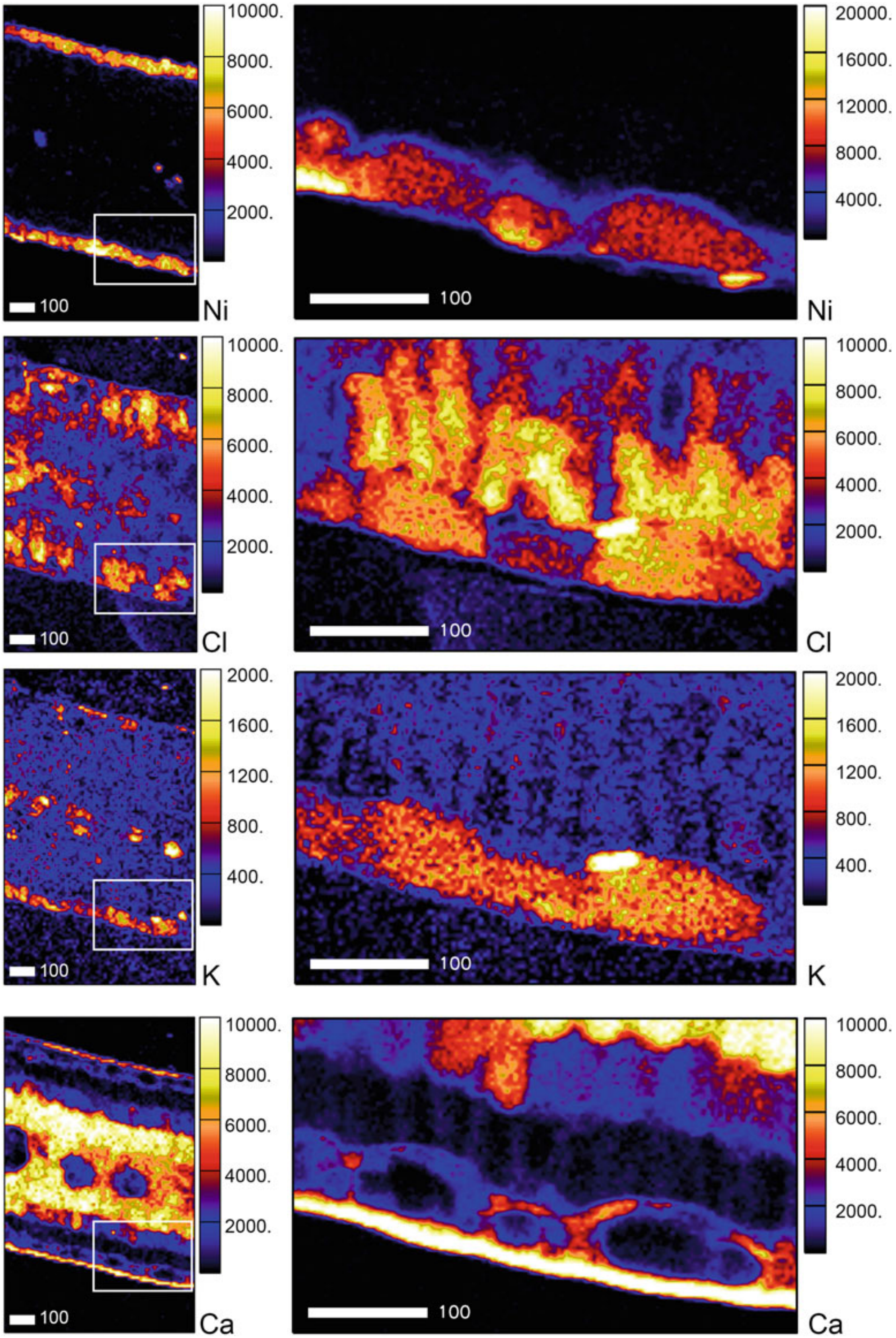

Fig. 9 Proton Induced X-ray Emission (PIXE) quantitative elemental maps of the leaf cross-section of *Senecio coronatus* (Asteraceae) analysed in frozen hydrated state. Maps on the right side are from the subsequent scan of the smaller section marked in maps on the left. Significant enrichment of Ni in the vacuoles of epidermal cells is well visible. Maps demonstrate that micro-PIXE is able to reveal elemental distribution at the sub-cellular level. All results are shown in µg g^{-1}. Accumulated charge was 0.5 µC in both cases. Scale bar is 100 µm (reproduced from van der Ent et al. 2018 with permission)

is largely linear. In contrast to NanoSIMS, the analysis takes place under ambient pressure. It is an advantage; however, there are some related drawbacks as well. The laser ablation process takes place in a He or Ar atmosphere with a certain flow rate which may cause dehydration of the sample during the measurement. For samples with high water content such as plant roots, metal redistribution and sample distortion may happen. Therefore, to keep the sample moist and intact is crucial in LA-ICP-MS analysis (Wu and Becker 2012). The technique is highly sensitive, down to at least μg g^{-1} levels, and particularly useful in bioimaging of elements such as Cd or Pb, that are difficult to measure with X-ray techniques at such low concentrations. Quantification is achieved by scanning matrix-matched standard reference materials together with the sample under the same conditions (the same laser conditions and the same ICP-MS conditions). If no certified standard reference is available, matrix-matched laboratory standards can be prepared using control/blank sample materials spiked with standard solution with known concentration. ^{13}C is typically used as an internal standard (Wu and Becker 2012; Becker et al. 2014). Using LA-ICP-MS, Tian et al. (2011) examined Cd distribution in *Sedum alfredii* using fresh (entire) leaves and freeze-dried sections taken from both leaves and stems. These authors reported preferential accumulation of Cd within parenchyma cells, especially in stems. Wu et al. (2009) used LA-ICP-MS to examine elemental distribution in fresh (hydrated) leaves of *Elsholtzia splendens*. Callahan et al. (2016) used this technique for elemental imaging of Ni, Zn and Cd in leaves from the hyperaccumulator *Noccaea caerulescens*. In addition to the comprehensive overview by Becker et al. (2014), reviews on the biological applications of LA-ICP-MS including hyperaccumulator studies can be found e.g. in Pozebon et al. (2014, 2017).

3.7 Autoradiography and MRI

The use of radioactive isotopes in autoradiography is a powerful technique for the visualisation of specific radiotracers within intact living plants. It offers high resolution and the ability for time-resolved studies, but application is limited primarily due to occupational health and safety concerns. The distribution of Cd has been elucidated in *N. caerulescens* and *Arabidopsis halleri* using the ^{109}Cd radiotracer (Cosio et al. 2005; Huguet et al. 2012). An alternative approach that does not involve radioactive isotopes, but has many of the same advantages, is Magnetic Resonance Imaging (MRI), which has been successfully demonstrated for in situ Ni distribution in the rhizosphere of *Berkheya coddii* (Moradi et al. 2009).

4 Histochemical Methods of Elemental Localization

4.1 Use of Chelating Dyes and Light Microscopy

Chelating dyes have also been used to stain metal(loid)s in plant tissues. Dimethylglyoxime ($C_4H_8N_2O_2$) forms a crimson/pink complex with Ni^{2+} and has been used as a histochemical stain for identifying Ni localization within tissues of hyperaccumulator plants (Mizuno et al. 2003; Bhatia et al. 2004). However, artefacts are evident using this method, as demonstrated by Bhatia et al. (2004) who reported re-distribution of Ni. Similarly, Zincon ($C_{20}H_{15}N_4NaO_6S$), which forms a blue complex with Zn^{2+}, has been used for staining Zn in *N. caerulescens* (Macnair and Smirnoff 1999; Kozhevnikova et al. 2016).

Autometallography is another histological technique by which target ions (such as Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}) are precipitated with sulfides upon which metallic Ag is subsequently deposited by use of a reductant (Danscher 1984). The Ag clusters can then be imaged by light microscopy or SEM/TEM. The main disadvantage of autometallurgy is that the sulfide reaction is non-specific and precipitates a range of transition elements, although at neutral pH the selective formation of ZnS nanocrystals is favoured (McRae et al. 2009). Selectivity may also be achieved using immuno-histochemical methods via monoclonal anti-bodies in order to specifically bind chelated target ions, as demonstrated for Cd^{2+} in plant tissue (Gao et al. 2015).

4.2 Use of Fluorophores and Confocal Microscopy

Fluorescence microscopy is sometimes employed for investigations of plant structure that may be pertinent for interpreting elemental distribution patterns in specific tissues. These include, for example, the identification of apoplastic barriers such as Casparian bands in roots stained with the fluorophore berberine ($C_{20}H_{18}NO_4^+$) (Brundrett et al. 1988). Ion-selective fluorophores have been used to image the distribution of Ni and Zn in hyperaccumulator plants. Zinpyr-1 ($C_{46}H_{36}Cl_2N_6O_5$) was used to image Zn^{2+} in *N. caerulescens* (Kozhevnikova et al. 2014, 2016). The high sensitivity of this fluorophore also enabled imaging of Zn^{2+} in non-hyperaccumulator plants, such as in the roots of *Arabidopsis thaliana* (Sinclair et al. 2007). Leadmium Green was used to map Zn^{2+} and Cd^{2+} in the hyperaccumulators *Sedum alfredii* and *Picris divaricata* (Lu et al. 2008; Hu et al. 2012). Newport Green ($C_{43}H_{30}Cl_2N_4O_8$) has been applied for the imaging of Ni^{2+} in cells of *O. chalcidica* (Agrawal et al. 2013) and *O. lesbiaca* (Ingle et al. 2008).

Fluorophores have a number of merits, including high selectivity, the ability to be used in live tissues in time-resolved visualization, and an inherently high resolution via visible light microscopy. However, there are also limitations related to unknown penetration and binding to target metal(loid) ions. Fluorophores will only bind to free ions that are not already strongly ligated; for example, Al^{3+} binds strongly to the cell wall but the fluorophore morin ($C_{15}H_{10}O_7$) does not bind to this wall-bound Al, hence this method was unable to identify the major compartment of Al (Eticha et al. 2005). Another concern relates to the application method of fluorophores to the sample: loading vibratome-sectioned samples in a solution with the fluorophore will ostensibly result in removal of any soluble metal(loid) ions present. Delivery of cell-permeable fluorophores into a nutrient solution, followed by uptake in the plant, could circumvent this issue, but avoiding re-distribution during sectioning remains difficult. Attempts have also been made at fixing mobile target ions (Cd^{2+} or Zn^{2+}) by in situ precipitation as metal sulphides or with strong chelators before microtoming and microscopy (Hu et al. 2009, 2012; Gao et al. 2015). There is a need for independently validating the use of fluorophores for mapping metal(loid) ions in hyperaccumulator plants, e.g. by mapping of fluorophore-labelled tissues using other micro-analytical techniques such as micro-XRF or micro-PIXE.

4.3 Light Microscopy and TEM

Knowledge of anatomical features is crucial for accurately interpreting elemental distribution in the hyperaccumulator plant tissues being studied (e.g. Mesjasz-Przybyłowicz et al. 2007, 2016), For bright-field light microscopy, samples are usually fixed in 1–3% glutaraldehyde ($C_5H_8O_2$) buffered in cacodylate or phosphate-buffered saline (PBS). Fixation is often followed by dehydration in a graded ethanol (C_2H_6O) series, with or without propylene oxide (C_3H_6O), followed by embedding in a low-viscosity resin (such as Spurr's 1969). Sections 0.5–2 µm thick can then be cut, stained with dyes, and imaged. If subcellular information is needed on the cells in which elemental distribution is being investigated, ultrathin sections of gold interference colour (80–90 nm) can be cut from the same resin blocks and mounted onto grids, then stained with 2% uranyl acetate ($UO_2(CH_3COO)_2$) and lead citrate ($(C_6H_5O7)_2Pb_3$) (Reynolds 1963), followed by TEM analysis. It is better, however, to use tissue samples that are rapidly frozen and then freeze-substituted and treated with a secondary fixative.

5 Outlook

The major limitation for researchers in using micro-XRF and micro-PIXE in experiments is the restrictive nature of access to synchrotron and accelerator facilities. There is, therefore, a need to bridge the gap between what is currently possible in the typical laboratory environment and the capability of large (and scarce) analytical facilities. Technological developments, including very high-flux X-ray sources based on metal jets (Hemberg et al. 2003), and even table-top accelerators based on Inverse Compton Scattering (Graves et al. 2014), will not replace synchrotron-based micro-XRF, but will permit combining their individual strengths, for example, by whole organism mapping at the local laboratory followed by investigation of target cells at a major synchrotron or accelerator facility.

Acknowledgements The authors have greatly benefited from the expertise in plant anatomy and microscopy from Prof Alban Barnabas over many years. Imam Purwadi is the recipient of an Australian Government Research Training Program Scholarship and an UQ Centennial Scholarship at The University of Queensland, Australia. Parts of this research was undertaken using the X-Ray Fluorescence Microscopy (XFM) beamline of the Australian Synchrotron, part of ANSTO. Research was also undertaken at the Centre for Microscopy and Microanalysis at The University of Queensland. W.J. Przybyłowicz and J. Mesjasz-Przybyłowicz are recipients of the South African National Research Foundation incentive grants No 114693 and 114694, respectively.

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Genesis and Behaviour of Ultramafic Soils and Consequences for Nickel Biogeochemistry

Guillaume Echevarria

Abstract

Ultramafic outcrops could represent up to 3% or more of the terrestrial surface and their unusual geochemistry makes them a global hotspot for biodiversity. Ultramafic soils are a peculiarity for soil scientists in all climatic zones of the world. These soils lack essential pedogenetic elements including Al, Ca, K and P. Whereas serpentinites will most likely give birth to Eutric Cambisols with little influence by climate, peridotites will induce an acceleration of weathering processes; this over-expressed weathering is due to their deficiency in Si and Al, abundance in Mg, and lack of secondary clay formation. Soils evolve towards Ferralsols in tropical conditions. Results from isotopic dilution techniques show that Ni borne by primary minerals is unavailable. Secondary 2:1 clay minerals (e.g. Fe-rich smectite) and amorphous Fe-oxyhydroxides are the most important phases that contain available Ni. Therefore, smectite-rich soils developed on serpentinite and poorly weathered Cambisols on peridotite (only in temperate climates) are the soils with highest Ni availability. Although soil pH conditions are a major factor in controlling available Ni, the chemical bounds of Ni to bearing phases are even more important to consider. Plants may take up significant amounts of Ni, and its biogeochemical recycling seems an essential factor that explains Ni availability in the surface horizons of ultramafic soils.

1 Introduction

Globally, ultramafic (or ultrabasic) outcrops represent more than 3% of the terrestrial surface, according to a recent estimation (Guillot and Hattori 2013). They have focused attention for decades because of their unusual geochemical composition at the surface of continents. This peculiar geochemistry (lack of most essential elements for plant nutrition: K, Ca and P) makes them a global hotspot for biodiversity. To soil scientists (Alexander 2009), ultramafic soils have also been of a particular interest because the pedogenetic processes involved in their formation are usually different from those taking place in soils developed on all other types of bedrock in the same area. It is quite common that ultramafic soils in warm regions display soil types that are

G. Echevarria (✉)
Laboratoire Sols et Environnement, Université de Lorraine-INRAE, Nancy 54000, France
e-mail: Guillaume.Echevarria@univ-lorraine.fr

G. Echevarria
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD, Australia

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_11

common in colder regions, or the opposite. Ultramafic soils are also the most important source of Ni and Co to terrestrial ecosystems (Estrade et al. 2015). Nickel and Co hyperaccumulation by plants has evolved a great deal from ultramafic soils, mostly in relation to metal bioavailability within these soils (Lange et al. 2017; van der Ent et al. 2016a, 2018a). This chapter discusses the most important trends of soil genesis and evolution on ultramafic bedrock worldwide. It then focuses on the main Ni-bearing phases resulting from soil genesis patterns in the most important cases, providing a foundation for a better understanding of the factors that control Ni availability in ultramafic soils.

2 Properties of Ultramafic Bedrock

2.1 What Are Ophiolites and Peridotites?

Ophiolites, which were long regarded as continental analogs of oceanic crust, are now viewed as out-of-place fragments of oceanic lithosphere (Dilek and Furnes 2009). They generally consist of some or all of the following components: lherzolite or harzburgite commonly altered to serpentinite, gabbro, 'sheeted' basic dykes, pillow-bearing basic volcanic rocks, chert, and sedimentary rocks (Vaughan and Scarrow 2003). Ultramafic outcrops occupy 1–3% of Earth's land surface (Fig. 1) but are locally abundant in ophiolite belts along tectonic plate margins (Coleman and Jove 1992; Vaughan and Scarrow 2003; Dilek and Furnes 2009, Guillot and Hattori 2013). One of the most studied ophiolite complexes is that of the ancient Tethyan Ocean, which spread from the Alps to the Himalayas, and was subducted onto the margin of the continental Eurasian plate from Jurassic to Cretaceous time (Dilek and Furnes 2009). Ophiolites worldwide, including upper mantle peridotites, vary in lithology and geochemistry owing to differences in compositions of magmas over time and according to regional conditions (Dilek and Furnes 2009).

2.2 Varieties of Ultramafic Rocks and Their Compositions

Peridotite (or ultramafic) rocks are defined as igneous rocks that contain more than 90 vol% mafic minerals (Le Bas and Streckeisen 1991), of which are olivine (nesosilicates), and pyroxene (inosilicates). There are several types of peridotites based on vertical position in the original ophiolite, being distinguished according to the ratio of olivine to clino- and ortho-pyroxene (Fig. 2) as officially defined by the International Union of Geological Sciences, i.e. IUGS (Le Bas and Streckeisen 1991). The most common ultramafic rocks found in outcrops include dunite, harzburgite, lherzolite and pyroxenite.

All of these rocks can undergo metamorphism in varying conditions according to the history of mid-ocean ridges and subduction zones (O'Hanley 1996; McCollom et al. 2016). This process includes deformation—including shearing—and of course serpentinization (Fornasaro et al. 2019), which essentially consists of a hydration of olivine and pyroxene that results in the formation of serpentine clay minerals; however, other minerals are also typically formed during the serpentinization process, e.g. magnetite, brucite, talc, chlorite, etc. (Coleman and Jove 1992; McCollom et al. 2016). As an example, the formation of serpentine through the weathering of olivine is described in Eq. (1) (McCollom et al. 2016):

$$\begin{aligned}&\underset{\text{Olivine(Forsterite)}}{\mathrm{Mg_{1.82}Fe_{0.18}SiO_4}} + \mathrm{wH_2O}\\&\rightarrow 0.5\underset{\text{Serpentine}}{\left(\mathrm{Mg, Fe^{II}, Fe^{III}}\right)_3\left(\mathrm{Si, Fe^{III}}\right)_2\mathrm{O_5(OH)_4}}\\&+ \mathrm{x}\underset{\text{Brucite}}{\mathrm{(Mg, Fe)(OH)_2}} + \mathrm{y}\underset{\text{Magnetite}}{\mathrm{Fe_3O_4}} + \mathrm{zH_2}\end{aligned} \tag{1}$$

Serpentines are 1:1 clay minerals and comprise three different types: chrysotile (asbestos form—Fig. 3c), lizardite, and antigorite (platy form). Chrysotile is one of the most common serpentine-group minerals and typically displays long fibres due to self-winding of the clay-like

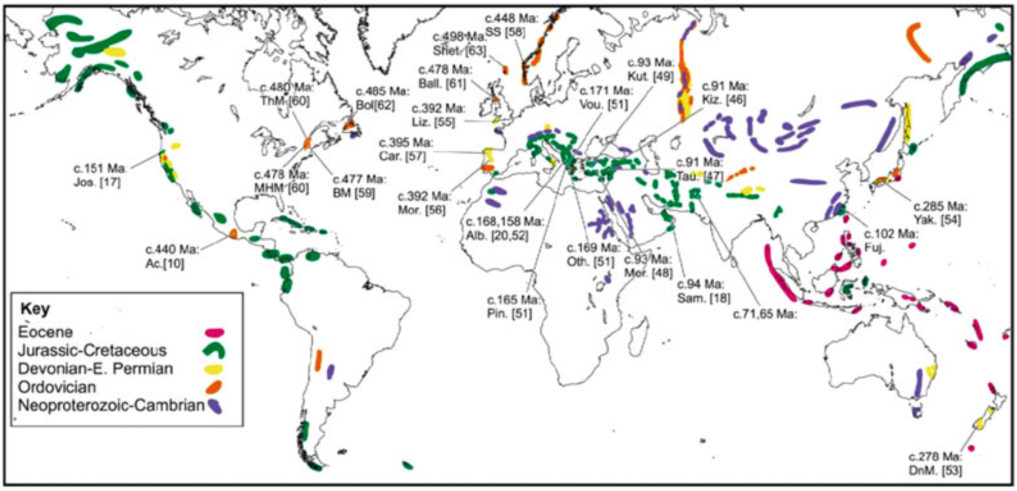

Fig. 1. Distribution of belts of Phanerozoic ophiolites cited in the text, including major Neoproterozoic and Phanerozoic occurrences. Note that ophiolites of several age pulses are represented in most belts. Scandinavian ophiolites [2], Mediterranean and western Asia ophiolite distribution [3], Central Asian ophiolites [4] apart from [5], western Pacific ophiolites [6], Uralian ophiolites [7], Alaskan ophiolites [8], western North American, Central American and Caribbean ophiolites [9] apart from [10,11], southern South American ophiolites [12,13]. Shet.: Shetland; Ball.: Ballantrae; ThM: Thetford Mines; MHM: Mont Ham Massif; BoI: Bay of Islands; BM: Boil Mountain; SS: Solund-Stavfjord; Car.: Careon; Ac.: Acatlán; Mor.: Morais; Liz.: Lizard; Yak.: Yakuno; DnM: Dun Mountain; Vou.: Vourinos; Oth.: Othris; Alb.: Albanian; Pin.: Pindos; Jos.: Josephine; Fuj.: Fujian; Sam.: Samail; Kut.: Kutahya; Mer.: Mersin; Tau.: Tauride; MB: Muslim-Bagh.

A.P.M. Vaughan, J.H. Scarrow / Earth and Planetary Science Letters 213 (2003) 407–416

Fig. 1 Distribution of ophiolite belts worldwide (from Vaughan and Scarrow 2003)

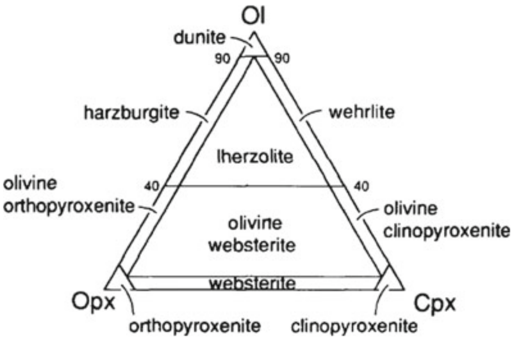

Fig. 2 Classification and nomenclature of the ultramafic rocks (mafic minerals >90%) based on the modal proportions of olivine (Ol), orthopyroxene (Opx) and clinopyroxene (Cpx) (from Le Bas and Streckeisen 1991)

sheets (Fig. 3). Where serpentinization results in the transformation of most olivine and pyroxene contained in the peridotite, then the rock is classified as serpentinite (O'Hanley 1996). The difference between peridotite and serpentinite is clear: the former is dark black with an oxidized weathering sheath (Fig. 3a), mainly due to Fe-oxide precipitation, whereas the serpentinite has a greener and uneven aspect (Fig. 3b) owing to particles of serpentine, talc, and other clay minerals, which produces a texture that resembles snake skin and gives the rock its name 'serpentine'. Because of its specific effect on the mineralogy, the serpentinization process is a key phenomenon in explaining the distribution of Ni and Cr among secondary mineral phases formed during the initial stages of rock weathering before complete pedogenesis (Fornasaro et al. 2019).

Secondary geological materials originally derived from ultramafic rocks such as mountain rock slides (Gasser et al. 1995; d'Amico et al. 2008), colluvium (Lee et al. 2004), sedimentary rocks (e.g. conglomerate, Fig. 4), and river alluvia (Estrade et al. 2015; Rinklebe et al. 2016), can also contribute to ultramafic soils and landscapes. Usually, these materials are not identified as ultramafic rocks on geological maps but are mentioned using different qualifiers based on geomorphological origin and period of deposition (e.g. glacial/fluvial deposits). According to the composition of the original minerals forming peridotites, ultramafic rocks have very high Mg (18–24 wt%) and high Fe (6–9 wt%), but very

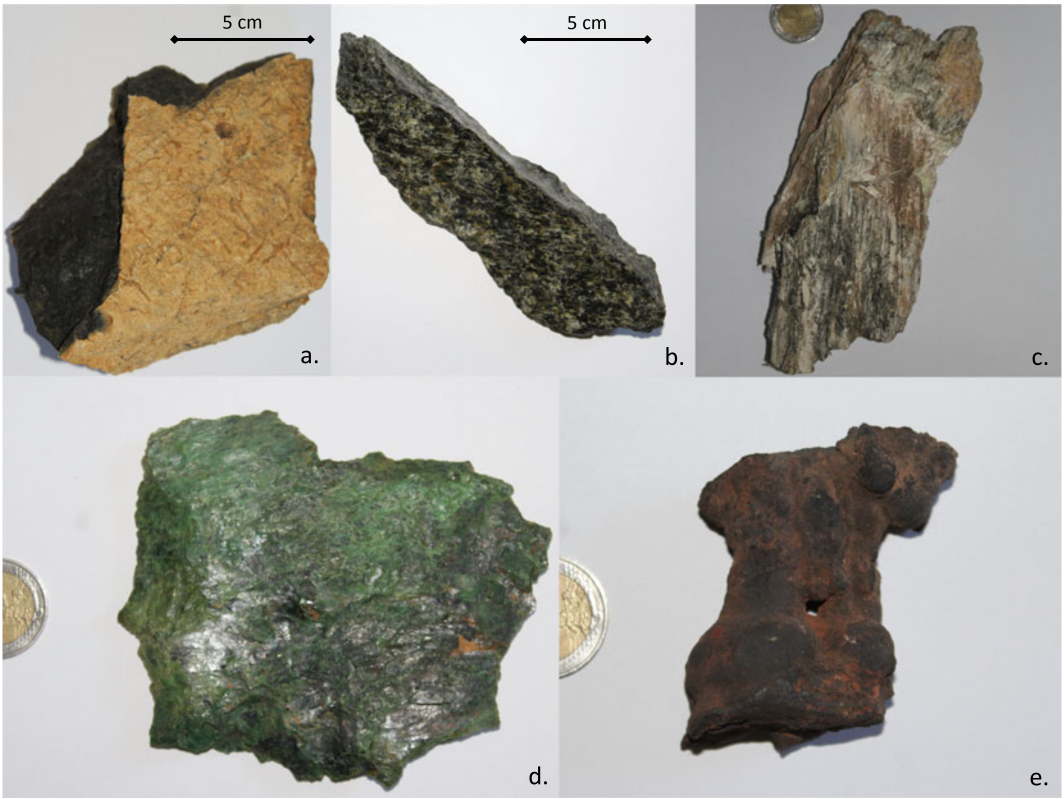

Fig. 3 **a** Olivine-rich peridotite with weathered surface from Shebenik (Albania). **b** Highly serpentinized peridotite from Pindus Mountains (Greece). **c** Large chrysotile fibres from the Barberton Greenstone belt (South Africa). **d** Garnierite: serpentine-like minerals with approx. 20% Ni (green colour) from the saprolite horizon of Geric Ferralsols in Vulcain Mine, La Tontouta (New Caledonia). **e** Fragment taken from the Petroplinthic horizon (ferricrete or iron pan) of a Geric Ferralsol in Yaté (New Caledonia) where two parallel earthworm galleries have been fossilised through Fe-oxide crystallization

low Ca (1–4 wt%) and Al (1–2 wt%) contents (Alexander 2004). The main weatherable minerals in peridotite and serpentinite (Lee et al. 2004; White and Buss 2014) are olivine, pyroxene, and clay minerals (mainly serpentine but also talc, chlorite, etc.).

3 Pedogenesis of Ultramafic Rocks and Soil Characteristics

The genesis, development, and functioning of soils developed on ultramafic materials have received a great deal of attention over the last 30 years. Firstly, ultramafic soils were studied due to their economic importance related to the genesis of nickeliferous laterites (Colin et al. 1990; Gleeson et al. 2003), but also because of their role in trace metal geochemistry (Becquer et al. 2006; Cheng et al. 2011; Bani et al. 2014; Kierczak et al. 2016). One of the major factors in influencing the genesis and characteristics of soils developed on ultramafics is the nature of the parent ultramafic rock (Alexander 2004, 2009; Alexander and DuShey 2011; Pędziwiatr et al. 2018; van der Ent et al. 2018a; Marescotti et al. 2019). Other major factors affecting soil genesis are climatic conditions, topographic situation (position in the toposequence), and vegetation cover (Bonifacio et al. 1997; Alexander and DuShey 2011; Chardot et al. 2007; Kierczak et al. 2007; Cheng et al. 2011; Bani et al. 2014; Hseu et al. 2018; van der Ent et al. 2018a). After compiling available descriptions on major

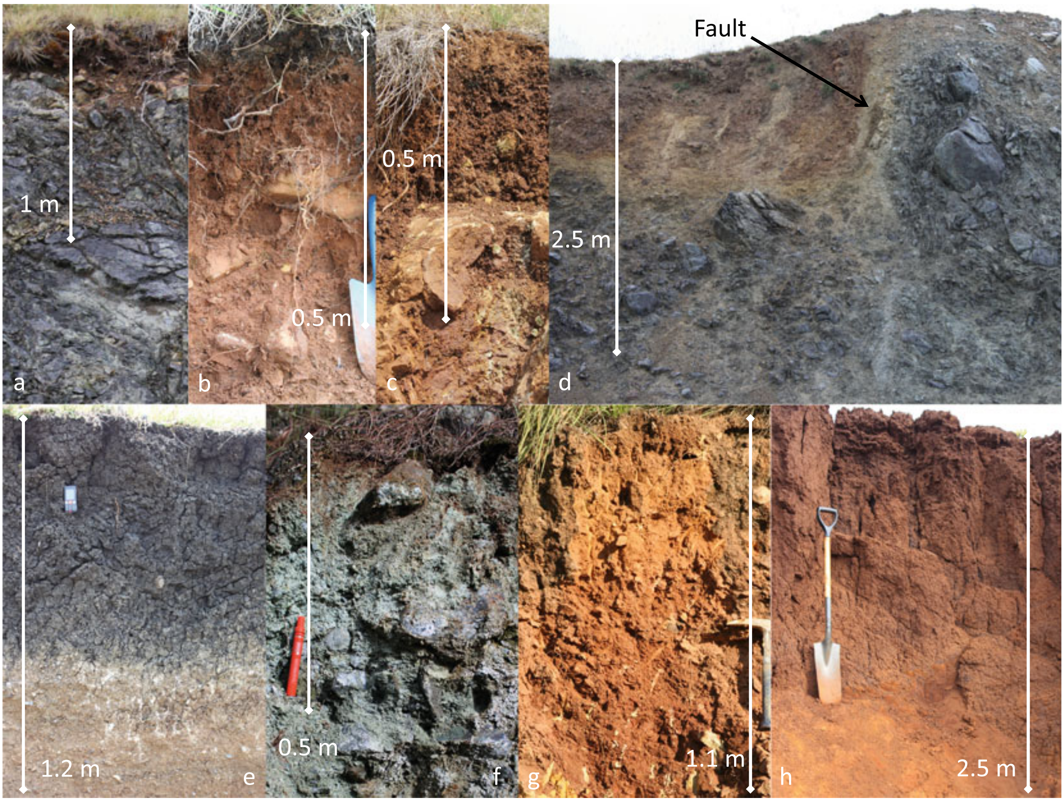

Fig. 4 Caracteristic pedons on ultramafic rocks globally: **a** Cambic Leptosol (Serpentinite 1700 m, Katara Pass, Greece). **b** Eutric Cambisol (Dunite, 1200 m, Qaftë Shtamë, Albania). **c** Chromic Cambisol (Dunite, 890 m, Trygona, Greece). **d** A fault between partially-serpentinized (left) and fully-serpentinized peridotite (right) (1700 m, Katara Pass, Greece). **e** Hypermagnesic Vertisol (Serpentinite, 650 m, Prrenjas, Albania). **f** Folic Leptosol (Serpentinite, 800 m, Kinabalu Park, Sabah, Malaysia). **g** Chromic Luvisol (Peridotite, 1100 m, Barberton, South Africa)—Geric Ferralsol (peridotite, 100 m, Goro, New Caledonia). Photographs: G. Echevarria

soils developed on serpentinite sensu stricto (Table 1), and on partially serpentinized peridotite (Table 2), it appears that most such soils reach a limited development stage, whatever the climatic conditions, which confers on them typical cambic and magnesic (exchangeable Ca/Mg < 1) properties with very few exceptions globally. These cambic properties are mainly derived from the formation of secondary clays of the 2:1 type—Fe-rich smectite and low-charge vermiculite—that form stable complexes in soils, typically with excess Mg ions (Bonifacio et al. 1997; Lee et al. 2004; Bani et al. 2014).

3.1 Common Steps of Pedogenesis on Ultramafic Bedrock

Olivine in the bedrock is not only weathered to clay minerals but a significant fraction transforms into Fe-oxyhydroxides with loss of Si and Mg. Formation of secondary 2:1 clay is the main difference between serpentinized and non-serpentinized ultramafic bedrock (Alexander 2009). The influence of draining conditions seems to strongly influence the outcome of pedogenesis: formation and accumulation of Fe- or Mg-rich smectite under poorly drained conditions (Lee et al. 2004; Bani et al. 2014), or

Table 1 Soil types derived from serpentinite according to the location, climatic conditions, mineralogy of the bedrock and position in landscape

Bedrock	Climate	Location	Mineralogy (order of predominance)	Soil type (WRB soil resources) (as described or adapted from descriptions)	References
Serpentinite	Semi-continental	Slovakia	Serpentine, magnetite	Technosol (hypermagnesic)	Massoura et al. (2006)
Augite-serpentinite	Sub-alpine	Swiss Alps	Serpentine, augite, spinels	Dystric Cambisol—rock slides	Gasser et al. (1995)
Serpentinite	Sub-alpine	Italian Alps	Serpentine, magnetite	Haplic Regosol (dystric)—mid-slope	D'Amico et al. (2008)
«	«	«	«	Epileptic Cambisol (protospodic)—high-solpe	«
Serpentinite	Temperate montane	Poland	Serpentine, spinels	Eutric cambic Leptosol (magnesic)	Kierczak et al. (2016)
Serpentinite	Semi-continental	Poland	Serpentine, spinels	Eutric skeletic leptic Cambisol (magnesic)	Kierczak et al. (2016)
Serpentinite	Semi-continental	Austria	Serpentine, chlorite, spinels	Skeletic hypereutric Leptosol (hypermagnesic)	Hseu et al. (2018)
Serpentinite	Semi-continental	Austria	«	Eutric Leptosol (magnesic)	Hseu et al. (2018)
Serpentinite	Temperate oceanic	France, Centre	Serpentine, chlorite, magnetite	Hypereutric Cambisol (hypermagnesic)	Caillaud et al. (2004)
Foliated serpentinite	Temperate oceanic	Italy, Western Alps	Antigorite, chlorite, and magnetite	Leptic skeletic hypereutric Cambisol (hyper)magnesic	Marescotti et al. (2019)
Serpentinite	Mediterranean montane	Albania	Serpentine	Chromic hypereutric Cambisol (magnesic)—slope	Bani et al. (2014)
«	«	«	Serpentine (alluvium)	Eutric cambic Vertisol (hypermagnesic)—downslope or alluvial terrace	Bani et al. (2014), Estrade et al. (2015)
Serpentinite	Mediterranean montane	California	Serpentine (no pyroxene)	Lithic mollic Leptosol (magnesic)—summit	Alexander and Dushey (2011)
«	«	«	«	Epileptic luvic Phaeozem (magnesic)—slope	
«	«	«	«	Endoleptic Luvisol (magnesic)—downslope	«
Serpentinite	Mediterranean	Italy	Serpentine, magnetite	Leptosol (hypermagnesic)—upslope	Bonifacio et al. (1997)
«	«	«	«	Leptic Cambisol (hypermagnesic)—backslope	«
«	«	«	«	Chromic Luvisol (magnesic)—downslope	«
«	«	«	«	Dystric Cambisol (magnesic)—bottom	«
Serpentinite	Warm temperate	Japan	Serpentine	Leptic Cambisol (hypermagnesic)	Hseu et al. (2018)

(continued)

Table 1 (continued)

Bedrock	Climate	Location	Mineralogy (order of predominance)	Soil type (WRB soil resources) (as described or adapted from descriptions)	References
«		«	«	Dystric Cambisol (magnesic)	«
Serpentinite	Temperate subhumid	Mexico, Oaxaca	Lizardite, antigorite, chlorite, talc, magnetite	Rhodic hypereutric Cambisol (magnesic)	Navarrete Gutiérrez et al. (2018)
Serpentinite	Dry subtropical (tropical subhumid)	Mexico, Puebla	Lizardite, antigorite, chlorite, talc, magnetite	Hypereutric rhodic Cambisol	«
Serpentinite	Humid subtropical	Taiwan	Serpentine, chlorite, enstatite	Cambic Leptosol (magnesic)—upslope	Cheng et al. (2011)
«	«	«	«	Eutric Cambisol (magnesic)—slope	«
«	«	«	«	Haplic Luvisol—downslope	«
«	«	«	Serpentine, amphibole, talc, chlorite	Haplic Vertisol—shoulder	Hseu et al. (2007, 2018)
Serpentinite	Tropical short dry season	Brasil, Minas	Serpentine, tremolite, chlorite	Eutric Regosol (magnesic)	Vidal-Torrado et al. (2006)
Serpentinite	Humid tropical (equatorial)	Sabah	Serpentine, magnetite	Eutric mollic Leptosol (hypermagnesic)—slope	van der Ent et al. (2016a, 2018a, b)
Serpentinite	Humid tropical (equatorial)	Indonesia, Sulawesi	Serpentine, olivine, augite	Posic Ferralsol (eutric, rhodic)	Anda (2012)
Serpentinite	Humid tropical (equatorial)	Indonesia, Kalimantan	Serpentine, chlorite	(Rhodic or chromic) Acrisol (us: typic hapludult)	Hseu et al. (2018)
Serpentinite	Humid tropical (equatorial)	Indonesia, Halmahera	Antigorite, lizardite, chromite	Leptic hypereutric rhodic Cambisol (magnesic)	Lopez et al. (2019)
Serpentinite	Humid tropical (equatorial)	Cameroon	Antigorite, magnetite, (olivine)	Geric Ferralsol	Yongue-Fouateu et al. (2006)
Serpentinite	Humid tropical	New Caledonia	Not described	Eutric Cambisol (hypermagnesic) > Vertisol	Proctor (2003)

Soil types are given according to the latest version of soil taxonomy (IUSS Working Group WRB 2014)

development of low-charge vermiculite in well-drained soils (Bonifacio et al. 1997). Serpentine-group minerals are commonly unstable in soil conditions and form secondary 2:1 phyllosilicates as well (Bonifacio et al. 1997; Lee et al. 2003; Cheng et al. 2011). Iron released from olivine and serpentine during weathering can be bound in a great range of secondary minerals according to temperature and drainage conditions. Under a temperate climate and even in the case of moderately weathered tropical soils, Mg plays a leading role among exchangeable cations although Ca binds preferentially to soil Cation Exchange Capacity (CEC; Lee et al. 2001; Chardot et al. 2007; Kierczak et al. 2007; Cheng et al. 2011) despite much lower concentrations than Mg in soils. Magnesium is partially leached during soil formation in temperate

Table 2 Soil types derived from non- or slightly-serpentinized peridotite according to the location, climatic conditions, mineralogy of the bedrock and position in landscape

Bedrock	Climate	Location	Mineralogy (order of predominance)	Soil type (WRB soil resources) (as described or adapted from descriptions)	References
Hornblende-peridotite	Semi-continental	Poland	Serpentine (32%), enstatite, forsterite, amphibole	Eutric skeletic leptic Cambisol (magnesic, siltic)	Kierczak et al. (2016)
Partially serp. harzburgite	Temperate montane	France, Vosges	Serpentine, olivine, pyroxene, chlorite	Hypereutric Cambisol (magnesic)—slope	Chardot et al. (2007)
Partially serp. lherzolite	Temperate oceanic	Italy, Western Alps	Antigorite, enstatite, clinochlore, olivine	Chromic leptic skeletic hypereutric Cambisol (hyper)magnesic	Marescotti et al. (2019)
Peridotite	Mediterranean montane	California	Olivines, pyroxenes	Abruptic Luvisol—summit	Alexander and Dushey (2011)
«	«	«	«	Endoleptic chromic Luvisol—downslope	
Peridotite	Humid mediterranean montane	«	«	Ferritic Luvisol (rhodic)	Alexander (2014)
Peridotite	Humid tropical	New Caledonia	Not described	Geric Ferralsol (pisolithic rhodic)—slope and downslope	Becquer et al. (2001)
Partially serp. peridotite	Humid tropical	New Caledonia	Olivines, serpentines, enstatite	Geric Ferralsol (pisolithic)	Dublet et al. (2014)
Peridotite	Humid tropical	Philippines	Not described	Geric Ferralsol	Fan and Gerson (2011)
Dunite	Tropical montane	Malaysia, Sabah	Olivines	Dystric folic Cambisol (magnesic)	van der Ent et al. (2016a, b)
Peridotite	Humid tropical (equatorial)	«	Forsterite, antigorite, lezardite, tremolite, spinels, diopside	Plinthic rhodic Ferralsol (magnesic)	van der Ent et al. (2018a, b)
Harzburgite	Humid tropical (equatorial)	Indonesia, Halmahera	Ni-rich olivine, Enstatite, chromite, antigorite	Geric plinthic rhodic Ferralsol	Lopez et al. (2019)
Dunite	Humid tropical (lower montane)	Indonesia, Halmahera	Ni-rich olivine, chromite	Geric rhodic plinthic Ferralsol (magnesic)	«
Pyroxenite	Tropical long dry season	Brazil, Cerrado	Enstatite, serpentine, spinel	Vertic eutric Cambisol (magnesic rhodic)—backslope	Garnier et al. (2009b)
«	«	«	«	Geric Ferralsol (pisolithic rhodic)—downslope	«
Pyroxenite	Tropical long dry season	Brazil, Cerrado	Enstatite, diopside	Geric Ferralsol with smectitic saprolite	Colin et al. (1990)
Dunite	«	«	Olivines	Geric petroplinthic rhodic Ferralsol	«

Soil types are given according to the latest version of soil taxonomy (IUSS Working Group WRB 2014) climates (incomplete hydrolysis), and a variety of secondary clay phases such as vermiculite and smectite, are produced; in contrast, in tropical lateritic soils Mg is nearly totally leached (complete hydrolysis) as is Si, leading finally to the accumulation of free Fe-oxides (Becquer et al. 2001).

Ultramafic soils share a number of chemical particularities including a low Ca/Mg ratio with Ca existing at significantly lower concentrations than in other soils of surrounding areas. The ultramafic soils also typically contain elevated levels of metals other than Fe, Mn, Ni, Cr and Co, which could induce toxicity for most plants. Serpentine soils are commonly deficient in essential plant nutrients such as N, K and P (Brooks 1987; Proctor and Woodell 1975). In ultramafic soils, Cr and Ni distribution and mineral speciation strongly depend on the mineralogy of the bedrock (Garnier et al. 2006, 2009b; Raous et al. 2013), as well as on climatic conditions (Massoura et al. 2006), position in the toposequence (Cheng et al. 2011), and more precisely on thermodynamic conditions of the soils (Antić-Mladenović et al. 2011). All these factors have been evidenced in a recent thorough work on the ultramafic soil cover of Kinabalu Park in Malaysia (van der Ent et al. 2018a). Among these, the degree of serpentinization is known to be a major discriminant factor in ultramafic pedogenesis (Alexander 2004, 2009; Pędziwiatr et al. 2018; van der Ent et al. 2018a). Photographs of the most common pedons mentioned in the following paragraphs are shown in Fig. 5.

3.2 Soils on Serpentinite Bedrock

Serpentinite is one of the most amazing bedrocks for soil development worldwide because soils developed on this substrate tend to be similar in pedogenesis and functioning regardless of latitude or elevation (Table 1): They are mostly classified as Cambisols or closely-related soils (van der Ent et al. 2018a). If we take a close look at the genesis and characteristics of these soils in both cold and temperate climates, they commonly range from high pH Regosols/Leptosols with cambic properties with CEC dominated by Mg over Ca, to Cambisols with neutral to slightly acidic pH (Caillaud et al. 2004; Chardot et al. 2007; Kierczak et al. 2007, 2016). Temperate ultramafic Cambisols typically contain serpentine as the predominant mineral phase, although 2:1 clays either derive from the bedrock (e.g. chlorite) or form from serpentine weathering and related loss of Mg (Caillaud et al. 2009; Chardot et al. 2007). Formation of Fe-rich smectite from serpentine is evidenced in temperate serpentinite soils (Caillaud et al. 2004, 2009). The Mg-chlorite is weathered to trioctahedral vermiculite (Caillaud et al. 2009). During weathering, free Fe is released and oxyhydroxides are partly crystallized (Chardot et al. 2007). The chroma from serpentinite soil in temperate conditions is generally redder than that in surrounding soils developed on non-ultramafic substrates, owing to the high content of free Fe (Chardot et al. 2007), although not as high as for soils developed on non-serpentinized peridotite (Kierczak et al. 2016; Marescotti et al. 2019).

In Mediterranean conditions, weathering intensity is higher and magnesic Cambisols have been shown to form after the loss of 85–90% of the initial bedrock material (Estrade et al. 2015). Also, further pedological evolution may occur if topographic conditions are suitable, including the formation of Luvisols where the pedons are thicker (Bonifacio et al. 1997). However, the soils remain dominated by magnesic Cambisols where landscapes are dominated by slopes. As in temperate environments (Caillaud et al. 2004, 2009), the primary serpentine is easily weathered to smectite (Bonifacio et al. 1997) that typically is Fe-rich (Ece et al. 1999; Caillaud et al. 2004; Bani et al. 2014). Further evolution of the soil transforms smectite into low-charge vermiculite if drainage conditions of the soil are favourable (Istok and Harward 1982; Bonifacio et al. 1997). In the absence of sufficient drainage (downslope or in alluvial zones), Vertisols form due to a significant accumulation of neoformed smectites (Lee et al. 2003; Bani et al. 2014), some of which are Mg-rich smectite that is not found in Cambisols (Bani et al. 2014). Upslope, the soil cover

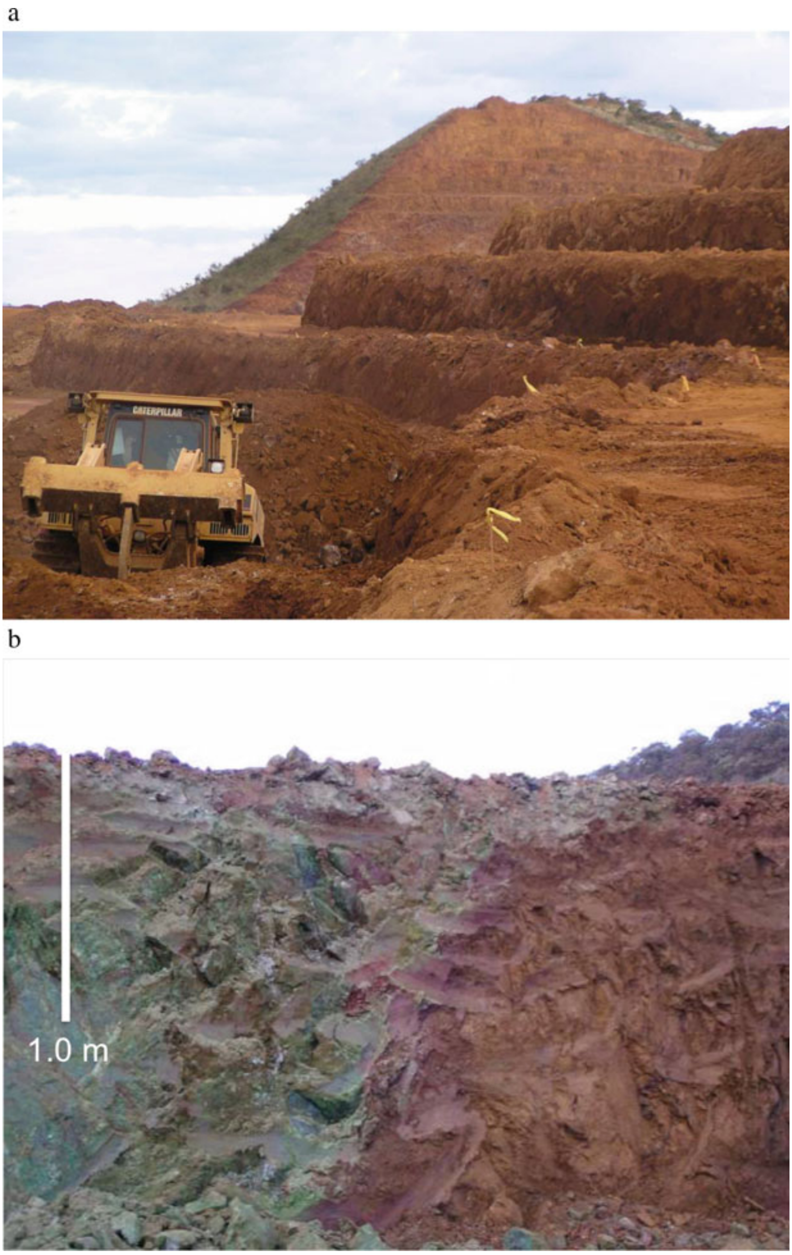

Fig. 5 **a** Formation of hematite on the upper part of the lateritic profile in the background hill due to surface dehydration of Fe oxyhydroxides (Niquelândia, Brazil). **b** A fault between pyroxenite (left) and dunite (right) allows seeing the difference of composition and mineralogy: Ni-rich smectites (left) and goethite (right) (Niquelândia, Brazil). Photographs: G. Echevarria

is still very similar as in colder regions (Bonifacio et al. 1997; Alexander and DuShey 2011), and Leptosols are still found on shallower portions of the toposequences (Marescotti et al. 2019).

In humid climates of subtropical to tropical areas, serpentinite soils are dominated by 2:1 clays (e.g. smectite) and mostly dominated by Cambic Leptosols/Cambisols having a relatively high pH and magnesic/hypermagnesic properties

(Cheng et al. 2011; Isnard et al. 2016; van der Ent et al. 2016b). These soils are typically developed on upslope and midslope landscape positions (Hseu et al. 2007, Cheng et al. 2011). Within these soils, the main pedogenic process is weathering of primary clay minerals (i.e. serpentines) to smectite after a significant loss of Mg (Hseu et al. 2007; Nguyen Thanh et al. 2017) and formation of a stable organo-mineral complex. As in temperate ultramafic soils di-octahedral Fe-rich smectites are usually formed, except in soil bottom layers with poor drainage and higher Mg contents (Nguyen Thanh et al. 2017). Vertic properties have been mentioned in many ultramafic Cambisols in such regions, because of the high proportion of 2:1 clays (e.g. smectite), including even the formation of Vertisols sensu stricto (Proctor 2003). Soils tend to be rejuvenated because of the Vertic properties and erosion where a slope is present; that could explain the predominance of Cambisol-related soils even in humid-tropical conditions. Under conditions that are favourable for leaching, the loss of Si from smectite/vermiculite can lead to accumulation of kaolinite and Fe-oxides (Hseu et al. 2007). Luvisols or other tropical leached soils (e.g. Acrisol) are present in such areas (i.e. downslope) where weathering products of the toposequence accumulate (Hseu et al. 2007, 2018; Cheng et al. 2011), which again shows a great similarity with those of colder regions (Bonifacio et al. 1997). Only one record of a Ferralsol has been reported to develop on pure serpentinite, in Sulawesi (Anda 2012), but the author did not mention if lateritic material had accumulated from the surrounding soil cover on peridotite, or if that particular soil was several millions of years old. According to Gleeson et al. (2003), laterite formation on wholly serpentinized peridotite is common and leads to smectite-rich saprolite owing to poor drainage of the serpentinite. The presence of deep Geric Ferralsols with possible accompanying ferricrete is reported on serpentinite that contains only traces of olivine (Yongue-Fouateu et al. 2006). The deep laterite profiles developed on this 240 km^2 outcrop in the Equatorial Forest in Cameroon have probably developed under stable climatic conditions for tens of millions of years; lateritization is still active. If the presence of serpentinite reduces the speed of lateritization, the endpoint of pedogenesis on ultramafic material (serpentinized or not) seems to be the formation of Petroplinthic Geric Ferralsols. However, this type of soil is seldom found in tropical regions because many local factors apparently block pedogenesis at an early stage (e.g. steep slopes, Vertic properties).

3.3 Soils on Non-serpentinized Peridotite

It is difficult to differentiate ultramafic soils developed on serpentinite versus peridotite in cold environments (Kierczak et al. 2007, 2016) because their geochemistry is generally similar and the end products of weathering (i.e. soils) do not differ much in chroma, soil depth, and weathering intensity. However, there are detectable differences in soil mineralogy and composition (Pędziwiatr et al. 2018). In warmer climates, the differences become more obvious (Alexander and DuShey 2011; van der Ent et al. 2018a) and in tropical environments, we face two different ecosystems (Proctor 2003; van der Ent et al. 2018a). Cuba is a good example, where Cambisols occur on serpentinites that are covered with maquis-like vegetation (high occurrence of Ni-hyperaccumulators); Ferralsols occur on peridotites, which host a rainforest. Unlike Cambisols on serpentinite, ultramafic Ferralsols generally contain very few hyperaccumulator species (van der Ent et al. 2018a).

In temperate environments, the predominant soil type on non- or slightly serpentinized peridotite is again Cambisol (Table 2) that displays slight differences with Cambisols developed on serpentinite (Pędziwiatr et al. 2018). One such difference is the qualifier ‘chromic’ that can be applied as a consequence of iron segregation (easily weatherable olivine can produce significant amounts of Fe-oxyhydroxides). Soil Chroma of these soils can be typical of those found on Mediterranean soils with values situated in the YR range (whereas surrounding soils are in the Y range), locally reaching YR 7.5 or redder (Chardot et al. 2007; Kierczak et al. 2016). In soils developed on serpentinite bedrock, the amount of 'free' Fe-oxides (amorphous and crystalline) measured by dithionite-bicarbonate-citrate extraction (McKeague and Day 1966) is significantly different from that of surrounding soils formed on other types of bedrock and can exceed 5% (Chardot et al. 2007). Also, the proportion of smectite in Bw horizons of peridotite derived Cambisols is less than in soils formed on serpentinite (Caillaud et al. 2004; Chardot et al. 2007).

In Mediterranean environments, Fe segregation becomes a significant pedogenic process on peridotite (Alexander and DuShey 2011). Amounts of free Fe in the well-drained soils of the Klamath Mountains transect (i.e. a typical ultramafic region of northern California) are related to the degree of serpentinization of the parent peridotite and its weathering extent (Alexander 2014). Goethite in the peridotitic soil is predominant in the wetter soil having higher free Fe contents (Alexander 2014). In the peridotitic soil, the accumulation of Fe-oxyhydroxides (up to 25%; Massoura et al. 2006; Alexander 2014) is associated with chroma values redder than YR 5; the soils can be defined as Chromic. Such soils are mostly Luvisols with properties that typically include Magnesic and Chromic/Rhodic. The high amount of free Fe, and the reduced concentrations of 2:1 clay minerals in the B horizons of these soils, induce the lack of CEC with net positive charges such as in Ferralic horizons that are typical of tropical lateritic soils. This feature found in Mediterranean climatic conditions on peridotite is unique for non-tropical soils (Alexander 2014) and means that pedogenesis on peridotite under Mediterranean conditions involves the first steps of lateritization.

Soil genesis on peridotite in tropical environments is probably the most peculiar and intense type of weathering of rock occurring on the surface of Earth. It has been extensively documented over the last 40 years because of the economic value of nickel laterites (Trescases 1975; Nahon et al. 1982; Colin et al. 1990; Gleeson et al. 2003). Ultramafic laterites are divided into three main categories that mostly differentiate in the saprolitic horizon, although the type of Ni laterite deposit is only partially controlled by lithology (Gleeson et al. 2003). Each of the three laterite classes may be developed on peridotite, but on dunite protoliths oxide deposits predominate. However, in terms of soil properties, these three types present only slight differences as all are classified as Geric Ferralsols. Only a few papers have focused on biological and active soil functioning at the toposequence level that provide elements of soil taxonomy (Becquer et al. 2001; Garnier et al. 2009b). Most ultramafic laterites studied worldwide are paleo-laterites that are not actively forming, such as Cuban or Dominican ultramafic laterites that developed in the Tertiary (Aiglsperger et al. 2016). Below we therefore emphasize on soil behaviour rather than trying to describe the complete formation and evolution of laterites. Some of the most developed ultramafic laterite profiles result in 60–100 m (Colin et al. 1990) of weathered material with the formation of a full lateritic horizon, i.e. goethite-dominant limonite that ranges in thickness from 4 m in Niquelândia (Brazil) on pyroxenite (Colin et al. 1990) to 30 m in Goro (New Caledonia) on peridotite (Dublet et al. 2014), or in Moa Bay (Cuba) on non-weathered harzburgite (Aiglsperger et al. 2016). On pyroxenite, the high silica content (twice as high as in other peridotite) allows the formation of smectite at a relatively shallow depth (Fig. 6b) and therefore limits the development of laterite. Such soils are relatively similar to those found on serpentinite where smectite minerals predominate (Gleeson et al. 2003; Garnier et al. 2009b), also showing a high CEC (high proportion of 2:1 clays) that is incompatible with Ferralic and Vertic properties due to the swelling-shrinking properties of smectite.

In the lateritic horizon usually described by miners as 'limonite' or 'laterite', the mineralogy is dominated by goethite. Hematite is formed at the surface ('red limonite or laterite') as a consequence of extremely hot temperatures reached at the soil surface where affected by sunlight (Fig. 5a). Mineralogical and geochemical changes of iron oxides occur throughout the thickness above the bedrock (Dublet et al. 2014). The formation of ferricrete (iron pan) after crystallisation (Fig. 3e) and dehydration of Fe-oxides

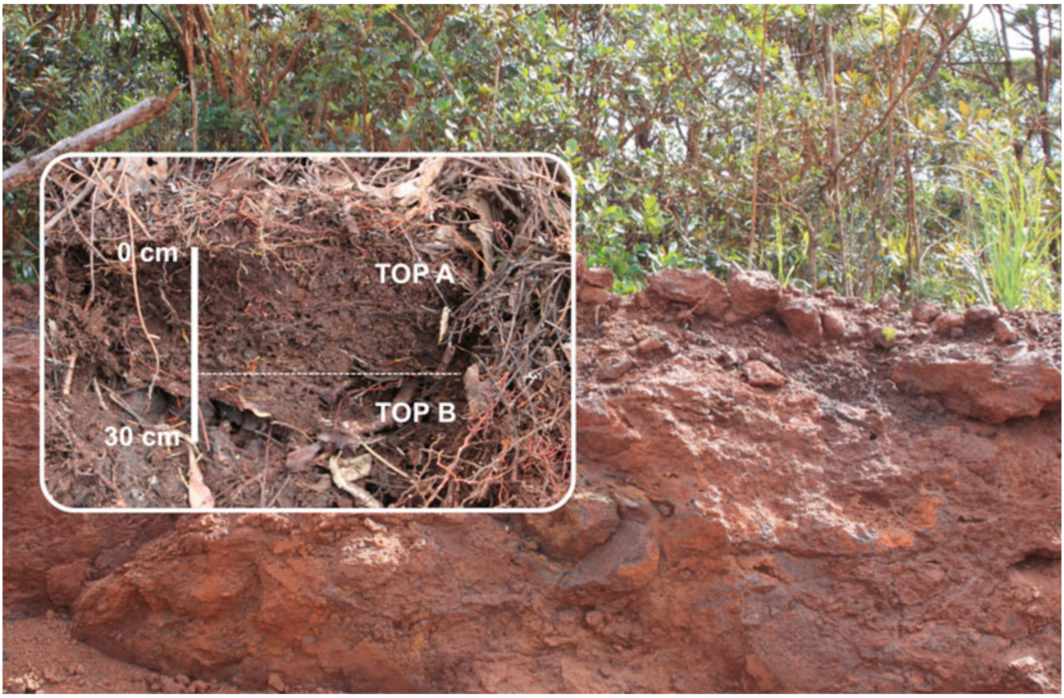

Fig. 6 Hyperskeletic mollic sideralic Leptosol on ancient laterites with a stunted forest dominated by *Gymnostoma* species (Goro, New Caledonia). Photographs: G. Echevarria

(typically as a result of successive dissolution-crystallization cycles during lateritization) results in a significant loss of Ni (Dublet et al. 2015) and a relative gain of trivalent elements (e.g. Al, Cr, Sc), especially in hematite particles (Aiglsperger et al. 2016). Where developed on dunite, the ferricrete can be continuous with hematite being the predominant mineral, whereas goethite is a secondary phase (Colin et al. 1990). On olivine-poor ultramafic bedrock, the formation of ferricrete does not seem to be significant (Colin et al. 1990). Ferricrete dominates in the southeastern part of the main island of New Caledonia where it is continuous over many square kilometres (Fig. 6). The formation of ferricrete is the final evolution of Petroplinthic Rhodic Geric Ferralsols and, after the sealing of soil surfaces, a landscape of Hyperskeletic Leptosols (Quintela-Sabarís et al. 2018), a few decimetres deep, develops on a large scale. A rather low vegetation (stunted secondary forest dominated by *Nothofagus* spp., *Arillastrum gummiferum*, and *Gymnostoma* spp.) develops on these soils (Isnard et al. 2016) where most of the nutrients are brought to plant roots by the recycling of decayed organic matter.

4 Mining Ultramafic Technosols

After removing the lateritic cover, mining operations are required to rehabilitate the stripped areas by preferentially using the spoil materials (Echevarria and Morel 2015; Quintela-Sabarís et al. 2018). The construction of mining Technosols aims at restoring the soil cover in order to fulfil essential ecological and environmental functions (Echevarria and Morel 2015). Ultramafic raw materials (spoils, stripped soils, tailings) are the main geochemical driving force of the new soil cover. Limonitic spoils composed mainly of goethite and hematite, and saprolitic spoil composed of a high fraction of clay minerals and chalcedony, are the main materials available for soil construction (Raous et al. 2013). Topsoil is also frequently used to cover the spoil materials after storage, for periods that may vary from a few days to several years (Echevarria and Morel 2015). The evolution of topsoil biogeochemistry and biology during storage has been thoroughly studied in a recent project called BIOTOP, which was funded by the New Caledonian Research Centre on Nickel (CNRT). The evolution of topsoil geochemistry after stripping and storing (0–24 months) showed several trends. Storage of topsoil developed on ferricrete does not affect strongly its chemical and biochemical fertility (Echevarria and Morel 2015; Quintela-Sabarís et al. 2018). Typically, organic amendments or even mineral fertilizers (hydroseeding) are also added for the reconstruction of soil cover on stripped land.

Globally, Serpentinite quarries are also in need of environmental rehabilitation and the constructed soil cover is strongly influenced by the chemical and mineralogical properties of serpentinites: high pH, high Mg/Ca ratios, lack of nutrients (Massoura et al. 2006; Cerdeira-Pérez et al. 2019; Quintela-Sabarís et al. 2019). Efforts to create new soil covers (Technosols) are currently being carried out, either by understanding the natural succession of spontaneous plants that re-colonize the mineral barrens (Quintela-Sabarís et al. 2019) and anticipate their needs to speed up revegetation, or, by planting Ni hyperaccumulator plants in order to implement Ni phytomining as well as to improve soil fertility (e.g. microbial functions, nutrient availability, organic matter content) (Cerdeira-Pérez et al. 2019).

5 A Global Model of Ultramafic Soil Evolution

Summarizing the pedogenetical features of all soils developed on ultramafic bedrock worldwide cannot be done on a single figure. However, in most sites from cold to tropical climates, it is possible to highlight the most significant pedogenic processes and predominant mineral phases that characterise the ultramafic soils known on both non-serpentinized peridotite and serpentinite (Fig. 7). A comparison of the different evolutionary patterns developed in cold, temperate, and Mediterranean regions, based on results from the literature, showed that Mg is extremely depleted and reduces from nearly 20% of the mass of the bedrock to less than a few percent (Kierczak et al. 2007). Silicon is also depleted, especially in Mediterranean conditions. As a result, the relative concentrations of Fe and Cr increase in the soil (with a strong impact on soil redness), as well as those of other stable metals (i.e. Ti and Nb), whereas Ni is slightly depleted relatively in temperate soils and is stable in Mediterranean soils. Importantly, 85–90% of the Ni is lost during pedogenesis (Estrade et al. 2015). The presence of serpentine considerably reduces the loss of Si, and most mineral phases will be primary and secondary clay minerals.

6 The Fate of Nickel During Ultramafic Soil Evolution

Nickel in ultramafic bedrock is borne in serpentine, olivine, and pyroxene grains with an average concentration of 1200 to 3800 $\mu g\ g^{-1}$ for serpentine (Table 3). In serpentine clays, it is located in the octahedral sheet (Siebecker et al. 2018). In magnesic Cambisols and related soils, soil organic matter (Hseu et al. 2018), poorly crystallized Fe-oxides (Chardot et al. 2007), possibly Mn-oxides (Alves et al. 2011), and secondary 2:1 clays such as Fe-rich smectite (Bani et al. 2014) will hold most of the Ni in the soil. However, weathered serpentines will contain higher concentrations of Ni than those, intact, present in the bedrock, i.e. 6000 $\mu g\ g^{-1}$ (Bani et al. 2014). Progressively, the formation of smectite produces Ni-rich particles that may in places reach several weight percent (Table 3), including under non-tropical conditions (Bani et al. 2014). Where lateritization occurs, Ni from the first few meters is leached down the profile where Fe-oxides crystallize, and is then transferred to clay minerals in deep saprolite (Colin et al. 1990; Dublet et al. 2014). These saprolite clays that trap Ni can be smectite (Colin et al. 1990; Raous et al. 2013), talc (Becquer et al. 2006), or serpentine (Dublet et al. 2012). On top of the saprolite layer, Mn oxides (e.g. asbolane,

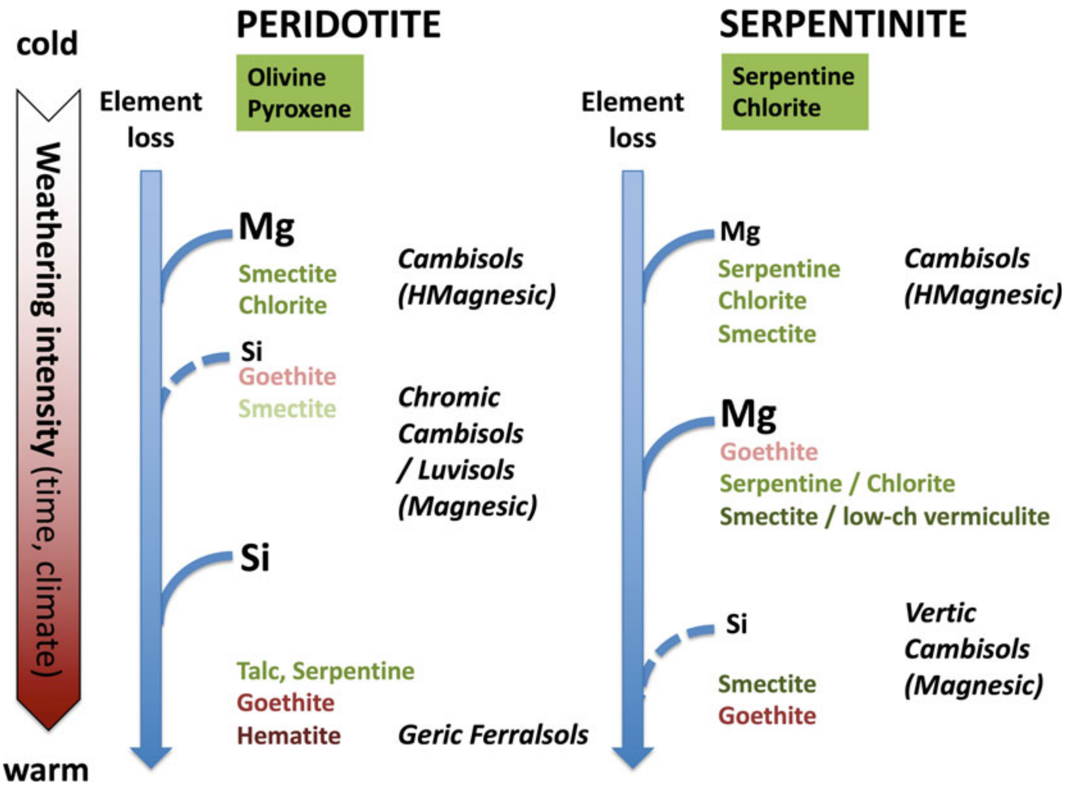

Fig. 7 A simplified description of soil genesis and evolution on ultramafic bedrock: non-serpentinized peridotite and serpentinite. In warmer climates, the difference between the two types of ultramafic bedrocks becomes more pronounced. The high abundance of serpentine minerals impedes the formation of a lateritic (Ferralic) horizon and gives to the soil Cambic and Vertic properties, mainly inherited from the high proportion of smectites. Pyroxenite will produce a similar type of pedogenesis than serpentinite

lithiophorite) may also trap Ni and Co (Llorca and Monchoux 1991; Dublet et al. 2014). However, Ni is also concentrated in goethite from the yellow limonite horizon that has not undergone such pervasive dissolution-recrystallization processes (Dublet et al. 2015).

7 Nickel Availability in Ultramafic-Bearing Minerals and Soils

7.1 Methods of Evaluating Nickel Availability

Current methods to assess and characterize Ni chemical availability in ultramafic soils are described by numerous workers (Echevarria et al. 1998, 2006; Chaney et al. 2005; Garnier et al. 2006, 2009a; Raous et al. 2013; Zelano et al. 2016a, b; van der Ent et al. 2018b), and include:

- Single chemical extractions using salts—$CaCl_2$ and $Sr(NO_3)_2$;
- Single chemical extractions using chelating agents—DTPA and EDTA;
- Sequential chemical extraction procedures; and
- Isotopic exchange and dilution techniques (IEK).

The last techniques are useful and complete tools for measuring the true phytoavailability of trace elements in soils (Echevarria et al. 1998; Massoura et al. 2004). Measured by IEK, isotopically exchangeable Ni during time t (E_t) refers mostly to the element retained through

Table 3 Element concentrations in leaves, litter and soils under the influence of the Ni-hyperaccumulator *Odontarrhenia chalcidica* grown on a Eutric Vertisol (Hypermagnesic) in Albania

Compartment	Nickel (g kg^{-1})	Calcium (%)	Potassium (%)
Leaves	19.0	3.57	1.50
Litter (current year)	9.22	2.06	0.53
Soil under litter at 0–3 cm	3.03 a	0.72 a	0.34 a
Bare soil (no litter) at 0–3 cm	2.84 b	0.69 a	0.32 a

Mean values of five replicates that are followed by different letters indicate a significant difference at the $p < 0.05$ level (ANOVA)

sorption and surface-complexation onto soil particles. Soil labile Ni − E_t—is highly dependent on the chemical state of the element, i.e. inclusion in crystal lattices (Massoura et al. 2006; Zelano et al. 2016a, b). The metal exchanged for a very short period of time ($E_{0-1\ min}$) corresponds to the fraction that is weakly bound to the soil solid phase (e.g. CEC). Isotopically exchangeable pools having longer periods of exchange (i.e. 1 min to 3 months) are typically linked to diffusion-limited sorption sites (Massoura et al. 2006; Zelano et al. 2016b).

The DTPA extraction of Ni in soils is a good field test for assessing ecotoxicological risk (L'Huillier and Edighoffer 1996; Echevarria et al. 1998, 2006; van der Ent et al. 2018b). The Ni pool extracted by DTPA was significantly correlated with medium-term, isotopically exchangeable, i.e. $E_{0-3\ months}$, which is the same source of Ni uptake for plants with contrasted demand for Ni (Echevarria et al. 2006). Recently, it was shown that DTPA-extracted Ni has a similar stable isotope composition as soluble Ni, whereas soil Ni has a much lighter isotope composition (Estrade et al. 2015). The DTPA extraction thus accesses only exchangeable and surface-complexed Ni that is eventually available and accessible to plants (Echevarria et al. 1998).

In 100 very contrasted soil samples that represent a range of origin and total content of Ni in soils worldwide, including ultramafic soils, IEK methods and DTPA extractions have shown that availability of Ni to plants is mainly controlled by soil pH (Echevarria et al. 2006). As expected, soil pH controls the intensity (i.e. Ni concentration in solution). In 16 naturally and anthropogenically Ni-rich soils selected from various weathering conditions, Ni was found to be present in either primary phyllosilicates (i.e. serpentine, chlorite, talc), secondary clay minerals, or Fe–Mn oxyhydroxides (Massoura et al. 2006; Echevarria et al. 2006). The availability of Ni from primary clay minerals was low and attributed to the presence of Ni within the crystal lattice (Zelano et al. 2016b). Nickel in secondary clay minerals (e.g. smectite) was probably sorbed onto the mineral surfaces with the result that its availability was very high (Massoura et al. 2006; Raous et al. 2010, 2013). Bioavailability of Ni in goethite-dominant soils was extremely poor, because these minerals act as a stable sink for the metal in both natural and contaminated Ni-rich soils (Massoura et al. 2006; Raous et al. 2010; 2013; Zelano et al. 2016a, b). The response of excluder, accumulator, and hyperaccumulator plants to Ni availability assessed by IEK methods showed that all plants took up Ni from the same labile pools of Ni in soils, regardless of the mechanisms of root uptake, translocation and storage (Massoura et al. 2004; Echevarria et al. 2006). This finding validates IEK as a universal method for assessing Ni availability in soils and other environmental solid matrices (Zelano et al. 2013, 2016a).

7.2 Main Ni-Bearing Phases in Ultramafic Soils and Ni Availability Properties

The use of isotope exchange kinetics to describe Ni availability properties of pure Ni-bearing minerals was introduced 15 years ago (Echevarria et al. 2006; Massoura et al. 2006), as Ni-bearing phases in ultramafic soils were shown to be nearly the sole control on Ni availability, with only a limited effect imposed by pH. The Ni availability properties of single pure minerals were described for chrysotile, which was purified from an asbestos mine spoil (Chardot-Jacques et al. 2013), and for Ni-rich synthetic goethite (Massoura 2003), and fully explained the resulting Ni transfer to plants in both cases. Recently, Zelano et al. (2016a) detailed isotopically exchangeable properties of single pure mineral phases that are commonly found in ultramafic soils, with E_t values being a first-order kinetic of time, and specific for each Ni-bearing phase. Furthermore, these workers modelled the bulk Ni availability of soils by computing the linear combination of properties of single minerals according to their relative proportion in the studied soils (Zelano et al. 2016a). It was verified, in most cases, that the computed values were accurate and that Ni-bearing phases contributed to soil Ni availability in this manner. This approach was successfully applied to natural ultramafic soils and the mining of Technosols (Zelano et al. 2016b), thus assigning to each phase its contribution in Ni availability.

No study has been performed on primary olivine and pyroxene assuming that the only release of Ni from these silicates comes from their dissolution (Chardot-Jacques et al. 2013; Pędziwiatr et al. 2018). Serpentine minerals in unweathered bedrock have been reported to contain from 0.12 to 0.38 wt% Ni (Caillaud et al. 2009; Yongue-Fouateu et al. 2006; Quantin et al. 2008; Bani et al. 2014; Kierczak et al. 2016), with a surprisingly high value of 1.99 wt% reported in specific conditions (Quantin et al. 2008). In cold temperate conditions, the serpentine Ni content in the Bw horizon remains similar to that in the bedrock: 0.18 wt% (Chardot et al. 2007). However, in the B horizons of Cambisols and Vertisols developed on serpentinite under Mediterranean conditions, it seems that the Ni content in serpentine minerals is slightly higher (e.g. 0.60–0.85 wt%) than in the original bedrock (0.30 wt%), probably because these serpentines have undergone incipient weathering (Bani et al. 2014). In the saprolite layers of Geric Ferralsols, serpentine minerals such as nepouite and garnierite can contain up to 20 wt% Ni as a result of neoformation (Fig. 3d). The availability of Ni in primary phyllosilicates such as chrysotile is very limited (Chardot-Jacques et al. 2013); when equilibrated with water, these minerals release Ni concentrations in solution that are very low at *ca.* a few µg L^{-1}, exchangeable sites that are very limited (a few % of total Ni), and a strong diffusion-limited component that slowly releases soluble and exchangeable Ni over time (Zelano et al. 2016a). Where plants are grown on this sole substrate, Ni absorption by roots is mainly fed by chrysotile dissolution instead of by labile pools (Chardot-Jacques et al. 2013).

Chlorite and talc in ultramafic soils can be both primary (Lee et al. 2003) or secondary (Becquer et al. 2006) minerals. Primary minerals in soils being the minerals derived from the bedrock or parent material, and secondary minerals the minerals that are transformed or neoformed during pedogenesis. Talc is a 1:1 clay mineral that is extremely resistant to pedological weathering and in places is the only clay mineral present in Ferralic horizons of ultramafic laterites (Becquer et al. 2006). The CEC of such talcs is almost null, which explains that the availability of Ni in talc (e.g. deweylite) is so scarce that it is lower than that of chrysotile (Zelano et al. 2016a). The availability of Ni in chlorite minerals is more than twice that of chrysotile (Zelano et al. 2016a), as it is a 2:1 clay mineral having much higher CEC. Although more common at the surface than in the Bw and C horizons of Hypereutric Cambisols in the Vosges Mountains of France (Massoura et al. 2006; Chardot et al. 2007), Ni availability is much higher in the lower horizons and negatively correlates with chlorite content. Chlorite is, therefore, not a significant contributor to Ni availability in ultramafic soils (Massoura et al. 2006).

Smectite is a high-exchange, high-specific, surface secondary clay. The Ni content of smectite in ultramafic soils is also highly variable and no specific trend in terms of geochemical composition (Mg-rich vs. Fe-rich), weathering intensity, or climatic conditions seems to influence the Ni content. Nickel concentrations in smectite range from 0.49–3.12 wt% in

Temperate B horizons (Caillaud et al. 2009; Bani et al. 2014), whereas the range is 0.40–14.9 wt% in a tropical Bw horizon of a Cambisol developed on pyroxenite (Garnier et al. 2009b). In the saprolite horizons of Geric Ferralsols, Ni contents typically range from 1.06–1.33 wt% (Yongue-Fouateu et al. 2006; Raous et al. 2013), as in temperate soils. But concentrations can exceptionally reach 13.3–25.9 wt% Ni in Fe-rich smectite of Geric Ferralsols developed on pyroxenite in Niquelândia, Brazil (Decarreau et al. 1987). Nickel-bearing smectite is a major source of available Ni in ultramafic soils (Bani et al. 2007; Raous et al. 2013; Zelano et al. 2016a), and its availability is like that of Mg released under similar conditions (Raous et al. 2010). The concentration of Ni measured in solution during IEK of smectite is one of the highest of all Ni-bearing minerals, amounting to 0.25–0.5 μg L^{-1} (Massoura 2003; Bani et al. 2007). Instantaneously exchangeable pools (i.e. E0-1 min) are also the highest, reaching more than 100 μg g^{-1} after 1 min (Massoura 2003; Bani et al. 2007; Zelano et al. 2016a) as a result of the high content of exchangeable Ni present in the CEC (Raous et al. 2013).

Iron- and Mn-oxyhydroxides are important scavengers of Ni in ultramafic soils. Where free Fe is released during the first stages of weathering, Ni is rapidly sorbed onto, and incorporated into, amorphous Fe-hydroxides (Chardot et al. 2007). Where goethite (a significant mineral in ultramafic Cambisols) is formed, the affinity to Ni seems to decrease (Massoura et al. 2006; Chardot et al. 2007; Bani et al. 2009) and the ratio of Nio/Nid is usually the double as Feo/Fed (Chardot et al. 2007); however, Ni contents in goethite can range from 0.8–4.3 wt% in these soils. Goethite in the lateritic horizons of Geric Ferralsols range from 1.7 wt% Ni% in upper layers to 3.8 wt% in deeper parts of the Ferralic horizon (Becquer et al. 2006). The same concentrations have been reported in other Geric Ferralsols (Yongue-Fouateu et al. 2006). Nickel concentration in Fe-oxides decreases from the bottom of the Ferralic horizons up to the surface, by about a factor 5–10 as a result of goethite aging (Dublet et al. 2015), a process involving dissolution and recrystallization. Hematite particles are even poorer in Ni than the surface goethite (Raous et al. 2013). The isotopically exchangeable pools of Ni in ultramafic soils were shown to correlate with concentrations of amorphous Fe-oxyhydroxide or with the ratio of amorphous to well-crystallized Fe-oxides (McKeague and Day 1966; Massoura et al. 2006). Manganese oxides, although a minor phase quantitatively in temperate soils, also may have significant concentrations of Ni that are suspected to be highly available (Alves et al. 2011). In some ultramafic areas of Serbia, the soils with highest available Ni are also those with highest available Mn (Mišljenović et al. 2018). In tropical laterites, Mn-oxides play a significant role in that Ni-bearing minerals and heterogenite can hold up to 20 wt% Ni, followed by lower Ni contents within lithiophorite and asbolane (Llorca and Monchoux 1991; Decrée et al. 2015). These minerals are the most important scavengers for cobalt; however, little is known about Ni availability in such well-characterized Mn-oxides.

Amorphous Fe-oxides are not easily isolated in pure form from bulk soils, so it is impossible to assess the specific availability of Ni in these phases. The available properties of Ni borne by goethite particles have been studied by several workers (Massoura 2003; Raous et al. 2013; Zelano et al. 2016a, b). Nickel availability associated with synthetic goethite can be nearly null (Massoura 2003), with no proof of isotopic exchange between Ni included in the crystal lattice and free Ni present in solution. This is confirmed by the absence of Ni in the CEC of limonite where Ni is contained mostly in goethite particles (Raous et al. 2013). Goethite has some potential to bear isotopically exchangeable Ni as surface-complexed Ni (Raous et al. 2013; Zelano et al. 2016a). However, Ni availability associated with synthetic goethite is extremely low relative to other ultramafic Ni-bearing phyllosilicates (Zelano et al. 2016a). Unsurprisingly, the availability of Ni from hematite is null (Zelano et al. 2016a).

Available Ni (DTPA-extractable) is strongly positively correlated to organic C in an ultramafic toposequence in Albania (Bani et al. 2014). The labile pool associated with organic matter in a Brazilian ultramafic soil also accounts for most isotopically exchangeable Ni of the whole soil, as 3 wt% of the soil mass provides 60 wt% of labile Ni (Zelano et al. 2016b). Little attention has been devoted to the role of organic-borne Ni in ultramafic soils, but recently it was shown that plants and litter are a major contributor to total Ni in surface soils (Estrade et al. 2015), which is in turn highly available. It is, therefore, crucial to describe better the pools of available Ni associated with organic matter and detail the speciation of Ni and the chemical bounds that may exist to organic matter.

8 Biogeochemical Cycling of Nickel in Ultramafic Soils

The use of nickel stable isotopes has recently been introduced in studies of ultramafic environments to elucidate the pathways and mechanisms of nickel biogeochemistry (Estrade et al. 2015; Ratié et al. 2015, 2019): mineral weathering, nickel fluxes, root uptake and plant translocation. Typically, the weathering of primary minerals during pedogenesis induces a loss of heavy isotopes by leaching processes, such that the resulting soil has isotopically lighter Ni than the parent bedrock. In several ultramafic soils, the soluble and available (i.e. DTPA-extractable) fraction of Ni is usually much heavier, isotopically, than Ni in the soil solid phase (Estrade et al. 2015; Ratié et al. 2015; Pędziwiatr et al. 2018). In a Chromic Cambisol, the upper A1 (0–3 cm) horizon presents a significantly lower degree of isotopic fractionation from bedrock than the deeper horizons (i.e. A/Bw and Bw). Similar observations were made on a Eutric Skeletic Cambisol on Serpentinite from Poland (Pędziwiatr et al. 2018) whereas a Eutric Leptosol from a partially serpentinized peridotite showed a continuous loss of available Ni in the surface, probably in relation to an intense weathering of Ni-bearing phases and a lack of CEC traps (lacking secondary high-charge clays) to prevent from the leaching of the heavier soluble fraction of Ni released during the dissolution of olivines (Pędziwiatr et al. 2018). The isotopic compositions of Ni in litter are all heavier than in corresponding rhizosphere soils, suggesting a significant contribution from decaying plant material that contains a much greater proportion of isotopically heavy Ni than the bulk soil (Estrade et al. 2015; Ratié et al. 2019). The reason for this is that plants take up soluble Ni from the soil, which is the heaviest isotopic pool of Ni present within the soil. In a ploughed ultramafic Vertisol, this relationship could not be observed because the organic matter residues in this soil are diluted in the Ap horizon (0–20 cm).

In May 2013, five plants of the Ni-hyperaccumulator species *Odontarrhena chalcidica* (syn. *Alyssum murale*) were sampled at flowering stage on a Eutric Vertisol (Hypermagnesic) in Prrenjas (Albania) where no other vegetation had grown for at least 12 months. Plants were distant from each other with at least a 1 m spacing distance (details in Bani et al. 2015). In this study, a careful sampling of plant leaves, deposited as litter beneath each plant, and surface soil samples clear of any litter particles (from a 0–3 cm depth), was carried out. In parallel, soil samples (from a 0–3 cm depth) were taken randomly in bare soils at a minimal distance (2 m) from each plant to ensure that there would be no influence of *O. chalcidica* on the geochemistry of the soil sample. All plant and litter samples were analyzed for total element concentrations after complete digestion with concentrated HNO_3. Soil samples were digested with concentrated HF for total dissolution of minerals. Digestion solutions were analyzed using ICP-MS after appropriate dilutions. The results (Table 3) clearly showed that total Ca, K, and Ni were significantly enriched in the soil beneath *O. chalcidica* after only one growth cycle of the plant (9–12 months). Moreover, the Ni contained in plant leaves after decayed material was leached away after only a few months (by almost 50%), which was the time equivalent to the age of the deposited litter. Potassium was even more affected by leaching (>66% of initial K). This experiment brings additional understanding to what had been previously shown with stable isotope studies on Ni in topsoil and litter (Estrade et al. 2015): the contribution of plants to the building up of Ni concentrations in topsoil. Furthermore, the majority of Ni present in the primary minerals is leached during pedogenesis (Kierczak et al. 2007; Estrade et al. 2015), thus resulting in a Ni pool that is enriched in lighter isotopes. Hence the existing Ni contents in topsoils, which have a Ni isotopic composition strongly influenced by plant recycling (Estrade et al. 2015), probably result from significant biogeochemical recycling. Nickel hyperaccumulator plants, which can be quite common in these environments, likely contribute in a major way to this phenomenon. More detailed studies should be carried out by tracing Ni fluxes using stable isotopes (Deng et al. 2016) in soil-plant systems that are representative of various edaphic situations worldwide, in order to shed more light on the importance of Ni biogeochemical cycling in ultramafic soils and related ecosystems.

9 Conclusions: Global Trends of Nickel Availability in Ultramafic Soils

At first glance, it seems that soils developed on serpentinite are much richer in available Ni than those developed on non-serpentinized peridotite (van der Ent et al. 2016a). However, it is not that simple, because extremely well-developed Geric Ferralsols may occur on serpentinite (Yongue-Fouateu et al. 2006), at the surface of which is a very low availability of Ni. At the same time, Cambisols with extremely high Ni availability may exist on non-serpentinized pyroxenite (i.e. peridotite) (Garnier et al. 2009b). Nickel is a relatively mobile metal in ultramafic environments (Raous et al. 2010) and its resulting total concentration in most temperate and Mediterranean soils, although of the same order of magnitude as the bedrock concentration, results from the loss of more than 85% of its initial mass in the bedrock (Kierczak et al. 2007; Estrade et al. 2015; Pędziwiatr et al. 2018). It is frequently seen that Ni is as easily released and mobilized as Mg during the steps of soil weathering and pedogenesis (Raous et al. 2010; Chardot-Jacques et al. 2013; Hseu et al. 2018). The difference between these elements is the fact that Ni forms stable complexes with organic matter and Fe- and Mn-oxyhydroxides (especially amorphous Fe-oxyhydroxides). Therefore, Ni is retained in a certain number of pedological situations in which Mg is not, thus explaining differences in losses between the two elements during pedogenesis (Kierczak et al. 2007; Hseu et al. 2018). The availability of Ni in soils is therefore favoured by the following mechanisms that may occur under various edaphic conditions:

Dissolution/weathering of primary minerals holding Ni (Chardot-Jacques et al. 2013).

- Uptake of released Ni by soil humic substances before significant amounts of secondary minerals that contribute strongly to soil CEC are produced (Hseu et al. 2018).
- Building up a CEC from the neoformation/transformation of primary silicates into 2:1 clays and from incorporation of organic matter in the substrate (Lee et al. 2003; Bani et al. 2014), and subsequent uptake of Ni released by mineral dissolution.
- Formation of amorphous Fe-oxyhydroxides that have a CEC at high pH values (typically above 7–8, which is the usual pH of slightly weathered ultramafic soils), and to which Ni is easily reversibly sorbed (Chardot et al. 2007) after being released during weathering.

Under tropical conditions, the intense loss of Si during lateritization favours the formation of Fe-oxides (e.g. goethite) (Hseu et al. 2018; van der Ent et al. 2018a) that incorporates high concentrations of Ni during the phases of dehydration of Fe-hydroxides, despite a lower affinity of Ni to crystallized Fe-oxyhydroxides (Massoura et al. 2006). Further evolution of goethite can lead to an important loss of Ni (Dublet et al. 2015) and Ni contents in resulting hematite may decrease further to reach those of the bedrock. Nickel is known to be unavailable from wellcrystallised Fe-oxides (e.g. hematite) that occur in ferricrete (Zelano et al. 2016a; Quintela-Sabarís et al. 2018). In the saprolite layers of Geric Ferralsols, Ni that is progressively leached by lateritization and the maturation of Ni-bearing Fe-oxides is accumulated to high concentrations in phyllosilicates (serpentine, talc, smectite), which commonly represent the best Ni lateritic ore. The prime example is saprolitic smectite (Decarreau et al. 1987) found on the pyroxenite of Niquelândia (Brazil), where Ni concentration reaches >20 wt%. The Ni borne by saprolitic smectite is one of the most available sources of Ni in soils globally (Raous et al. 2013). Vertic Cambisols (Hypermagnesic) described on many serpentinite outcrops in the tropics, such as in Cuba, Borneo, and New Caledonia (Proctor 2003), also display very high Ni availability, for the same geochemical and mineralogical reasons (van der Ent et al. 2018a). Where natural erosion or mining has removed the lateritic horizons of ultramafic Ferralsols, the resulting soils have similar geochemical properties as the natural Vertic Cambisols (Hypermagnesic) found on serpentinite (Garnier et al. 2009b). Tropical Ni hyperaccumulators are generally native to these two types of environments (van der Ent et al. 2016a, 2018a). Geric Ferralsols having well-established ferricrete lack hyperaccumulator species owing to the absence of available Ni (Raous et al. 2010, 2013; Zelano et al. 2016a; van der Ent et al. 2016a).

Finally, vegetation and possibly Ni-hyperaccumulators are very active at recycling Ni in topsoil (Estrade et al. 2015; Ratié et al. 2019). As such, hyperaccumulator plants provide organic matter that increases the CEC and releases significant amounts of Ni through litter and the decay of organic matter. The result is a very mobile pool of Ni in the surface of soils (Bani et al. 2014). The role of biogeochemical recycling for maintaining sufficient levels of essential elements in acidic forest topsoils have been elucidated recently (Laclau et al. 2010); it is probable that the same processes control Ni geochemistry in ultramafic soils and ecosystems worldwide.

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Diversity and Role of Endophytic and Rhizosphere Microbes Associated with Hyperaccumulator Plants During Metal Accumulation

Emile Benizri, Séverine Lopez, Alexis Durand, and
Petra S. Kidd

Abstract

Phytomining can be limited by low biomass productivity by plants or limited availability of soil metals. Ongoing research attempts to overcome these potential constraints and to make phytomining a successful commercial technique in the recovery of metals from polluted or naturally metal-rich soil by (hyper) accumulating plants. Recently, the benefits of combining phytoremediation with bioremediation, which uses beneficial microorganisms such as endophytic or rhizosphere bacteria and fungi for metal removal from soils, have been demonstrated. Metal-resistant microorganisms play an important role in enhancing plant survival and growth in these soils by alleviating metal toxicity and supplying nutrients. Furthermore, these beneficial microorganisms are able to enhance metal bioavailability in the rhizosphere of plants. An increase in plant growth and metal uptake increases the effectiveness of phytoremediation processes coupled with bioremediation. Here, we discuss how abiotic factors, such as the presence of metals in polluted sites or in naturally metal-rich (ultramafic) soils modulate activities of soil microbial communities. Then we introduce the concept of microbe-assisted phytomining and underline the role of plant-associated microorganisms in metal bioavailability and uptake by host plants that has attracted growing interest over the last decade. Finally, we present various techniques, including phenotypic, genotypic, and metagenomic approaches, which allow for characterizing soil microbial community structure and diversity and endophytic community in polluted or naturally metal-rich soils.

1 Introduction

Soil microbes including bacteria, archaea, and fungi, play diverse and often critical roles in soil nutrient cycling and the provision of ecosystem services. Compared to bulk soil, the rhizosphere soil is characterized by higher concentrations of nutrients and labile organic C (Duineveld et al. 2001). Indeed, between 40 and 90% of the carbon transferred to the root is lost through rhizodeposition in the soil and represents an important flux of C in the rhizosphere (Nguyen 2003). Five kinds of rhizodeposits are

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E. Benizri (✉) · S. Lopez · A. Durand
Laboratoire Sols et Environnement—INRAE,
Université de Lorraine, Nancy 54000, France
e-mail: emile.benizri@univ-lorraine.fr

P. S. Kidd
Instituto de Investigaciones Agrobiológicas de
Galicia (IIAG), Consejo Superior de Investigaciones
Científicas (CSIC), 15706 Santiago de Compostela,
Spain

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_12

recognized: diffusates, secretions, lysates, gases, and mucilage (Nguyen 2003). Diffusates are water-soluble compounds of low molecular weight such as sugars, organic acids, or amino acids that diffuse passively through the cell envelope or among epidermal cells, owing to concentration gradients between the exterior and interior of the root. Secretions are made up of high-molecular weight compounds that are actively secreted by the roots. Lysates are the organic material released into the soil by dead cells after autolysis. Gases are mainly ethylene, carbon dioxide, and hydrogen cyanide. Mucilage, used for improving root penetration into soil, is composed of polysaccharides. Consequently, the rhizosphere represents a unique 'hot spot' in terms of microbial ecology, because soil microorganisms are stimulated by root activity (Baudoin et al. 2003; Hinsinger et al. 2006). Soil microorganisms drive, via their enzyme activities, the cycling of all major elements (e.g. C, N, P and S). This cycling affects the structure and functions of soil ecosystems as well as the ability of soils to provide ecosystem services.

Microbial enzyme activities in the rhizosphere can be of intracellular origin, released after microbial cell disruption, and may be associated with soil colloids and cell debris. Alternatively, such enzymes may be actively secreted by root-associated microorganisms. Soil enzyme activities have been reported to be used as a unique integrative biochemical assessment of soil function and condition (Naseby and Lynch 2002), and to be useful indicators of soil functional diversity (Bending et al. 2002; Sowerby et al. 2005; Epelde et al. 2008). The polymeric C and N components of plants (and microbes and animals) are both structurally complex and highly diverse, and their breakdown requires the combined activities of many different microorganisms. Some enzymes only facilitate the breakdown of organic matter (e.g. hydrolase, glucosidase), whereas others are involved in nutrient mineralization (e.g. amidase, urease, phosphatase, sulfatase) (Table 1). Rhizosphere microorganisms increase the ability of plants to acquire nutrients, by either increasing extent of the root system (through fungal hyphae) or by solubilizing macronutrients such as P or S (Smith and Read 1996). In natural systems, the action of soil microorganisms is a major determinant of efficient nutrient cycling. However, soil microorganisms also influence plant-pathogen interactions and/or plant growth by producing antibiotics or hormone compounds (discussed in the following sections). The activities of soil microorganisms are influenced by various environmental parameters such as soil type, nutrient status, pH, texture, organic matter content, moisture, and their interactions, as well as by plant factors such as species, age, root zone, or rhizodeposition (Baudoin et al. 2002).

Among abiotic factors, metals present in contaminated sites or in naturally metalliferous (such as ultramafic) soils also modulate activities of soil microorganisms and interactions among them (Krumins et al. 2015). The interaction of metals with native soil communities and their activities is an important area of research as scientists strive to understand the effects of metal pollution or enrichment on soil properties (Hagmann et al. 2015). Effects of trace elements (TE) on enzyme activity in the rhizosphere are complex and contrasting effects can be observed (Egamberdieva et al. 2011). Kandeler et al. (2000) found that C-acquiring enzymes (cellulase, xylanase, β-glucosidase) were the least affected by soil pollution, phosphatase and sulfatase being the most affected; finally, N-acquiring enzymes (urease) had an intermediate response. In a study on metal-polluted grassland soil (Kuperman and Carreiro 1997), β-glucosidase was the most depressed, whereas phosphatase and endocellulase activities were the least. Renella et al. (2011) reported that soil arylesterase activity was negatively correlated with exchangeable and soluble Cd fractions, although soil pH values and organic matter composition were important covariates. Nonetheless, in general, arylsulfatase appears to be most sensitive to metal pollution, whereas acid phosphatase and urease are less sensitive (Dick 1997).

An excess of metals can negatively affect the metabolic function of soil microorganisms. For instance, both the decomposition of organic matter and extent of microbial activities were

Table 1 Role of soil enzymes

Enzyme	Organic matter substances	End product	Significance	Predictor of soil function
	Acted on			
Beta glucosidase	Carbon compounds	Glucose (sugar)	Energy for microorganisms	Organic matter decomposition
FDA hydrolysis	Organic matter	Carbon and	Energy and nutrients for microorganisms, measure	Organic matter decomposition
		Various nutrients	Microbial biomass	Nutrient cycling
Amidase	Carbon and nitrogen compounds	Ammonium (NH_4)	Plant available NH_4	Nutrient cycling
Urease	Nitrogen (urea)	Ammonia (NH_3) and	Plant available NH_4	Nutrient cycling
		Carbon dioxide (CO_2)		
Phosphatase	Phosphorus	Phosphate (PO_4)	Plant available P	Nutrient cycling
Sulfatase	Sulfur	Sulfate (SO_4)	Plant available S	Nutrient cycling

Modified from Tabatabai (1994), Dick (1997), Bandick and Dick (1999)

inhibited in soils polluted with TE (Fritze et al. 1997). The addition of TE such as Cd, Cr, Cu, Ni, Pb and Zn to soil significantly inhibited N mineralization and nitrification (Liang and Tabatabai 1978). Influence of TE on the soil microbial activities varies with the kind of metal and with soil type and properties. Doelman (1985), who reviewed the literature on the effects of Cd, Cr, Cu, Hg, Ni, Pb and Zn on soil microbial activities, showed that the effect of Hg was the most significant and that of Pb was the least significant. These variations may be ascribed to differences in toxicity of the metals to soil microorganisms and the form of the metals in the soil. High Cd concentrations (40 µg Cd g^{-1} soil) inhibited soil acid phosphatase, alkaline phosphatase and urease activities (Renella et al. 2006). In another study, the presence of Cd (12 µg g^{-1} soil) and Zn (300 µg g^{-1} soil) negatively affected ammonifying microorganisms in loamy sand soil (Wyszkowska et al. 2009). A higher concentration of Cu (150–450 µg g^{-1} soil) significantly inhibited the activities of soil dehydrogenase, urease, and catalase (Wyszkowska et al. 2008).

However, such toxic effects may not always be observable owing to limited soil metal bioavailability or to development of metal resistance by the bacterial community (Nannipieri et al. 2012). Niklinska et al. (2006) found no differences between Zn- and Cu-polluted and non-polluted sites in terms of the activity of microbial communities. Similarly, Pessoa-Filho et al. (2015) concluded that activities of enzymes related to the C, P and S cycles were unaffected by high Ni concentrations; changes in soil microbiological functioning observed in their study were more related to organic matter content than to Ni availability. Epelde et al. (2008) confirmed that metal pollution did not cause a clear inhibition of soil enzyme activities. Metals can be toxic to living organisms, depending of their concentrations. This toxicity is primarily due to their protein-binding capacity and hence ability to inhibit enzymes (Dick 1997). However, the nature and degree of this inhibition is strongly related to soil type, pH, organic matter content, and interactions with other soil minerals and organic matter (Tate 2002). Moreover, Hattori (1989) reported that the influence of Cd on the decomposition of organic matter in soil varied with the content of water-soluble Cd in soil.

Rhizobacteria are the most studied plant-associated bacteria and are often found to have beneficial effects on plant growth, via the provision of essential elements, inhibition of colonization by pathogenic microorganisms, or by helping the plant to overcome environmental stresses (Mastretta et al. 2009). Similarly, plant endophytic bacteria play a key role in plant growth and survival, especially under stressful conditions (Vandenkoornhuyse et al. 2015; Rho et al. 2018). Indeed, endophytes have been demonstrated to be able to improve plant growth and vitality under salt (Piernik et al. 2017), alkali (Chen et al. 2018), water (Kavroulakis et al. 2018), and nutritionnel stresses (Krell et al. 2018). Moreover, it was shown that certain endophytic bacteria increase plant resistance to pathogens and even to herbivores (Azevedo et al. 2000; Reiter et al. 2002).

The polluted or naturally TE-enriched soils constitute a major stress for the plant as well as for plant endophytic bacteria, even if endophytes have the advantage of being relatively protected from the high-stress environment of the soil (Sturz and Nowak 2000; Guo et al. 2010). However, as for rhizosphere bacteria, it is known that metal resistance systems in endophytes are abundant and widespread, and that the frequencies range from a few percent of the isolates in non-contaminated environments to nearly all isolates in a heavily polluted environment (Zouboulis et al. 2004).

2 The Concept of Microbe-Assisted Phytomining

2.1 Rhizosphere Microbes

Experimental evidence clearly shows that root proliferation and effective root uptake mechanisms are among the key processes that distinguish metal hyperaccumulator plants from ‘normal’ plants. On the other hand, the role of plant-associated microorganisms in bioavailability of TE and their uptake by host plants is less well known and has attracted growing interest over the last decade. Toxic concentrations of TE have often been suggested to reduce microbial densities and diversity in metal-enriched soils (Pal et al. 2005). In fact, microorganisms are ubiquitous in soils to which metal-hyperaccumulating plants are native, despite the presence of high concentrations of some metals (Abou-Shanab et al. 2003a). During the late 1990s and early 2000s, several studies suggested that the accumulation of metals by (hyper)accumulating plants was influenced by their rhizosphere microflora (Mengoni et al. 2001; Lodewyckx et al. 2002a; Abou-Shanab et al. 2003a). Comparisons between sterile and non-sterile systems showed that metal accumulators in plants only reached their full accumulation capacity in the presence of indigenous rhizosphere microflora. Since then, many studies have indicated that microbiota are essential players during metal phytoextraction or phytomining (Lebeau et al. 2008; Kidd et al. 2009; Glick 2010; Becerra-Castro et al. 2013; Sessitsch et al. 2013). Moreover, it appears that the plant host can select metal-tolerant microbes out of the enormous pool and diversity of bacteria present in the bulk soil (Mengoni et al. 2001; Becerra-Castro et al. 2009; Álvarez-López et al. 2016a). Plants can also promote the abundance of beneficial microorganisms (such as plant growth-promoting bacteria, PGPB) in their surroundings (Vessey 2003; Thijs et al. 2016). Plants employ various strategies to increase metal bioavailability, such as secretion of phytosiderophores or carboxylates, and acidification of the rhizosphere (Kinnersley 1993), but soil microorganisms are also known to influence biogeochemical cycling of soil metals. The potential benefits of microbes associated with metal-accumulating plants led to the concept of microbial-assisted phytoextraction or phytomining (MAP) (Thijs et al. 2016). MAP aims to enhance metal recovery rates by inoculating metal (hyper)accumulating plants with plant-associated microbes, which are able to increase metal accumulation capacity of the phytoextracting plants by (i) improving plant biomass and thus metal yield, and/or (ii) increasing the bioavailability of metals and thus plant uptake and bioaccumulation.

2.2 Endophytic Microbes

Endophytes are, as rhizosphere microbes, plant partner able to mitigate environmental stresses. Knowing that some hyperaccumulating plants are especially adapted to recover metals from TE-enriched soils, plant-endophyte partnerships also seem to be a promising field of study for improving the phytoremediation of contaminated or naturally metal-rich soils (Weyens et al. 2013). Indeed, inoculation of endophytes could mitigate stresses caused by metal-contaminated soils, promote plant growth, and increase metal translocation in hyperaccumulators. These endophytic bacteria that produce organic acids, siderophores, or other metal-chelating agents can increase metal availability and uptake for themselves, but also for their host plant (Saravanan et al. 2007; Braud et al. 2009). Indeed, in a recent study, several Plant Growth Promoting Endophytes (PGPE) were isolated from the Zn ± Cd hyperaccumulator plant *Sedum plumbizincicola* and improved plant growth (Ma et al. 2015) In this work, one particular strain, *Bacillus pumilus* strain E2S2, significantly increased the plant Cd uptake, and increased plant root and shoot lengths, as well as the fresh and dry biomass compared to non-inoculated plants. Other experiments showed that endophytes that produce siderophores improved Ni uptake by the Ni-hyperaccumulator plant *Odontarrhena serpyllifolia* (Ma et al. 2011b). However, some reported results have shown that plant growth promotion caused by endophytic bacterial inoculation was sometimes accompanied by an overall decrease in the concentration of heavy metals in plant tissues (Mesa et al. 2015).

Characterizing the structure and genetic diversity of both rhizosphere and endophytic microbial communities associated with hyperaccumulators, and unravelling the complex plant-microbial interactions occurring in the rhizosphere and in plant tissues, will contribute towards our understanding of the metal hyperaccumulation process and may help enhance phytomining technology. In this chapter we focus mainly on the bacterial dimension of the plant-associated microbiome.

3 The (Hyper)accumulator Plant-Associated Microbial Community

Despite the long history of interest in ultramafic flora and metal hyperaccumulating plants, the attention of microbiologists towards bacteria from ultramafic and metal-enriched soils is more recent, with the prominent exception of Lipman (1926). Soil bacterial community structure and diversity can be categorized on a broad scale using various techniques, including phenotypic, genotypic, and metagenomic approaches.

3.1 Cultivation-Dependent Approaches

Several studies have shown that metals influence microorganisms by adversely affecting their growth, morphology, and biochemical activity, resulting in decreased biomass. In their study of microbial flora of ultramafic soils in Andaman Islands (India), Pal et al. (2005) used culture-dependent methods to show a lower range of microbial density ($6.2–11.3 \times 10^6$ colony-forming units (cfu) g^{-1} soil) in ultramafic soil than in ultramafic-free outcrops ($7.9–19.5 \times 10^6$ cfu g^{-1} soil). The ultramafic microbial population was dominated by bacteria that represented 5.12 to 9.5×10^6 cfu g^{-1} of soil, whereas the fungal population ranged from 0.17 to 3.21×10^6 cfu g^{-1} of soil. These results were confirmed by the study of Abou-Shanab et al. (2003a) in which total culturable microbial populations in the rhizosphere of *Odontarrhena chalcidica* (formerly *Alyssum murale*) and bulk soil were compared: they found 1.4×10^4 fungi and 5.5×10^9 bacteria g^{-1} of rhizosphere soil (and 1.5×10^3 and 1.8×10^5, respectively, in the bulk soil). Similarly, Aboudrar et al. (2007) reported that the ultramafic population of *Noccaea caerulescens* harboured various types of microorganisms with a predominance of bacteria. Lucisine et al. (2014) showed that the size of the culturable bacterial community appeared stable regardless of the rhizosphere soil type, when different covers of hyperaccumulator plants (*Bornmuellera* (formerly *Leptoplax*) *emarginata*, *Noccaea tymphaea* and *O. chalcidica*) were compared. They found no significant difference between the different species (mean values of 1.5×10^7 cfu g^{-1} dry soil). Moreover, Rue et al. (2015) determined that the ratio between the number of microorganisms in rhizosphere soils (R) and the corresponding number of microorganisms in the unplanted soils (S), the R/S quotient (Benizri et al. 2007), was the highest for a mixed multi-species cover of four hyperaccumulator plants, relative to the mono-species covers and the bulk soil (Rue et al. 2015).

Concerning endophytic bacteria, a decrease in bacterial number from the rhizosphere to the shoot is frequently observed and considered an indication that colonization was mainly taking place through the roots (Weyens et al. 2010, 2011). For example, Weyens et al. (2013) studied rhizosphere and endophytic bacterial communities isolated from willow clones growing on a metal-contaminated site, and showed that for the 'Belgisch Rood' clone (having a moderate metal extraction capacity), the number of cultivable bacteria recovered was the highest in the rhizosphere (6.02×10^6 cfu g^{-1} fresh weight), slightly lower in the roots (1.85×10^6 cfu g^{-1} fresh weight), and again lower in the shoots (2.95×10^5 cfu g^{-1} fresh weight).

On the other hand, several reports have shown the presence of highly metal-resistant bacteria in ultramafic soils, which suggests a potential adaptation of the indigenous bacterial populations (Abou-Shanab et al. 2007). Different Ni salts added to culture media were used to isolate Ni-resistant bacteria, including $NiCl_2$ (Abou-Shanab et al. 2003b, 2007; Turgay et al. 2012) or $NiSO_4$ (Aboudrar et al. 2007; Álvarez-López et al. 2016a; Durand et al. 2016). The concentrations used generally ranged from 0 to 40 mM. Such Ni concentrations might seem elevated (400–1000 times greater) when compared with natural Ni concentrations in the pore waters of ultramafic soils (Bani et al. 2015); however, it is known that a significant amount of added Ni is adsorbed onto agar and complexed by other culture medium components in Petri dishes. Pal et al. (2005) showed that bacterial isolates from core ultramafic areas were more resistant to Ni than those from peripheral areas. Analysis of relative resistance in bacteria revealed that 62.3% of ultramafic isolates were able to grow at 4 mM Ni, whereas it was only 24.8% grew for non-ultramafic isolates. At the highest concentration of Ni (8 mM) only 19 bacterial strains (11.7%) from core ultramafic outcrops showed visible growth. In their study, Durand et al. (2016) reported that the ratios of Ni-resistant bacteria to total bacteria steadily decreased from 81% at 0.5 mM of Ni to 0.76% at 10 mM. Similarly, Aboudrar et al. (2007) found that the ratio of the number of Ni-resistant bacteria (cfu on TSA plate with 1, 3 or 10 mM Ni) to the total number of bacteria (cfu on TSA plate) varied between 4 and 100%, and was about two to three times higher in the rhizosphere than in the bulk soil. Because metal availability and toxicity in soil are related to soil pH, soil acidification in the rhizosphere of *O. chalcidica* and also *N. caerulescens* has been hypothesized to explain the higher proportion of Ni-resistant bacteria in the rhizosphere compared to bulk soil (Abou-Shanab et al. 2003a). However, it should be noted that many studies have shown an increase in soil pH in the rhizosphere of hyperaccumulators growing in ultramafic soils alongside metal-resistant bacteria (Álvarez-López et al. 2016a).

Changes in microbial communities can be studied using sole-carbon-source tests. The method first described by Garland and Mills (1991) involves a commercially available microtiter plate (Biolog), which can be used to simultaneously test the utilization of 95 substrates as sole carbon sources. Carbon source utilization is indicated by color development of a redox indicator dye, and changes in the overall patterns of carbon source utilization rates can be assessed by multivariate statistics. The technique has been used to detect differences between microbial communities in soil and the rhizosphere (Baudoin et al. 2001), but only in a few cases have effects of metal pollution been studied (Cd, Cu or Zn according to Knight et al. (1997); Cu according to Fritze et al. (1997)). The Biolog EcoPlate system was more recently used to characterize the metabolic profile of microbial communities. It contains 31 of the most useful carbon sources for soil community analysis, allowing for community level physiological profiling of heterotrophic bacterial assemblages. This technique has been widely used to assess the toxicological impacts of different pollutants, including different heavy metals. By studying rhizosphere microbial communities under mono- or multi-species hyperaccumulator plant cover in an ultramafic soil, Rue et al. (2015) showed that the phenotypic structure of the bacterial communities appeared to be specific to the type of cover. Rhizosphere bacteria associated with *O. chalcidica*, *N. tymphaea*, or a mixed cover of four hyperaccumulator plants (*B. emarginata/N. tymphaea/O. chalcidica/B. tymphaea*) catabolized carbohydrate substrates more intensely than did bacteria from other rhizosphere soils. Similarly, bacteria from the rhizosphere of *B. emarginata* seemed to prefer both carboxylic acids and polymers. Aboudrar et al. (2007) also demonstrated that rhizosphere microorganisms isolated from *N. caerulescens* tended to show a greater utilization of some carboxylic acids than did the bulk soil microorganisms. Interestingly, Epelde et al. (2008) observed that ketobutyric acid and D-malic acid were utilized to a significantly greater extent by the culturable portion of the soil microbial community in metal-polluted and planted soil than in all other treatments (unplanted soil with or without metals and planted soil without metals). In hyperaccumulators, it has also been shown that *N. caerulescens* has constitutively high concentrations of malic acid/malate in their tissues (Boominathan and Doran 2003). Based on Biolog EcoPlate values, some authors have estimated the average well color development (AWCD), determined by calculating the mean of every well absorbance value at each reading time, and also estimated different diversity indexes such as S (richness), H′ (Shannon's diversity), and J′ (Shannon's evenness). Based on the AWCD technique, contradictory results were found. Indeed, Epelde et al. (2009) showed lower AWCD values in metal-polluted than in control unpolluted soils, but the same authors highlighted the point that diversity indices were higher in polluted soils with or without plants (Epelde et al. 2008).

Microbial community characterization by biomolecules other than nucleic acids such as lipids has been used without relying on culturing (Banowetz et al. 2006). Fatty acids are present in a relatively constant proportion of the cell biomass, and signature fatty acids exist in microbial cells that can differentiate major taxonomic groups within a community. The emerging pattern is then compared to a reference FAME database, in order to identify the fatty acids and their corresponding microbial signatures by multivariate statistical analyses. For example, Kozdrój and van Elsas (2001a, b) studied the structural diversity of microbial communities in arable soils of a heavily industrialized area.

Generally, rhizosphere bacteria found to be associated with hyperaccumulators using cultivation-dependent methods mainly comprise the Gram-positive genera *Arthrobacter*, *Microbacterium*, *Bacillus*, and *Curtobacterium*, and the Gram-negative genera *Pseudomonas, Sphingomonas*, and *Variovorax* (Mengoni et al. 2001; Oline 2006; Pal et al. 2007; Turgay et al. 2012). Álvarez-López et al. (2016a) found the culturable rhizobacterial community of the Ni-hyperaccumulator *Odontarrhena serpyllifolia s.l.* to be dominated by the phyla *Proteobacteria* and *Actinobacteria*. Isolates were affiliated with members of genera, such as *Arthrobacter*, *Streptomyces*, *Rhodococcus*, or *Microbacterium*, which have been frequently described among soil bacteria. Importantly, however, differences were observed in the bacterial communities associated with different populations of the same Ni-hyperaccumulating species.

Certain genera have been found in both the rhizosphere and the endosphere of hyperaccumulators regardless of the specific metal composition of the soil (Visioli et al. 2015a). Indeed, many studies also focused on the hyperaccumulator-associated endophytes, such as *O. bertolonii* (Barzanti et al. 2007), *O. serpyllifolia s.l.* (Ma et al. 2011b), *Alnus firma* (Shin et al. 2012), *Brassica napus* (Sheng et al. 2008b), *Nicotiana tabacum* (Mastretta et al. 2009), *N. caerulescens* (Lodewyckx et al. 2002b), and *N. goesingense* (Idris et al. 2004), from which many metal-resistant endophytes were isolated. The reported metal-resistant endophytes belonged to a wide range of taxa; in bacteria, these include *Arthrobacter*, *Bacillus*, *Clostridium*, *Curtobacterium*, *Enterobacter*, *Leifsonia*, *Microbacterium*, *Paenibacillus*, *Pseudomonas*, *Xanthomonadaceae*, *Staphylococcus*, *Stenotrophomonas*, and *Sanguibacte*, and in fungi *Microsphaeropsis*, *Mucor*, *Phoma*, *Alternaria*, *Peyronellaea*, *Steganosporium* and *Aspergillus* (Li et al. 2012).

3.2 Cultivation-Independent Approaches

Because only a minor percentage of naturally occurring microorganisms can be cultured, cultivation-independent approaches are now often used to analyze plant-associated microflora. The molecular techniques generally involve extraction of nucleic acid from soil. These approaches are independent of culture, and according to sensitivity can detect taxonomic groups, families, genera, and even down to species level (Rastogi and Sani 2011). Such techniques have been classified into two major categories depending on their capability of revealing the microbial diversity structures and functions: (i) partial-community analysis approaches, and (ii) whole-community analysis approaches (Fig. 1). Despite the recent interest paid to the study of the microbial communities in naturally metal-rich soils, few studies have focused on characterizing the genetic diversity of rhizosphere and endophytic bacterial communities of metal-hyperaccumulator plants (Kumar et al. 2009).

3.2.1 Partial-Community DNA Analysis

Partial genetic approaches have been developed as a result of the polymerase chain reaction (PCR) of specific marker genes. Indeed, these techniques offer direct access to the genomes of the different microbial communities inhabiting a given environment, targeting the genes encoding the different subunits of the ribosomes. The 16S gene is the most used marker and codes for the small ribosomal subunit in prokaryotes, whereas the 23S gene codes for the large subunit. The intergenic spacer (IGS) between the two ribosomal genes can also be used to characterize the microbial community structure within one or more samples. Indeed, the length and sequence of the ribosomal IGS is variable depending on the species (between 60 and 1500 base pairs) and on the alleles of different species (Gürtler and Stanisich 1996). Therefore, by using specific primers to the conserved sequences of ribosomal genes 16S and 23S, the IGS can be amplified by PCR to analyze the microbial community structure present in a soil sample (Table 2).

Clone Library

Bacterial populations can also be characterized by cloning and sequencing of 16S rRNA genes. The most widely used method to analyze PCR products amplified from an environmental sample is cloning and then sequencing the individual gene fragments (DeSantis et al. 2007). Obtained sequences are compared to known sequences in a database such as GenBank, Ribosomal Database Project (RDP), SILVA, and Greengenes. Typically, cloned sequences are assigned to phylum, class, order, family, subfamily, or species at sequence similarity cut-off values of 80, 85, 90, 92, 94 or 97%, respectively (DeSantis et al. 2007).

Genetic Fingerprinting

Genetic fingerprinting generates a profile of microbial communities based on direct analysis of PCR products amplified from environmental DNA (Muyzer 1999). These techniques include, for example, DGGE/TTGE, SSCP, RAPD, T-RFLP, RISA and ARISA, and produce a community fingerprint based on either sequence polymorphism or length polymorphism. In denaturing-gradient gel electrophoresis (DGGE), the PCR products are obtained from environmental DNA using primers for a specific molecular marker (e.g. 16S rRNA gene), and are

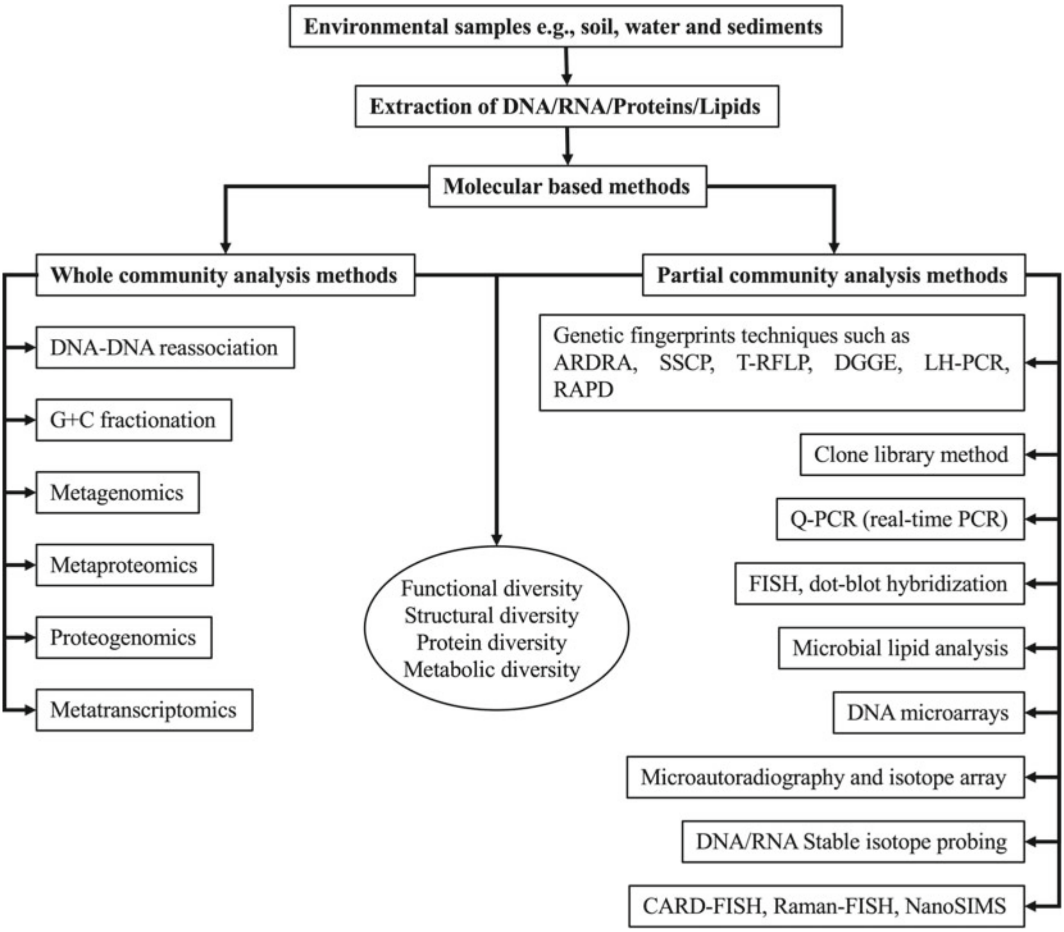

Fig. 1 Culture independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment (Rastogi and Sani 2011)

separated by electrophoresis on a polyacrylamide gel containing a linear gradient of DNA denaturant such as a mixture of urea and formamide (Muyzer 1999). Temperature-gradient gel electrophoresis (TTGE) is based on the same principle of DGGE, except that a temperature gradient rather than a chemical denaturant is applied. In single-strand conformation polymorphism (SSCP), the environmental PCR products are denatured, followed by electrophoretic separation of single-stranded DNA fragments on a non-denaturing polyacrylamide gel. Separation is based on subtle differences among sequences, which results in a different folded secondary structure leading to a measurable difference in mobility in the gel. The RAPD technique uses short random primers (about 10 bp) that anneal at different places on the genomic DNA, generating PCR products of various lengths that are further resolved on agarose or acrylamide gel. This technique was demonstrated to be rapid and sensitive for revealing differences among prokaryotic genomes of similar complexity (Ranjard et al. 2000). The terminal restriction fragment length polymorphism (T-RFLP) technique uses one 5′ fluorescently labelled primer during the PCR reaction. PCR products are digested with restriction enzyme(s), and terminal restriction fragments (T-RFs) are separated on an automated DNA sequencer. Only the terminal

Table 2 Advantages and inconveniences of the main analytical techniques used in microbial ecology studies dedicated to ultramafic or metal-polluted soils

Techniques	Advantages	Inconveniences	Focus of the study	Main results	References
Partial community DNA analysis					
T-RFLP	– Highly reproducible – Allows studying simultaneously a large number of samples	– Difficult to access to the microbial diversity – Cannot detect the dominant microbial populations – The sequences of the marker gene from two different organisms can share the same restriction site	*Thlaspi goesingense* rhizosphere	In rhizosphere T-RFLP profiles, a total of 112 fragments with fluorescence intensities higher than 50 were detected	Idris et al. (2004)
Establishment of 16S rRNA gene clone libraries and clones sequencing	– High phylogenetic resolution	– Time consuming – A very small number of samples can be processed simultaneously – Use several microbiological techniques	*Thlaspi goesingense* rhizosphere	*Holophaga/Acidobacterium* (27%), α-*Proteobacteria* (22%), high-G + C gram-positives (16%), *Cytophaga/Flexibacter/Bacteroides* (10%), γ- and ß-*Proteobacteria* (8 and 4%)	Idris et al. (2004)
			Alyssum bertolonii rhizosphere	Dominance of *Proteobacteria*	Mengoni et al. (2004)
			Ultramafic soils from Northern California and Southern Oregon	*Actinobacteria* (17%), *Acidobacteria* (17%), *Alphaproteobacteria* (11%), *Verrucomicrobia* (8.7%), Green-nonsulfur-bacterium related (8.7%), *Gemmatimonadetes* (8.0%), *Planctomycetes* (7.3%), *Bacteriodetes* (6.6%), *ß-proteobacteria* (5.3%), *δ-proteobacteria* (4.6%)	Oline (2006)
			Environments located at the Ni-mining sites in New Caledonia	Predominance of *Acidobacteria* in mining sites (without any vegetation), but not in sites revegetated with native plants, where *Proteobacteria* was the most abundant phylum	Herrera et al. (2007)
			Ultramafic soils from a tropical Savanna (Brazil)	Nine phyla, two of which—*Acidobacteria* and *Actinobacteria*—were the most abundant followed by *Proteobacteria*	Pessoa-Filho et al. (2015)

(continued)

Table 2 (continued)

Techniques	Advantages	Inconveniences	Focus of the study	Main results	References
DGGE/TGGE	– Microbial diversity can easily be assessed by excision and sequencing of bands – Theoretical discrimination possible between two sequences having a difference of only one nucleotide	– Comparison between two gels difficult – Technique poorly reproducible – Multiple bands for a species can be maintained due to a micro-heterogeneity within the genome – Possible bias related to PCR amplification	Soils exposed to different metal pollutants	An increase in *Acidobacterium and* decrease in terrestrial non-thermophilic *Crenarchaeota*	Kozdrój and van Elsas (2001a, 2001b)
			Polluted soils from an explosives factory	*Bacteroidetes* (0 to 26%), *Actinobacteria* (3.4 to 16%), *Firmicutes* (44 to 69%), *β-proteobacteria* (0 to 6.5%), *γ-proteobacteria* (5.6 to 50%)	Ellis et al. (2003)
			Cu-, Zn-, and Cd-contaminated paddy soils	A decrease in microbial community diversity	Li et al. (2006)
			Soil collected from a lead and zinc mine	A decrease in microbial community diversity due to the presence of heavy metals such as Cd and Pb	Hu et al. (2007)
			Soil sample was added with Cd and Pb	The structure of the bacterial community changed in heavy metal-amended soil samples	Khan et al. (2010)
			Ultramafic soils	*Ascomycota, Verrucariales, Hypocreales, Pleosporales, Chaetothyriales* and *Capnodiales* were the more represented orders	Daghino et al. (2012)
			Polluted Rhizosphere soil with chromium of the metallophyte *Silene vulgaris*	Under Cr pollution, a shift in the relative abundance of specific taxa with dominant phylotypes such as *Variovorax*, *Chitinophaga niastensis*, *Pontibacter* sp., *Ramlibacter* sp.	García-Gonzalo et al. (2016)
SSCP			*Odontarrhena chalcidica, Bornmuellera emarginata, Bornmuellera tymphaea, Noccaea tymphaea*	The bacterial genetic structure depended on the plant cover composition (mono- or multi-species covers)	Lucisine et al. (2014) Rue et al. (2015)

(continued)

Table 2 (continued)

Techniques	Advantages	Inconveniences	Focus of the study	Main results	References
RISA/ARISA	– Highly reproducible	– More of one sequence can be generated for the same organism – A similar size IGS between different organisms may lead to an underestimation of the microbial richness – Possible bias related to PCR amplification	Ultramafic neocaledonian soils	Emergence of a bacterial group closely related to the *Ralstonia/Oxalobacter/Burkholderia* group in the ß-Proteobacteria and also related to *Actinomycete* group	Héry et al. (2006)
			Polluted soils with copper	ARISA fingerprinting showed slight but significant modifications of bacterial and fungal communities suggesting a short-term effect of Cu stress	Ranjard et al. (2006)
Whole genomic community DNA analysis approaches					
Metagenomic	High resolution, very fast	– Requires knowledge of bioinformatics and powerful computers – Expensive equipment and consumables	Cu-polluted field site in Hygum, Denmark	*Proteobacteria*, *Bacteroidetes*, *Verrumicrobia*, *Chloroflexi*, *WS3*, and *Planctomycetes*, decreased with increasing bioavailable Cu, while members of the dominant phylum, the *Actinobacteria*, showed no response and members of the *Acidobacteria* showed a marked increase in abundance	Berg et al. (2012)
			Soil pollution with trace metals (Pb, Zn, Cd, or Cu, and Cr) in the vicinity of mines, smelters, and other industrial facilities (Southern Poland)	*Proteobacteria* were the most abundant phylum followed by *Acidobacteria* and *Actinobacteria*. Other major phyla were *Bacteroidetes*, *Chloroflexi*, and *Gemmatimonadetes*. The most abundant classes were *α-proteobacteria, Actinobacteria, Acidobacteria, γ-proteobacteria, Sphingobacteria*, and *ß-proteobacteria*	Gołębiewski et al. (2014)
			Ultramafic soils (New Caledonia)	The most abundant bacterial groups were *Proteobacteria* (41%) and *Acidobacteria* (18%), followed by *Actinobacteria* (10%),	Bordez et al. (2016)

(continued)

Table 2 (continued)

Techniques	Advantages	Inconveniences	Focus of the study	Main results	References
				Planctomycetes (10%), *Verrucomicrobia* (6.2%) and *Chloroflexi* (< 6%)	
			Ultramafic rhizosphere soils (Spain)	*Proteobacteria* was the most represented phylum (38%), followed by *Acidobacteria* (14%) and *Actinobacteria* (13%)	Saad et al. (2018)
			Ultramafic rhizosphere soils (Albania)	The most abundant bacterial phyla were *Proteobacteria* (32%), *Acidobacteria* (19%), *Actinobacteria* (16%), *Gemmatimonadetes* (10%), *Chloroflexi* (7.8%), and *Bacteroidetes* (7.4%)	Lopez et al. (2019a)
			Ultramafic rhizosphere soils (Indonesia)	*Proteobacteria* phylum was the most represented amongst all the rhizosphere soils (33%), followed by *Acidobacteria* (21%), *Actinobacteria* (19%) and *Chloroflexi* (8.4%)	Lopez et al. (2019b)
			Ultramafic rhizosphere soils (Malaysia)	The most abundant bacterial phyla were *Proteobacteria* (46%), *Acidobacteria* (21%), *Actinobacteria* (6.3%), *Rokubacteria* (5.5%) and *Bacteroidetes* (4.3%)	Lopez et al. (2020)
			Hg-enriched soil from industrial tailings dumps (France)	*In bulk soils, t*he most abundant bacterial phyla were *Proteobacteria (33%), Actinobacteria (26%)*, *Chloroflexi* (26%), *TM7* (4%) and *Acidobacteria* (3%) *In root t*he most abundant bacterial phyla were *Actinobacteria* (42%), *Proteobacteria* (40%), *Chloroflexi* (7%), *Bacteroidetes* (5%) and *TM7* (4%)	Durand et al. (2018)

fluorescent-labelled restriction fragments are detected. The ribosomal intergenic spacer analysis (RISA) technique allows the analysis of ribosomal intergenic region after PCR amplification and migration on acrylamide gel. The automated version (ARISA) consists, for its part, in the use of a fluorescence-labelled forward primer, and intergenic spacer fragments are detected automatically by a laser detector. These two methods of analysis of ribosomal IGS are considered highly reproducible and provide a community specific profile, with each band corresponding to at least one organism in the original community. However, these methods require a large amount of microbial DNA and are slow to develop (Kirk et al. 2004).

3.2.2 Whole Genomic Community DNA Analysis Approaches

The latest advances in molecular biology have allowed, since the early 2000s, the provision of next generation sequencers (NGS). Unlike Sanger sequencing (Sanger et al. 1977), the next-generation sequencers enable access to the structure and diversity of microbial communities from diverse environments by high-throughput sequencing and simultaneous, multi-million sequences obtained from many samples (Rastogi and Sani 2011). These high-throughput sequencing technologies were developed by different companies (Roche Diagnostics Corp., Illumina, Life Technologies Corp., mainly), and thus allow rapid access, by the analysis of a specific marker gene, to the structure and genetic diversity of microbial communities in any environment (Visioli et al. 2015a). NGS has been used to analyze the bacterial community in soils polluted with heavy metals and to determine the impact of heavy metal pollution on the composition of the microbial community (Table 2).

Concerning natural Ni-rich soils, analysis of rhizosphere microbial communities is rather recent despite the long history of interest in ultramafic flora and metal hyperaccumulators. Indeed, microbiologists have paid little attention to the bacterial communities specific to these environments. Concerning rhizosphere bacteria from natural Ni-rich soils, Saad et al. (2018) characterized the potential benefits for phytoextraction efficiency of the Ni-hyperaccumulator *O. chalcidica* (Brassicaceae) in an ultramafic zone in northwest Spain. In their study, *Proteobacteria* was the most represented phylum (38%), followed by *Acidobacteria* (14%) and *Actinobacteria* (13%), (Table 2). Similarly, Lopez et al. (2019a, b, 2020) characterized the structure and diversity of bacterial communities in several countries, such as Albania, Indonesia, and Malaysia, (Table 2). In Albania, 14 phyla of bacteria were identified. The major lineages (>1%) of total sequences were in the following order: *Proteobacteria* (32%) > *Acidobacteria* (19%) > *Actinobacteria* (16%) > *Gemmatimonadetes* (10%) > *Chloroflexi* (7.8%) > *Bacteroidetes* (7.4%) > *Nitrospirae* (2.4%) (Lopez et al. 2019a). In Indonesia (Halmaera Island), *Proteobacteria* phylum was the most represented amongst all rhizosphere soils (33%), followed by *Acidobacteria* (21%), *Actinobacteria* (19%) and *Chloroflexi* (8.4%), but relative abundances of the bacterial phyla in plant species' rhizospheres were location specific, even if the relative abundances of α-*Proteobacteria* subphylum (36%) and δ-*Proteobacteria* subphylum (28%) were the highest regardless of the plant species and sampled sites considered (Lopez et al. 2019b). In Malaysia, among the ten most abundant phyla, *Proteobacteria* (46%) and *Acidobacteria* (21%) were best represented at all sites and regardless of plant species. After that, the most abundant phyla were *Actinobacteria* (6.3%), *Rokubacteria* (5.5%), and *Bacteroidetes* (4.3%) (Lopez et al. 2020). The studies of Lopez and collaborators (Lopez et al. 2019a, b, 2020), focusing on rhizosphere of spontaneously developed hyperaccumulating plants from natural ultramafic environments, underlined differences among the regions, with large relative abundances of *Gemmatimonadetes* and *Bacteroidetes* observed for samples from Albania in temperate climate, whereas in Indonesia and Malaysia, under tropical climate, *Planctomycetes* and *Nitrospirae* were relatively abundant. Moreover, whatever the region (temperate or tropical), the most abundant bacterial phyla were *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*; these results corroborated other studies using cultivation-based approaches (Abou-Shanab et al. 2010; Álvarez-López et al. 2016a), but also works based on molecular techniques using high-throughput sequencing techniques. Indeed, these studies confirmed that these three phyla were the dominant ones in both contaminated and uncontaminated soils (Roesch et al. 2007; Buée et al. 2009; Chu et al. 2010, Zhao et al. 2014; Bordez et al. 2016). Numerous studies suggest that *Proteobacteria* and *Actinobacteria* are the most common phyla in the rhizosphere of many plant species (Fierer et al. 2007; Lienhard et al. 2014), because these copiotrophic bacteria are known to prefer C-rich environments, such as rhizosphere (Buée et al. 2009; Yang et al. 2017). In the same way, Durand et al. (2018) reported that bacterial communities close to poplar roots in an Hg-rich soil were significantly enriched with *Proteobacteria* and *Actinobacteria* compared to bulk soil communities, whereas reciprocally, bulk soil was enriched with *Chloroflexi* (Table 2). *Acidobacteria* phylum is also known to be one of the most abundant bacterial phyla in terrestrial ecosystems (Barns et al. 1999), playing a crucial role in the C cycle and particularly in degradation of compounds commonly found in rhizosphere environments, such as cellulose and lignin (Ward et al. 2009). However, soil pH has a major influence on members of the phylum *Acidobacteria*, which is commonly among the most abundant phyla in soil communities in low-pH (<6) soils, but rare or absent in soils with pH > 6.5 (Sait et al. 2006).

Microorganisms able to penetrate and invade the internal tissues of roots form the endosphere microbiota. In the vast majority of land plants, the root endosphere is mainly colonized by arbuscular mycorrhiza (Vandenkoornhuyse et al. 2002), bacteria (Reinhold-Hurek and Hurek 2011), and, to a lesser extent, *Archaea* (Sun et al. 2008). Whichever plant is considered, microbial diversity is much lower than that estimated for microbial communities outside the root (Bulgarelli et al. 2012). Comparisons between various plant endophyte communities are difficult, however, because the plant endophyte population depends on many variables such as plant growth stage, the plant tissue analyzed, health of the plant, nutritional state of the plant, the type of soil and its conditions (including pH and moisture content), altitude, and temperature (Idris et al. 2004; Hardoim et al. 2008). In general, the phylum *Proteobacteria*, including the classes α-, β-, and γ-*Proteobacteria*, are reported to be dominant in diversity analysis of endophytes, although members of the *Firmicutes* and *Actinobacteria* are also among the classes most consistently found as endophytes. Other classes such as *Bacteroidetes*, *Planctomycetes*, *Verrucomicrobia*, and *Acidobacteria* are less commonly reported as endophytes. The most commonly found genera of bacterial endophytes are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus*, *Pantoea* and *Microbacterium* (Hallmann et al. 1997; Rosenblueth and Martínez-Romero 2006; Marquez-Santacruz et al. 2010; Romero et al. 2014; Shi et al. 2014). All of these genera, described as bacterial endophytes, are also common inhabitants of the rhizosphere. Therefore, it has been suggested that the endophyte microbiome may be a subpopulation of the rhizosphere-inhabiting bacteria (Germida et al. 1998; Marquez-Santacruz et al. 2010). However, despite the broad application of molecular approaches for the analysis of bacterial communities in a wide range of natural habitats, little information is available on the diversity of bacterial endophytes within hyperaccumulators growing at heavy-metal contaminated sites, and particularly in natural Ni-rich soil (Idris et al. 2004). Idris et al. (2004) studied the shoot-associated (endophytic) bacteria colonizing *T. goesingense* that grow in an ultramafic soil in eastern Austria. Bacterial populations were characterized by terminal-restriction fragment length polymorphism (T-RFLP), as well as by cloning and sequencing of 16SrRNA genes. The majority (67%) of clones were clustered with the α-, β- and γ-*Proteobacteria*. This result agrees with previous studies showing that the interior of plants is preferentially colonized by *Proteobacteria* (Chelius and Triplett 2001; Garbeva et al. 2001; Sessitsch et al. 2002).

Transcriptomics and proteomics can be useful for revealing the ability of microbes to respond to particular stimuli, and for showing how functions of the majority of microbial species are expressed (Visioli et al. 2015a). These tools focus on the messenger RNAs produced by the microbial community from an environment. By analyzing these messenger RNAs (called meta-transcriptomic), information relating to the structure and functional diversity of different populations present in a sample are obtained. Finally, meta-proteomics focuses on the study of all of the proteins produced by the microbial community and provides access to functional diversity.

4 Isolation of Plant-Associated Microorganisms and Application in Phytomining

4.1 Improving Metal Removal Through Plant Growth Promotion

Microorganisms can enhance plant growth and resistance to biotic and abiotic stresses by various mechanisms (Lebeau et al. 2008; Gadd 2010). Well-known examples include the nitrogen-fixing rhizobia and mycorrhizal fungi that facilitate phosphorus uptake (Richardson and Simpson 2011). The importance of symbionts such as mycorrhizal fungi in supplying nutrients and minerals to the plant, in soil physical structure and aggregate stability, and in the suppression of soil-borne plant pathogens is well recognized and documented (Kapulnik and Douds 2000; van der Heijden and Sanders 2002). Documented mechanisms by which plant growth-promoting rhizobacteria (PGPR) can benefit plant growth and physiology include the provision of plant nutrients, release of plant hormones or growth regulators, or suppression of plant-stress ethylene levels. PGP bacteria directly influence plant growth by increasing the availability of essential nutrients such as N, P or Fe, thus improving plant nutrition in nutrient-deficient soils. For example, diazotrophs use the enzyme nitrogenase to fix atmospheric N into ammonia, which can then be absorbed by plant roots. Diazotrophic bacteria occur in the form of nodules (symbionts) or free-living cells (Beattie 2007). Another mechanism involves the release of organic acids and/or phosphatases that solubilize inorganic phosphates into plant-available forms (Richardson and Simpson 2011). Inoculating plants with P-solubilizing microorganisms has been shown to improve plant P accumulation and growth (Richardson and Simpson 2011). Microorganisms are also known to exude high-affinity Fe (III)-specific chelating agents (generally < 1000 Da) or siderophores, in response to limited Fe supply, which can mobilize Fe associated with soil solid-phase minerals or that complexed by organic matter (Crowley and Kraemer 2007).

Bacterial indoleacetic acid (IAA) stimulates root hair formation while increasing the number and length of lateral and primary roots when in an ideal concentration range (Duca et al. 2014). Some PGP bacteria are also known to synthesize cytokinins, which have been suggested to enhance plant growth under stress conditions (Glick 2010). An important mechanism of plant stress alleviation is through the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. ACC deaminase-producing bacteria can reduce the production of stress ethylene in plants (Dell'Amico et al. 2008). Improvements observed in the growth of plants under various stresses, including flooding, drought, high salinity, phytopathogens, or the presence of organic pollutants and metals, have been associated with the activity of ACC deaminase-producing PGP bacteria (Glick 2010). Bacterial production of volatile compounds can also significantly promote plant growth (Ryu et al. 2003). Several studies have focused on screening bacterial-liberated volatile compounds for their effects on the growth of *Arabidopsis thaliana* (Ryu et al. 2003; Blom et al. 2011). A diverse array of bacterial volatile compounds was detected (over 100), and amongst these, compounds such as 2,3-butanediol, acetoin, indole, 1-hexanol, and pentadecane were found to have a strong beneficial effect on the growth of this plant species.

The activity of some bacteria can also indirectly inhibit or suppress plant diseases. Biocontrol traits include competion with pathogenic bacteria for nutrients and space (niche exclusion), production of antimicrobial compounds (such as hydrogen cyanide (HCN)), biosurfactants with antimicrobial activity or chitinolytic enzymes (such as quitanases), or the induction of plant defence mechanisms (Compant et al. 2005; Lemanceau et al. 2007). Compant et al. (2005) showed that PGP bacteria could deprive pathogens of Fe through the production of Fe-chelating siderophores. In a study by Kurek and Jaroszuk-Scisel (2003), siderophore-producing strains of *P. fluorescens* and *P. putida* strain B10 successfully suppressed activity of the fungus *Fusarium* sp. Many bacterial genera produce antimicrobial compounds and most of these have been shown to have broad-spectrum activity. Antibiotics produced by antagonistic bacteria include compounds such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), kanosamine, phenazines, oomycin A, pyrrolnitrin, viscosinamide, pyoluteorin, butyrolactones, pantocin A and B, xanthobaccins, and zwittermycin A (Raaijmakers et al. 2002). The antimicrobial compounds released by these bacterial biocontrol agents have been shown to act on four main targets: cell wall synthesis, protein synthesis, nucleic acid replication, and cellular membranes (Raaijmakers et al. 2002). Many bacterial biocontrol agents show hyperparasitic acitivty and can attack pathogens through the production of cell wall hydrolases, such as by quitinases or glucanases (Compant et al. 2005). Other PGP bacteria are able to detoxify the virulence factors produced by pathogens (Thangavelu et al. 2001). *Pseudomonas fluorescens* strain Pf10, isolated from the rhizosphere of banana, detoxifies fusaric acid produced by the wilt pathogen, *Fusarium oxysporum* f. sp. *cubense* (Thangavelu et al. 2001). Finally, some bacterial strains can induce plant systemic resistance (ISR) without causing visible symptoms of stress on the host plant, and often simultaneously enhance plant growth (van Loon et al. 1998). Examples of rhizobacteria-mediated ISR were given by van Loon et al. (1998). Various bacterial determinants of ISR have been described, including lipopoly-saccharides, siderophores, salicylic acid, and other macromolecules (van Loon et al. 1998).

Plant growth-promoting endophytes (PGPE) may also influence plant growth and physiology through direct or indirect methods. Inoculation of bacterial endophytes implies physiological changes in plant including accumulation of osmolytes and osmotic adjustment, stomatal regulation, reduced membrane potentials, as well as changes in phospholipid content in the cell membranes (Compant et al. 2005). Endophytic bacteria confer to the plant higher tolerance to different stresses and can stimulate host plant growth through several mechanisms. These mechanisms include nitrogen fixation. Indeed, N-fixing endophytic bacteria have been found in association with non-nodulating plants such as cereals, willow, poplar and other non-legume plants such as rice (Kandel et al. 2015; Dent and Cocking 2017). These endophytes belong to several genera such as *Aureobacterium*, *Azoarcus*, *Bacillus*, *Burkholderia*, *Gluconobacter*, *Herbaspirillum*, *Klebsiella*, *Pantoea*, *Paenibacillus,* *Phyllobacterium*, *Pseudomonas* and *Rahnella* (Kandel et al. 2017). Endophytes may also improve mineral nutrients and water uptake (Ryan et al. 2008). Indeed, it has been shown that bacterial endophytes, with the capacity of phosphate solubilization when inoculated by soaking young plants of *Noccaea caerulescens* and *Rumex acetosa*, significantly increased values of acid phosphatase activity of the metal-contaminated soil of the mesocosms (Burges et al. 2017). Moreover, antagonisms between endophytes and plant bio-aggressors lead to reduction of plant herbivory and diseases, thus improving plant fitness. For instance, it has been reported that Salicaceae endophytes acted as biocontrol agents against various soil-borne plant pathogens including *Rhizoctonia solani* AG-8, *Fusarium culmorum*, *Gaeumannomyces graminis* var. *tritici* and *Pythium ultimum* (Kandel et al. 2017). In the same way, endophyte bacteria of *Nicotiana glauca* were able to inhibit *Fusarium oxysporum* f. sp. *lycopersici* growth *via* production of lytic enzymes, such as chitinases and/or proteases, among other substances (Abdallah et al. 2016). The strong suppressive effect against *Fusarium* wilt was achieved using two endophytic isolates leading to 92–96% lower disease severity compared to a pathogen-inoculated and untreated control.

Some bacterial endophytes produced volatile organic compounds (VOCs) and especially pyrazine derivatives, produced by *Paenibacillus* spp., that suppress the growth of other bacteria, fungi, and yeast under laboratory conditions (Rybakova et al. 2016). Among biocontrol properties, some endophytes can, as PGPR, stimulate ISR and improve plant resistance against a wide range of pathogens and insects or nematodes and herbivores (Dalal et al. 2015; Rashid and Chung 2017; Hu et al. 2017). In several cases, treatments with endophytes significantly have elevated the levels of phenols, peroxidase, phenylalanine ammonia-lyase, polyphenoloxidase, β-1,3-glucanase, and chitinase involved in ISR in plants. For example, systemic resistance induced by the endophytes *B. pumilus* strain SE34, *Serratia marcescens* strain 90-166, and *Pseudomonas fluorescens* strain 89B-61, has been shown to reduce the severity of blue mold on tobacco, caused by *Peronospora tabacina* (Zhang et al. 2002).

Endophytes have the potential to synthesize IAA, gibberellic acid, cytokinin, and abscisic acid (Patten and Glick 2002; Pirttilä et al. 2004; Feng et al. 2006; Sgroy et al. 2009; Shi et al. 2009), which influences the plant metabolism, more precicely cell elongation and/or division, and regulates the level of stress phytohormones. Endophytes also produce siderophores to retrieve essential metals such as iron (Fe^{3+}) in their environment, where metal bioavailability is a limiting factor (Ahmed and Holmström 2014).

The use of microorganisms influencing the availability of plant nutrients (e.g. N, Fe or P) in agriculture as ‘biofertilizers’ to enhance uptake of plant nutrients and alleviate nutrient deficiencies is well established (Vessey 2003). However, the idea of using plant-associated microbes to enhance metal uptake by plants as part of phytoremediation or phytomining strategies is more recent. In fact, over the last decade a growing number of studies have been dedicated to the search for bioinoculants with potential application in phytoremediation (Table 3). The discovery of new microbes can contribute greatly towards optimizing the efficiency of these technologies (Haslmayr et al. 2014).

Trace metal-polluted soils or natural metal-enriched soils can be a potential source of metal-tolerant bacteria, including strains that promote plant growth. Numerous metal-tolerant and plant growth-promoting microbial strains have been isolated from TE-polluted mine tailings, and their use as inoculants in phytoextraction is documented (Becerra-Castro et al. 2012; Pereira et al. 2015). For Ni phytomining, ultramafic soils represent a source of potentially beneficial plant-growth promoting and Ni-tolerant microorganisms (Durand et al. 2016; Álvarez-López et al. 2016a; Cabello-Conejo et al. 2014). Schlegel et al. (1991) found that bacterial strains isolated from ultramafic soils tolerated up to 10–20 mM Ni (in the culture medium), whereas strains from other soil types tolerated only 1 mM Ni. Turgay et al. (2012) reported that bacterial strains, isolated from Turkish ultramafic soils, could tolerate up to 34 mM Ni in the growth medium. Furthermore, as described above, the rhizosphere bacterial communities associated with Ni-hyperaccumulating plants have been shown to differ from those of non-accumulating plants growing at the same site or of non-vegetated soil, but are also known to host a higher number of Ni-tolerant bacteria (Schlegel et al. 1991; Mengoni et al. 2001; Abou-Shanab et al. 2003b; Becerra-Castro et al. 2009; Álvarez-López et al. 2016a). Schlegel et al. (1991) reported a higher occurrence of Ni-resistant bacteria in soil samples collected with increasing proximity to the Ni-hyperaccumulating tree *Pycnandra acuminata*. Mengoni et al. (2001) also found a higher proportion of Ni-resistant cfu in proximity to the Ni-hyperaccumulator *O. bertolonii* than in non-vegetated soil. These authors observed simultaneous resistance to a set of metals and highest resistance from isolates of the rhizosphere. Becerra-Castro et al. (2009) found higher proportions of Ni-tolerant bacteria in the rhizosphere of *O. serpyllifolia*. This selective enrichment in

Table 3 Effects on plant metal accumulation of plant-associated bacteria isolated for phytoextraction or phytomining purposes

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
Alnus firma	*Bacillus* sp. *MN3-4* PGPE	Siderophore; IAA; Pb-tolerance	Pb	↑ Pb tolerance ↑ root elongation ↑ Pb uptake	Shin et al. (2012)
Odontarrhena chalcidica	*Microbacterium oxydans AY509223*	Acid; Siderophore	Ni	↑ leaf [Ni]	Abou-Shanab et al. (2006)
O. chalcidica	*Sphingomonas macrogoltabidus, Microbacterium liquefaciens, Microbacterium arabinogalactanolyticum*	Acid (*M. l, M. a*); PO_4 (*M. l, M. a*); Siderophore (*M. l*)	Ni	↑ soil $[Ni]_{Sr(NO3)2}$ (*M. a*); ↑ shoot [Ni]	Abou-Shanab et al. (2003a)
Odontarrhena serpyllifolia s.l.	*Microbacterium* sp., *Arthrobacter* sp., *Streptomyces* sp.	Acid; IAA; Siderophore; PO_4	Ni	↑ shoot [Ni]; ↑ plant biomass; ↑ Ni yield	Cabello-Conejo et al. (2014)
Amaranthus hypochondriacus Amaranthus mangostanus	*Rahnella sp. JN27* PGPE	Cd-tolerance; Cd-mobilization	Cd	↑ plant biomass ↑ plant Cd uptake	Yuan et al. (2014)
Intercropping of *Bornmuellera tymphaea–Noccaea tymphaea* and *B. tymphaea–Odontarrhena chalcidica*	*Variovorax paradoxus*	IAA; ACCD; Siderophore	Ni	↑ plant biomass; ↑ plant Ni uptake	Durand et al. (2016)
Brassica juncea	*Enterobacter aerogenes* NBRI K24, *Rahnella aquatilis* NBRI K3	IAA; Siderophore; ACCD; PO_4	Cr, Ni	↑ plant growth/biomass; ↑ [Cr, Ni] uptake	Kumar et al. (2009)
B. juncea	*Enterobacter* sp. *CBSB1* PGPE	Bifunctional glutathione (GSH) synthase gene *gcsgs*	Cd, Pb	↑ GSH contents in shoot ↑ plant length/biomass ↑ [Cd] and [Pb] shoot	Qiu et al. (2014)
B. juncea	*Achromobacter xylosoxidans* Ax10	IAA; ACCD; PO_4	Cu	↑ plant growth/biomass; ↑ [Cu]root/shoot	Ma et al. (2009c)
B. juncea	*Bacillus biosubtyl, Bacillus licheniformis, Bacillus thuringiensis*	nd	Cd, Cr, Se	↑ Cd accumulation (*B.l*)	Hussein (2008)
B. juncea	*Enterobacter* sp. NBRI K28	IAA; Siderophore; ACCD; PO_4	Cr, Ni	↑ plant biomass; ↑ [Cr, Ni, Zn] uptake	Kumar et al. (2008)
B. juncea	*Pseudomonas* sp. Ps29C, *Bacillus megaterium* Bm4C	IAA; Siderophore; ACCD; PO_4	Ni	↑ plant biomass plant protection from Ni toxicity	Rajkumar and Freitas (2008a)

(continued)

Table 3 (continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
B. juncea	*Pseudomonas* sp. PsA4, *Bacillus* sp. Ba32	IAA; ACCD; PO_4	Cr	↑ plant growth protection of Cr toxicity	Rajkumar et al. (2006)
B. juncea	*Azotobacter chroococcum* HKN-5, *Bacillus megaterium* HKP-1, *Bacillus mucilaginosus* HKK-1	N-fixing (*A. c*); PO_4 (*B. me*); K solubilization (*B. mu*)	Cu, Pb, Zn	↑ removal of Cu, Pb and Zn	Wu et al. (2006)
B. juncea	*Bacillus subtilis* strain SJ-101	IAA; PO_4	Ni	↑ plant growth/biomass; ↑ [Ni] shoot	Zaidi et al. (2006)
B. juncea	11 bacteria strains, *included:* *Variovorax paradoxus, Rhodococcus* sp., *Flavobacterium* sp.	Cd-tolerance; IAA; ACCD; Siderophore	Cd	↑ Co, Cu, Ni, Zn tolerance ↑ root elongation	Belimov et al. (2005)
B. juncea	Se-tolerant rhizobacteria (strains BJ1, BJ2, BJ2, BJ4)	Produce heat-labile bioactive compound	Se	↑ [Se] shoot	De Souza et al. (1999)
B. juncea, Brassica oxyrrhina	*Bacillus* sp. (strains SN3, SN9, SRS5, SRS15, SRI4, SRI11, SRI14) *Pseudomonas* sp. SRI2, *Psychrobacter* sp. SRS8	Ni-solubilization; IAA; Siderophore (except SRS5); ACCD (SN9, SRI2, SRI4, SRI11, SRI14); PO_4	Ni	↑ plant biomass (mainly SRI2, SRS8, SN9) ↑ [Ni] shoot and root (SN9)	Ma et al. (2009a)
B. juncea, B. oxyrrhina	*Psychrobacter* sp. (strains SRA1, SRA2), *Bacillus cereus* sp. (strains SRA10, SRP4), *Bacillus weihenstephanensis* SRP12	IAA; Siderophore (SRA1, SRA10, SRP4, SRP12); ACCD; PO_4	Ni	↑ plant biomass (SRA2) ↑ Ni solubilization (SRA1, SRA10) ↑ [Ni] shoot and root (SRA1, SRA10)	Ma et al. (2009b)
B. juncea, Lycopersicon esculentum, Zea mays L. var. Denhai-11	*Burkholderia* sp. J62	Metal-resistance; Antibiotic resistance; IAA; Siderophore; ACCD; PO_4	Cd, Pb	↑ plant biomass (*L. esculentum, Z. mays*); ↑ Cd, Pb uptake (*L. esculentum, Z. mays*);	Jiang et al. (2008)
B. juncea, Brassica napus, L. esculentum	*Kluyvera ascorbata* SUD165, *Kluyvera ascorbata* SUD165/26	Siderophore (*K. a. SUD165/26)*	Cu, Ni, Zn	↓ growth inhibition caused by metals	Burd et al. (2000)
Brassica napus	*Arthrobacter* sp. MT16, *Microbacterium* sp. JYC17, *Pseudomonas chlororaphis* SZY6,	IAA; Siderophore; ACCD; PO_4	Cu	↑ root length	He et al. (2010a)

(continued)

Table 3 (continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
	Azotobacter vinelandii GZC24, *Microbacterium lactium* YJ7				
B. napus	*Firmicutes* sp, *Actinobacteria* sp, *Proteobacteria* sp.	IAA; Siderophore; ACCD; arginine decarboxylase production	Cu	↑ plant biomass ↑ [Cu] shoot	Sun et al. (2010)
B. napus	*Sphingomonas* SaMR12 PGPE	Promote antioxidative enzymes	Cd	↑ plant biomass ↑ [Cd] root and shoot ↓ ROS damage	Wang et al. (2020)
B. napus	*Pseudomonas tolaasii* ACC23, *Pseudomonas fluorescens* ACC9, *Alcaligenes* sp. ZN4, *Mycobacterium* sp. ACC14	Cd-resistance; IAA; ACCD; Siderophore	Cd	↑ root elongation ↑ shoot and root growth	Dell'Amico et al. (2008)
B. napus	*Ralstonia* sp. J1-22-2, *Pantoea agglomerans* Jp3-3, *Pseudomonas thivervalensis* Y1-3-9 PGPE	Cu-resistance; IAA; ACCD; Siderophore; PO_4	Cu	↑ plant biomass ↑ Cu uptake	Zhang et al. (2011)
B. napus	*Pseudomonas fluorescens* G10, *Microbacterium* sp. G16	IAA; Siderophore; ACCD; PO_4	Pb	↑ plant biomass (root elongation) ↑ Pb uptake (shoot)	Sheng et al. (2008a)
B. napus	*Pseudomonas putida* UW4	ACCD	Ni	↑shoot biomass ↑ Ni tolerance	Farwell et al. (2007)
B. napus	*Pseudomonas putida* UW4, *Pseudomonas putida* HS2	ACCD High Ni-tolerance (*P. p* HS2)	Ni	↑ plant growth ↑ Ni yield	Farwell et al. (2006)
B. napus	Not determined	Cd-resistant	Cd	↑ [Cd] shoot	Sheng and Xia (2006)
B. napus, *L. esculentum*, *Z. mays*, *Sorghum sudanense*	*Bacillus* sp. J119	Metal-resistance; Antibiotic resistance; Biosurfactant; IAA; Siderophore	Cd	↑ shoot/root biomass of *L. esculentum*; ↑ [Cd] shoot (*B. napus*, *L. esculentum*); ↑ [Cd] root (*B. napus*, *L. esculentum*, *Z.mays*)	Sheng et al. (2008b)
Heliathnus annuus	*Bacillus weihenstephanensis* SM3	IAA; PO_4; Cu, Ni, Zn mobilization	Cu, Ni, Zn	↑ plant biomass ↑ Cu, Zn uptake	Raj kumar et al. (2008)
H. annuus	*Pseudomonas fluorescens*	IAA; Siderophore	As	↑ shoot biomass; ↑ [As] shoot; ↑ phloem fluxes	Shilev et al. (2006)

(continued)

Table 3 (continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
Leptochloa fusca (L.) Kunth	*Pantoea stewartii* ASI11, *Enterobacter* sp. HU38, and *Microbacterium arborescens* HU33 PGPE	Metal-resistance	U, Pb	↑ plant biomass and root length ↑ chlorophyll content ↑ U and Pb uptake	Ahsan et al. (2017)
Lycopersicon esculentum	*Pseudomonas* sp. RJ10, *Bacillus* sp. RJ16	IAA; Siderophore; ACCD; Cd, Pb mobilization	Cd, Pb	↑ plant biomass and root length ↑ Cd, Pb uptake	He et al. (2009)
Medicago sativa	*Pseudomonas fluorescens* (strains Avm, U), *Rhizobium leguminosarum* bv *phaseoli* (strains CPMex44, CPMex46), *Azospirillum lipopherum* (strains UAP40, UAP154)	Siderophore Growth promotion	Cu	↑ Cu, Fe root-shoot translocation (*CPMex46*, *Avm*)	Carrillo-Castañeda et al. (2002)
Nicotiana tabacum	*Sanguibacter* sp., *Enterobacter* sp., *Pseudomonas* sp.	nd	Cd	↑ plant biomass (*Sanguibacter* sp S-d2 and consortia) ↑ Cd translocation ↑ Cd, Fe uptake	Mastretta et al. (2009)
Noccaea caerulescens	*Arthrobacter* sp. Ncr-1, *Microbacterium* sp. Ncr-8 PGPE	IAA; ACCD	Ni	↑ root shoot length biomass ↑ Ni, uptake together with that of Fe, Co, and Cu	Visioli et al. (2015b)
Orychophragmus violaceus	*Bacillus subtilis, B. cereus, Flavobacterium* sp., *Pseudomonas aeruginosa*	Zn-tolerance	Zn	↑ plant biomass and root length ↑ Zn solubilization and uptake	He et al. (2010b)
Pteris vittata	*Rhodococcus* sp. TS1, *Delftia* sp. TS33, *Comamonas* sp. TS37, *Delftia* sp. TS41, *Streptomyces lividans* sp. PSQ22	As-reducing	As	↑ plant biomass ↑ As uptake ↑ As solubilization ↓ As leaching	Yang et al. (2012)
Populus deltoides	*Agrobacterium radiobacter*	As-tolerance; IAA; Siderophore	As	↑ plant biomass ↑ chlorophyll, enzymatic activity ↑ [As] root, stem, leaf ↑ As uptake and translocation	Wang et al. (2011)
Ricinus communis	*Pseudomonas* sp. PsM6, *P. jessenii* PjM15	IAA; Siderophore; ACCD; Cu, Ni, Zn mobilization	Cu, Ni, Zn	↑ plant biomass ↑ Zn translocation and uptake	Rajkumar and Freitas (2008a)

(continued)

Table 3 (continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
Salix caprea	*Agromyces* sp. AR33, *Streptomyces* sp. AR17	nd	Cd, Zn	↑ soil extractable-Cd/Zn; ↑ plant growth ↑ Cd/Zn uptake	Kuffner et al. (2008)
Salix viminalis and *S. alba* x *alba*	*Rahnella* sp., *Sphingobacterium* sp., *Caulobacter* sp., *Curtobacterium* sp., *Pseudomonas* sp.	IAA; ACCD	Cd, Zn	↑ Twig biomass, ↑ Cd/Zn extraction potential (with *Rahnella* sp.)	Janssen et al. (2015)
Sedum alfredii	*Burkholderia* sp. D54	IAA; Siderophore; ACCD; PO_4	Cd, Pb, Zn	↑ plant biomass; ↑ [Cd] shoot and root; ↑Cd, Pb, Zn uptake	Guo et al. (2011)
S. alfredii	*Sphingomonas SaMR12* PGPE	Promote antioxidative enzymes	Zn	↑ plant biomass ↑ root surface area ↑ exudation of oxalic acid ↑ [Zn] root and shoot ↓ ROS damage	Chen et al. (2014a)
S. alfredii	5 bacterial strains (unidentified)	nd	Cd, Cu, Pb, Zn	↑ plant biomass ↑chlorophyll and nutrient content ↓ Cd, Cu, Pb, Zn toxicity ↑ Cd, Cu, Pb, Zn uptake from contaminated water	Xiong et al. (2008)
S. alfredii	*Buttiauxellasp. SaSR1* PGPE	Promote antioxidative enzymes	Cd	↑ plant growth ↑ chlorophyll contents ↑ [Cd] shoot and root	Wu et al. (2018)
S. alfredii	*Burkholderia cepacia*	nd	Cd, Zn	↑ plant biomass; ↑ [Cd/Zn] shoot; ↑ metal tolerance; ↑ $[Cd/Zn]_{leaf}$: $[Cd/Zn]_{root}$ ratio	Li et al. (2007)
Sedum plumbizincicola	*Phyllobacterium myrsinacearum* RC6b	Metal-resistance; Metal mobilization; IAA; Siderophore; ACCD; PO_4	Cd, Pb, Zn	↑ plant biomass ↑ [Cd, Zn] root and shoot	Ma et al. (2013)
Solanum nigrum L.	*Pseudomonas* sp. Lk9 PGPE	Biosurfactants; siderophores; organic acids	Cd, Cu, Zn	↑ Fe and P mineral nutrition ↑ metal availability ↑ organic acids secretion ↑ soil microbial biomass C ↑ plant biomass ↑ [Cd],[Cu], [Zn] uptake	Chen et al. (2014b)

(continued)

Table 3 (continued)

Plant species	Bacterial strain	Characteristics	Metal	Effect	Reference
S. nigrum	*Serratia* sp. RSC-14 PGPE	IAA; PO_4; Cd-tolerance	Cd	↑ plant biomass ↑ chlorophyll contents ↑ [Cd] uptake	Khan et al. (2015)
Sorghum bicolor, Z. mays	*Bacillus subtilis, B. pumilus, Pseudomonas pseudoalcaligenes, Brevibacterium halotolerans*	nd	Cu, Cr, Pb, Zn	↑ plant biomass (with inoculum mixture); ↑ shoot biomass (*Br. h*, *P. p*); ↑ solubility Cr, Cu (*B. s*, *B. p*); ↑ [Cu, Cr, Pb, Zn] shoot on Cu-rich soil; ↑ [Cr]shoot on Cr-rich soil	Abou-Shanab et al. (2007)
S. bicolor	*Pseudomonas monteillii*	nd	Cd	↑ plant biomass ↑ Cd uptake	Duponnois et al. (2006)
Thlaspi caerulescens	Mixed inoculum: *Microbacterium saperdae*, *Pseudomonas monteilii*, *Enterobacter cancerogenes*	nd	Zn	↑ $[Zn]_{\text{water-soluble}}$; ↑ [Zn] shoot	Whiting et al. (2001)
Thlaspi caerulescens	Mixed inoculum: *Bacillus pumilus* and *Micrococcus* sp.	Ni-resistant	Ni	↓$[Ni]_{\text{water-soluble}}$; ↑plant biomass; ↑Ni yield	Aboudrar et al. (2013)
Trifolium arvense	*Pseudomonas azotoformans* ASS1 PGPE	IAA; Siderophore; ACCD; PO_4; resistance to heavy metals, drought, salinity, antibiotics and extreme temperature	Cu, Ni, Zn	↑ chlorophyll content ↑ [Cu], [Ni], [Zn] uptake ↓ ROS damage	Ma et al. (2017)
Trifolium repens Linn.	Bacterial strain mix (*Bacillus cereus*)	Metal-tolerance; IAA	Cd	↑ plant biomass and phytoextraction	Azcón et al. (2009)
Trifolium hybridum, Alopecurus pratensis, Poa pratensis, Hordeum violaceum, Ranunculus kotschyi, Cerastium sp.	*Bacillus megaterium* var. *phosphaticum*	Nutrient-solubilization; Pathogens control	B, Ni, Mn, Pb, Zn	↑ Ni, Pb, Fe, Zn, Na, B desorption from the soil ↑ Pb, Ni, B, Mn, Zn uptake	Gullap et al. (2014)
Zea mays	*Pseudomonas aeruginosa, Pseudomonas fluorescens, Ralstonia metalidurans*	Siderophore	Cr, Pb	↑ Cr, Pb exchangeable fraction in the soil (*P. aeruginosa*) ↑ [Cr, Pb] shoot	Braud et al. (2009)

Ni-tolerant bacteria in the rhizosphere was correlated with an increase in soil Ni availability (Becerra-Castro et al. 2009). Álvarez-López et al. (2016a) confirmed higher densities of Ni-tolerant bacteria associated with the Ni-hyperaccumulators *O. serpyllifolia s.l.* but observed significant differences in this selective enrichment amongst different plant populations across the Iberian Peninsula.

In the same way, studying endophytic bacteria associated with *N. goesingense*, Idris et al. (2004) found that for eight endophytes out of 22 strains, the minimum inhibitory concentration was 5 mM; the remaining isolates were able to tolerate 10 to 12 mM Ni. In general, endophytes tolerated larger concentrations of Ni than rhizosphere bacteria, indicating an adaptation of the endophytic microflora to the large heavy metal concentrations present in *N. goesingense* shoots. In another study, several bacterial endophytes were isolated from the hyperaccumulator *Sedum plumbizincicola*, five isolates of which exhibited metal resistance and were able to grow in a medium supplemented with 100 mg L^{-1} of Cd or Zn (Ma et al. 2015). In their study, Barzanti et al. (2007) isolated endophytic bacteria from the Ni-hyperaccumulator *O. bertolonii*. Most of the bacterial isolates were resistant to 5 mM Ni. Interestingly, in stem and lower root tissues, two isolates of *Curtobacterium* and one of *Bacillus* were found to be resistant up to 15 mM Ni. In fact, although Ni concentrations are three times higher in leaves than in stem, most of the Ni present in the leaf is likely stored intracellularly in the vacuoles, whereas in stem and root tissue it is mainly present in xylematic solutions. Consequently, xylem-inhabiting bacteria could likely cope with the higher free Ni concentrations than those living in parenchymatic tissues of leaf (and root) tissues.

The screening of bacterial isolates associated with metal (hyper)accumulating plants has led to the identification of candidate inoculants for application in phytomining. Bacterial strains are commonly characterized for the presence of PGP traits, such as the capacity to produce phytohormones (IAA), to solubilize inorganic P or K, to fix atmospheric N_2, to release siderophores, or for their ACC activity. Most bioaugmentation studies have evaluated the effects of re-inoculating host plants with their associated isolates (Abou-Shanab et al. 2003a, 2006; Li et al. 2007; Cabello-Conejo et al. 2014). However, the specificity of these plant-bacterial combinations is not always clear, and some inoculants have been shown to have beneficial effects on a wide range of plant hosts (Grandlic et al. 2008; Ma et al. 2011a; Becerra-Castro et al. 2012). The main objective of these studies has been to improve metal yields during phytoextraction. In this technique, a simple improvement in biomass can result in an increase in overall TE removal (Sessitsch et al. 2013). In a meta-analysis of the literature carried out by Sessitsch et al. (2013), based on more than 70 publications and 738 individual cases, the authors found that bacterial inoculation was generally more successful in promoting plant growth and biomass production (60% of total cases analyzed) than in influencing shoot TE accumulation (only 30% of cases showed a significant increase in shoot TE concentration, and 16% a decrease in TE concentration).

Ma et al. (2009a, b) reported that inoculation with PGP rhizobacteria (*Psychrobacter* sp. SRA1, *Bacillus cereus* SRA10, and *Achromobacter xylosoxidans* strain Ax10) improved the growth of *Brassica juncea* and *B. oxyrrhina*, enhanced soil Ni availability, and increased Ni accumulation in both species. Similarly, Rajkumar and Freitas (2008b) observed that inoculation with the PGP rhizobacterial strains *Pseudomonas* sp. PsM6 and *P. jessenii* PjM15, isolated from an ultramafic soil in *R. communis*, caused an increase in above-ground biomass, mainly due to IAA production and phosphate solubilization, and consequently enhanced the phytoextraction efficiency. Zaidi et al. (2006) demonstrated that inoculation with *Bacillus subtilis* strain SJ-101 not only stimulated the growth and Ni accumulation in *B. juncea*, but also protected the plant from Ni toxicity. Various authors also reported increases in Ni uptake by *B. juncea* and other non-hyperaccumulating plant species (*B. napus*, *Ricinus communis*, *Poa pratensis*, *etc.*) after bacterial inoculation

(Rajkumar and Freitas 2008a, b; Kumar et al. 2009; Gullap et al. 2014). With regards to Ni-hyperaccumulating species, Abou-Shanab et al. (2003b) showed that the strain *Microbacterium arabinogalactanolyticum* isolated from the rhizosphere of *O. chalcidica* significantly increased availability of Ni in the soil and enhanced Ni accumulation by *O. chalcidica*. In agreement with these results, a posterior study with *A. murale* grown in artificially Ni-polluted soils demonstrated that inoculation with selected rhizobacteria strains increased Ni extraction from the soil and Ni uptake by *O. chalcidica* (Abou-Shanab et al. 2006). These authors considered the presence of such rhizobacteria to be an important factor influencing metal hyperaccumulation. Becerra-Castro et al. (2013) used two strains of *Arthrobacter* (SBA82 and LA44), as an inoculum for *O. serpyllifolia* growing in ultramafic soil, and observed an increase in plant biomass and shoot Ni concentrations. Durand et al. (2016) isolated PGPR from the rhizosphere soil of two hyperaccumulator plant associations: *B. tymphaea–N. tymphaea* (NB) and *B. tymphaea–O. chalcidica* (AB), both being characteristic of an ultramafic outcrop in Greece. The screening of the isolates revealed two PGPR strains (AB30 and NB24), which were affiliated to *Variovorax paradoxus* and that were used to inoculate the same plant associations growing in mesocosms. Biomass (root and shoot), shoot Ni uptake, and Ni removal by the *B. tymphaea–N. tymphaea* plant association inoculated with strain NB24 was significantly higher than that of the respective non-inoculated association.

It has been demonstrated that some metal-resistant PGPE can also improve metal uptake and accumulation in plants. Ma et al. (2011b) found that inoculation with the PGPE *Pseudomonas* sp. A3R3 significantly increased Ni content in *O. serpyllifolia*. However, there are some opposing viewpoints suggesting that the presence of metal-resistant endophytes decreases metal uptake and accumulation in plants. For instance, Lodewyckx et al. (2001) found that the inoculation of Ni-resistant *Herbaspirillum seropedicae* into *Lolium perenne* resulted in a significant decrease of the Ni concentration in the roots (11%) as well as in the shoots (14%). Similarly, Madhaiyan et al. (2007) reported that inoculation with the endophytic bacteria *Methylobacterium oryzae* and *Burkholderia* sp. reduced the Ni uptake and accumulation in the roots and shoots of tomato plants. This effect was attributed to increased metal biosorption and bioaccumulation by bacterial strains (Ma et al. 2011a).

In 2017, Ma et al. showed that the inoculation of the endophyte *Pseudomonas azotoformans* ASS1 enhanced chlorophyll content and growth of *Trifolium arvense* growing in an ultramafic soil, and Cu, Zn, Ni uptake by this plant. Indeed, the inoculation significantly improved accumulation, total removal, the bio-concentration factor, and biological accumulation coefficient of Ni, whereas decreasing the translocation factor of Cu. This increase of metals uptake was related to alleviation of damage by reactive oxygen species (ROS). Inoculation of the endophyte enhanced proline and antioxidant enzymes, and decreased malondialdehyde (MDA) content in plant leaves. These results are corroborated by the recent work of Wang et al. (2020) showing that the endophytic bacterium *Sphingomonas* SaMR12 alleviates Cd stress in the Cd- ± Zn-hyperaccumulator *Sedum alfredii* through regulation of the GSH-AsA cycle and antioxidative enzymes. In this work, the authors point out that the endophyte strain inoculation decreased shoot hydrogen peroxide (H_2O_2) content by up to 38% and MDA content by up to 60%, but also reduced proline content by 7–30% in shoots and 17–32% in roots, compared to the levels observed in non-inoculated plants. Additionally, the inoculation promoted the activities of superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase and facilitated the relative gene expression levels of dehydroascorbate reductase and glutathione reductase involved in the glutathione (GSH)-ascorbic acid cycle. Those endophyte strains that may alleviate ROS damage in plants tissues seem to be of great interest for phytomining applications, and because the bacterial mechanisms involved are not yet well known, this opens interesting research opportunities.

Benefits to plants of mycorrhizal inoculation in highly stressed and nutrient-poor environments are well documented. Inoculation with arbuscular mycorrhizae in TE-polluted mine tailings has proven particularly effective in enhancing plant survival and performance during restoration (Orłowska et al. 2011; Kohler et al. 2015; Maltz and Treseder 2015). However, fewer studies have found inoculating with mycorrhizal fungi species or strains to enhance metal uptake and accumulation. In fact, until the early 2000s, hyperaccumulating plants were generally considered non-mycorrhizal (Gonçalves et al. 1997; Leyval et al. 1997; Pawlowska et al. 2000). Since then, several studies have found that hyperaccumulators can form symbioses with arbuscular mycorrhizal fungi (AMF). *Pteris vitatta*, an As-hyperaccumulating fern, tended to show higher mycorrhizal colonization on As-polluted soil. The authors suggested that enhanced P uptake could be linked to increased As absorption and transport (Leung et al. 2007). Mycorrhizal colonization has been observed in *Noccaea praecox*, a Cd- and Zn-hyperaccumulator of the *Brassicaceae* family (generally considered to be non-mycorrhizal) growing on metal-polluted soil in Slovenia (Vogel-Mikus et al. 2005). *Berkheya coddii* was the first Ni-hyperaccumulating plant in which the presence of arbuscular mycorrhizal (AM) symbiosis was reported (Turnau and Mesjasz-Przybylowicz 2003). Arbuscular mycorrhizal fungi isolated from ultramafic soil significantly enhanced growth and survival of the Ni-hyperaccumulator *B. coddii* where growing in the same soil. AMF inoculation reduced shoot and root Ni concentrations (Orłowska et al. 2011). However, due to the higher biomass, the total Ni yield was up to 20 times higher in mycorrhizal plants compared to the non-mycorrhizal ones. The authors suggested that AMF enhancement of Ni uptake may help improve nickel phytomining techniques.

4.2 Improving Soil Metal Removal Using Metal-Mobilizing Microbial Strains

Metal-hyperaccumulating plant species have an extraordinary capacity for TE accumulation. Many authors have considered the possibility that such species actively mobilize TE from non-labile soil metal pools, as a means of increasing the labile concentrations and consequently plant uptake and accumulation of TE. In accordance, several studies have shown a higher labile fraction of hyperaccumulated metals, such as Ni, at the rhizosphere of these plants compared to non-accumulating plant species or the surrounding non-vegetated soil (Puschenreiter et al. 2005). However, most authors have concluded that this effect is not a result of specific metal-mobilizing mechanisms employed by hyperaccumulators. It seems more likely, therefore, that the pronounced depletionof TE in the rhizosphere, caused by the high rate of uptake of the element from the soil solution, induces their replenishment from the less-mobile fraction to the mobile pools, and maintains a sustained high metal concentration in the soil solution. Root activity of *N. goesingense* has been proposed to participate in an enhanced dissolution of Ni-bearing minerals, contributing to higher Ni uptake (Puschenreiter et al. 2005). Similarly, mineralogical studies showed the presence of smectite in the rhizosphere of *O. serpyllifolia*, which was attributed to more intense weathering of Ni-rich ferromagnesium minerals (chlorite, serpentine) and an increase in labile Ni (Kidd et al. 2009). Chardot-Jacques et al. (2013) found that growth of the Ni-hyperaccumulator *B. emarginata* increased the dissolution of chrysotile (a silicate from the serpentine group having low Ni solubility). These authors suggested that the high Ni uptake by the plant causes a decrease in water-soluble Ni, which in turn induces chrysotile dissolution. Whether this phenomenon is plant- or microbial-induced, or the result of complex plant-microbial interactions, is unknown. It is well established that microbial transformation of soil minerals leads to the solubilization of metals together with essential nutrients, and to modification of their form and distribution in the solid phase (Quantin et al. 2002). Soil metal availability greatly influences the success of phytomining and its long-term sustainability, and bioaugmentation with metal-mobilizing, plant-associated bacteria could enhance the viability of this technique (Kidd et al. 2009).

Microbes are intimately associated with the biogeochemical cycling of metals. Microbial activity can result in metal mobilization or immobilization depending on the mechanism involved and the microenvironment where the organism(s) are located (Gadd 2010; Violante et al. 2008; Ehrlich and Newman 2009) (Fig. 2). Some metal-tolerant bacterial strains associated with (hyper)accumulating plants have been shown to mobilize metals in soils, and consequently increase the phytoavailable metal fraction in the soil, as well as plant uptake and accumulation. Muehe et al. (2015) found *Arabidopsis halleri* accumulated 100% more Cd and 15% more Zn when grown on natural Cd-polluted soil than on the same soil that had undergone gamma-irradiation. Gamma irradiation affected neither plant growth nor soil metal availability, but strongly altered the composition and density of the soil microbial community. Bacteria can modify TE mobility and bioavailability through several mechanisms: the release of chelating agents (such as organic acids, phenolic compounds, and siderophores), and acidification or redox changes in the rhizosphere (Lloyd 2003; Glick 2010). Sessitsch et al. (2013) reviewed the potential mechanisms for microbial

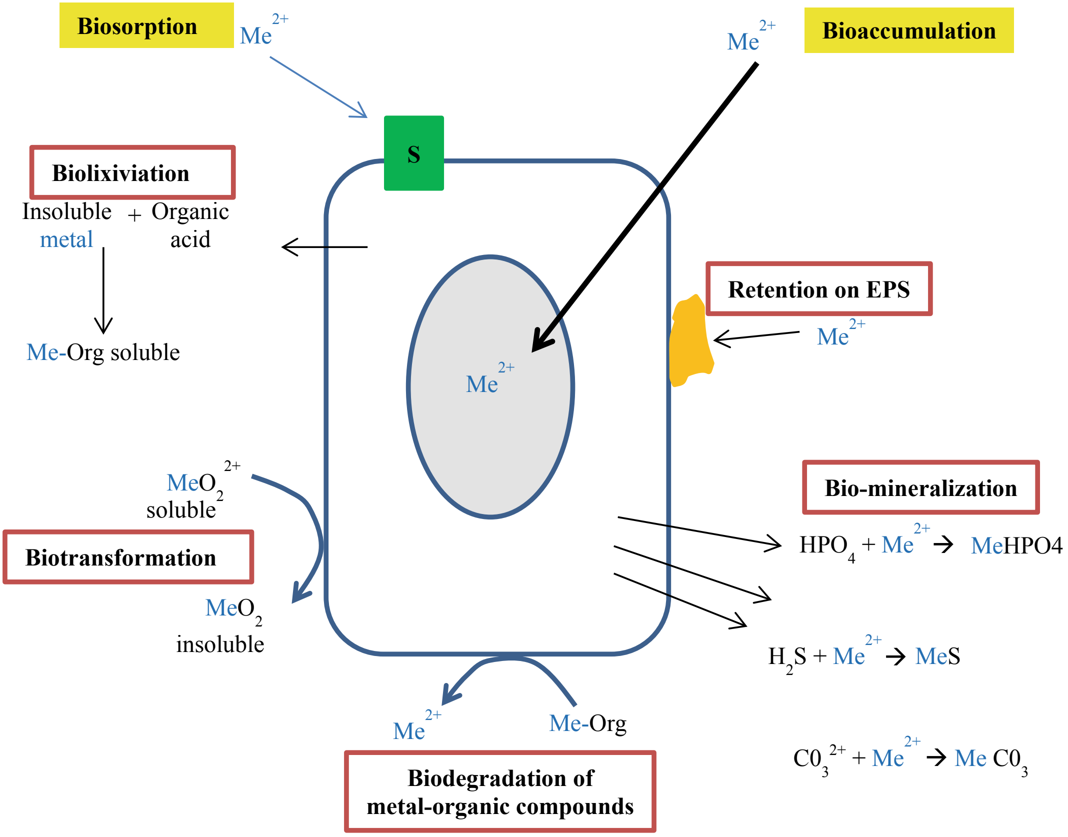

Fig. 2 Interactions between metals and bacteria (adapted from Ledin 2000). S corresponds to the reactive groups present on the bacterial cell wall. Me^{2+} represents a cation metallic. Org corresponds to an organic compound

effects on bioavailability in the rhizosphere environment. Sorbed, precipitated, and occluded TE can be solubilized by acidification, chelation, and ligand-induced dissolution. To date, two groups of bacterially produced natural chelators are known: organic acids and siderophores. Low-molecular mass carboxylic acids can play an important role in the chemical attack on minerals, providing protons as well as a metal-chelating anion (Jacobs et al. 2002; Huang et al. 2004; Lian et al. 2008).

Oxalic acid can leach metals that form soluble oxalate complexes, including Al and Fe (Strasser et al. 1994). Bacteria producing TE-chelating organic acids, such as citric, oxalic or acetic acid have been shown to mobilize various TE in soil (Becerra-Castro et al. 2013). As mentioned above, siderophores form high-affinity complexes with Fe(III), but can also form complexes of lower stability with other TE (Al, Cd, Cu, Ga, In, Ni, Pb and Zn), thus affecting their bioavailability (Schalk et al. 2011; Rajkumar et al. 2012; Sessitsch et al. 2013). Plants can then take up metals from siderophores *via* various mechanisms, such as chelate degradation, direct uptake of siderophore–metal complexes, or by a ligand exchange reaction (Schmidt 1999). Braud et al. (2009) reported that inoculating soils with siderophore-producing *P. aeruginosa* significantly increased the concentrations of bioavailable Cr and Pb compared with non-inoculated controls. Enhanced heavy-metal uptake was correlated with the increased production of siderophores. Siderophore-producing bacteria that are present in metal-polluted or metal-enriched soil, and their interaction with hyperaccumulators, could be used in microbial-assisted phytoextraction or phytomining (MAP). Several siderophore-producing bacterial strains associated with plants, such as the non-hyperaccumulator *Brassica juncea*, and the hyperaccumulators *N. goesingense*, *O. bertolonii, and O. chalcidica*, have been isolated and characterized from metal-rich soils. They belong to different species such as *Staphylococcus* sp., *Microbacterium* sp., *Pseudomonas* sp., *Curtobacterium* sp., *Bacillus* sp., *Arthrobacter* sp., *Paenibacillus* sp., *Leifsonia* sp., *Methylobacterium mesophilicum*, *Methylobacterium extorquens*, *Methylobacterium* sp., *Burkholderia terricola*, *Okibacterium fritillariae*, *Rhodococcus fascians* and *Rhodococcus* sp. (Idris et al. 2004; Barzanti et al. 2007). Becerra-Castro et al. (2013) evaluated the weathering capacities of, and Ni mobilization by, two rhizobacterial strains associated with the Ni-hyperaccumulators in *O. serpyllifolia s.l.* A minimal culture medium containing ground ultramafic rock was inoculated with either of two *Arthrobacter* strains: LA44 (indoleacetic acid [IAA] producer) or SBA82 (siderophore producer, PO_4 solubilizer, and IAA producer). Strain LA44 is a more efficient Ni mobilizer, apparently solubilizing Ni associated with Mn-oxides, a result that appeared to be related to the exudation of oxalate. On the other hand, strain SBA82 also led to the release of Ni and Mn, albeit to a much lower extent. In the latter case, the concurrent mobilization of Fe and Si reflects preferential weathering of Fe-oxides and serpentine minerals, possibly related to siderophore production capacity of the strain. However, some conflicting results showed that the presence of siderophore producers decreased the uptake of metals by plants. Siderophores produced by *Pseudomonas* sp., *Serratia marcescens* and *Streptomyces* sp. had either no effect or negatively affected Zn and Cd uptake by *Salix caprea* (Kuffner et al. 2010). These contrasting effects suggest that mechanisms underlying metal uptake are largely plant dependent, but that the efficiency of siderophore producers to either mobilize or immobilize heavy metals from soils is also dependent on several factors, such as binding form of the heavy metals present and charge of the siderophores, as well as pH of the soil and its mineral composition and organic content.

The influence of organic matter content in soil on metal availability and uptake by plants has been widely studied. It was reported that metal adsorption onto soil constituents declined with a decreasing organic matter content in the soils (Zeng et al. 2011). Moreover, dissolved organic matter in soils may increase the mobility and uptake of heavy metals by plant roots (Du Laing et al. 2008). In fact, organic matter decomposition appears to be one of the most important microbial activities in the rhizosphere, and it is well known that microbes, mainly bacteria and fungi, utilize a wide spectrum of organic compounds such as sugars, organic acids, and amino acids to more complex molecules such as cellulose, pectin, lignin, lignocellulose, chitin, and starch. Consequently, organic matter decomposition by soil microorganisms releases metals such as Fe, Mn, Zn, Cu, Mo, Ni, Co and Se, all of which are typically found in organism tissues.

5 Final Remarks and Future Directions

It seems clear that the rhizosphere microbiome plays an important role in plant TE bioaccumulation, as well as endophytic microbes, given experimental evidence suggesting that by reshaping this microbiome, we can further enhance the efficiency of phytoextraction and phytomining. However, the efficacy of plant-associated bioinoculants is dependent on a complex array of interacting factors: plant-microbe specificity, soil type and properties, TE concentration and type (mono- or poly-metallic), proliferation and survival of the inoculant, etc. Moreover, from the literature it can be seen that inoculation methods differ greatly among studies (inoculation of seed/plant/soil, frequency/timing of inoculation events, bacterial cellular densities, etc.) and these aspects are likely to influence whether or not a beneficial microbial-induced effect is observed.

Bioaugmentation can be challenged by strong competition encountered in the soil when a selected microbe is introduced. Also, the selected host plant species may not necessarily be compatible with the inoculated bacterial or fungal strain, since these are not naturally selected for by the host (Thijs et al. 2016). Indeed, the importance of effective root colonization in beneficial plant-microbe interactions is underlined by several studies as contributing towards the success of bioaugmentation (Lugtenberg and Dekkers 1999). Moreover, inoculation methods should be optimized in order to enhance success of the introduced microorganisms (Álvarez-López et al. 2016b). Álvarez-López et al. (2016b) and Pereira et al. (2015) showed that the size of the inoculum applied in the soil rhizosphere, and the mode of application (seed or soil inoculation), are important factors that should be taken into account when planning MAP strategies. Endophytes do not have to compete with the great abundance and diversity of soil microorganisms that may enhance their chances of establishing a stable and active population. To date, the field of MAP has focused on the use of individual bioinoculants. However, promising results have been found when using mixtures or consortia of different PGP-strains with complementary actions. Visioli et al. (2015a) reported that co-inoculating the Ni-hyperaccumulator *Noccaea caerulescen*s, when growing in serpentine soil with two root endophytes belonging to the *Arthrobacter* and *Microbacterium* genera, had a more positive effect on plant growth, soil Ni removal, and Ni translocation, than when inoculated individually. Both strains were strong IAA producers and presented ACC deaminase activity. Moreira et al. (2016) showed the benefits of combined inoculation of AMF and PGPR for the growth of maize as an ‘energy-crop’ in metal-polluted soils and their potential application in phytomanagement strategies. Moreover, it could be also of great interest to select PGPR and/or PGPE with the capacity to tolerate or resist high concentrations of different pollutants, with the aim to improve phytoremediation in multi-metal-contaminated soils. Although the mechanisms of plant-growth promotion between endophytes and rhizosphere bacteria can be very similar, it seems that most research work has been done in rhizosphere bacteria, assuming that the mechanism is similar in endophytes, even if the rhizosphere environment is quite different from that of internal plant tissues. Therefore, it is possible that in the future new mechanisms of PGP, unknown in rhizosphere bacteria, may be discovered in bacterial endophytes.

Ultramafic rocks occupy <3% of Earth's land surface and these outcrops have the potential to provide multiple ecosystem services and contribute to producing renewable raw materials and energy (i.e. agromining). In the same way, polluted soils are an ever-increasing environmental concern due to increased industrialization. Phytoremediation coupled with bioaugmentation could be a solution towards recovering soil quality, underlining the role of endophytes and rhizosphere microbes associated with metal accumulation in hyperaccumulator plants.

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The Integration of Hyperaccumulator Plants into Mine Rehabilitation in the Asia Pacific Region

Peter D. Erskine, Gavin Lee, Bruno Fogliani,
Laurent L'Huillier, and Stéphane McCoy

Abstract

The identification and use of hyperaccumulator plants in mining projects has been recognized as an important component of life of mine planning at several sites around the world. Mine planning that includes hyperaccumulator plants requires operators to maximize the biological resources of a site by discovering and utilizing these unusual plant species at the early stages of a mine's development. These locally adapted hyperaccumulator species can then be used for rehabilitation in and around the mine area or be utilised for their potential to extract ecocatalysts and metals. These opportunities should be more widely explored so that these unique plants can become an integrated and valuable part of the mining process. This chapter concentrates on the experiences of integrating hyperaccumulating plants into mine rehabilitation projects in Indonesia and New Caledonia to highlight some of the opportunities and challenges encountered when attempting to incorporate these species into the mining cycle.

1 Introduction

Metal hyperaccumulating plants have the potential to add significant value to the metal mining cycle and thus create a more sustainable minerals industry. With metal reserves declining in quality (ICMM 2012), and with cost and technical challenges being faced in the processing of low-grade ores, agromining techniques (i.e. the process of cultivating, harvesting and processing metal hyperaccumulating plants to produce metals and ecocatalysts), in concert with conventional mineral ore extraction and processing, could improve the efficiency of recovery of metal from existing ore reserves. The use of the metal enriched biomass from agromining to derive Ni, Mn and Zn salts using a 'green technology' appears to be an opportunity for mining companies to utilise sustainable approaches to mineral extraction (Losfeld et al. 2012). The unique opportunity provided by hyperaccumulator plants to naturally bio-chemically concentrate

P. D. Erskine (✉)
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, Australia
e-mail: p.erskine@uq.edu.au

G. Lee
OceanaGold Corporation, Macraes Operation, Otago, New Zealand

B. Fogliani
Institute of Exact and Applied Sciences (ISEA), University of New Caledonia, BP R4, Nouméa Cedex 98851, New Caledonia

L. L'Huillier
New Caledonian Agronomic Institute, BP 73 Port Laguerre, Paita 98890, New Caledonia

S. McCoy
Vale New Caledonia, BP 218, Nouméa 98845, New Caledonia

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_13

and purify metal from ultramafic soils (Kelly et al. 1975; Jaffré et al. 1976, 1979; L'Huillier and Edighoffer 1996; Chaney et al. 2007) and increase recovery of metal from reserves, can also facilitate multiple additional benefits including:

- Increased metal productivity per unit of biodiversity loss,
- Improved soil fertility in mined out soils hence available for a wider range of metal sensitive plants,
- Remediation of contaminated wastes materials,
- Reduced erosion on mined out soils and wastes, and
- A more productive and viable post-mining land use.

These benefits are important and meaningful contributions to mitigating environmental and social impacts from modern mining operations. The use of metal hyperaccumulator plants to increase mineral recovery prior to conventional strip mining and after mine extraction, can also provide a range of low-cost opportunities that facilitate generation of a sustainable vegetation cover with biodiversity values.

This chapter assesses some of the processes required for the seamless integration of hyperaccumulator plants into mine site rehabilitation and agromining. It covers the multiple stages of the mining cycle and addresses important issues that need to be considered when approaching a new mineral ore body, particularly in the highly biodiverse tropical regions. It then uses examples from the Asia Pacific region to illustrate the process of how hyperaccumulator discovery and the mining cycle can be combined.

2 The Mining Cycle and Agromining Development

The conventional mining cycle can generally be described by a number of specific but overlapping processes in which minerals are found and their quantity assessed (Exploration and Development), the means for extraction of the minerals identified, costed and a mechanism for funding the development established (Feasibility and Financing), the infrastructure is prepared for extraction and processing of the minerals (Construction), the extraction, processing and sale of the mineral product into the market (Operations), rehabilitation of disturbed land and finally the cessation of extraction and processing (Closure).

If the benefits of hyperaccumulator plants are to be attained, then processes need to be developed that identify the potential of these species, demonstrate their effectiveness, ensure their accessibility/viability and realize their potential to contribute to the mining cycle. There are three steps in this agromining process. *Screening and Prioritization* is to identify local variants of hyperaccumulator species, and which species have the potential to be used in agromining. The *Conservation and Propagation* stage ensures the long-term availability and viability of the selected hyperaccumulator species as assets. Finally, the *Recovery and Restoration* stage enables the materialisation of the asset and hence the creation of value for the Project.

Figure 1 presents the *Mining Cycle* and integration of agromining development within the cycle. The stages of agromining development within the *Mining Cycle* remain fluid and can vary significantly from the conceptualization of Fig. 1. However, the commencement of the *Screening and Prioritization* stage to identify potential hyperaccumulating species as early as possible within the *Mining Cycle* ensures that subsequent stages can be appropriately planned later in the *Mining Cycle*. This increases the possibility of the strategic objectives of agromining (i.e. maximizing the recovery of metals from existing ore) being met and the associated benefits being realized.

Agromining extends the recovery of target metals not accessible by conventional mining (e.g. ultramafic soils below cut-off grades) that often make up a large proportion of mining leases. It provides an opportunity to involve locally communities in a 'metal farming' cooperative supported by industry for the required research and development and off-take of the harvested

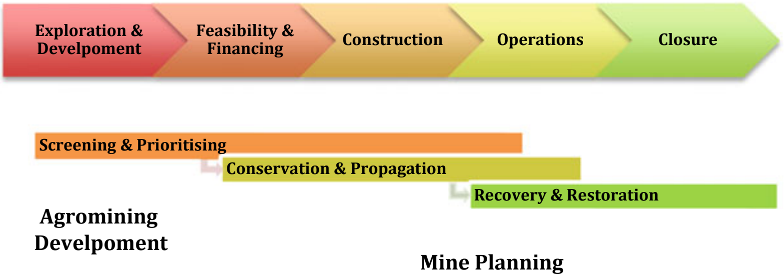

Fig. 1 The relationship between mine planning process and the steps required for agromining

bio-ore. The development of hyperaccumulating vegetation that reduces the metal content of the soil through agromining harvests over time will improve fertility by allowing more sensitive species to establish biomass because of the decrease in metal availability. Such a role can be regarded as a significant agronomic or ecosystem service if this is the aim of the rehabilitation. On the contrary, reducing concentrations of metals in environments where the flora is highly adapted to these local conditions may also provide habitat for invasive species (B. Fogliani, pers. obs.).

3 Screening and Prioritizing

The screening and prioritising phase should start as early as possible in the *Mining Cycle*. Where the spatial extent of nickel ore bodies is significant, and multiple pit development occurs over a temporal scale, it would be expected that screening could continue until well into the Operation Stage. Where a mining project has made commitments towards assessing biodiversity losses, it remains likely that flora surveys would be required to be ongoing and as such provide an opportunity for continuation of the screening and prioritizing phase.

3.1 Environmental Impact Assessments and Biodiversity Baselines

Environmental Impact Assessments (EIAs) are now a standard regulatory requirement for the mining industry. A relatively recent trend has been the emergence of EIAs for financing, where the regulatory framework is considered not rigorous enough to cover environmental and social risk adequately for investors. Institutional finance investment agencies, such as International Finance Corporation and Asia Development Bank, encourage their clients to make bold commitments towards management of environmental and social management. Such commitments then place a premium on the development of detailed EIAs. Recognition of the value of local Ni hyperaccumulator species in the EIA process through production and growth trials can provide further proof that the Project Owner is serious in achieving their stated commitments.

EIAs are generally conducted as part of the *Finance and Feasibility* stage, although planning for the EIA may take place in the *Exploration and Development* stage of the *Mining Cycle*. A key component to the planning of an EIA is the development of the biodiversity baseline. One of the initial steps in the generation of a biodiversity baseline is the desktop review. Where the flora of a particular location is already well understood, and the data are accessible—either through previous EIA studies or in regulatory or other open source databases—the species list can be cross-checked against known lists of hyperaccumulator species. Given that this is a relatively new field of research and the range of families known to contain hyperaccumulating species is already exceptionally diverse, any species list generated based on known hyperaccumulator species should be taken as a tentative baseline for further fieldwork to determine abundance and habitat preference. Floristic surveys conducted as part of the biodiversity baseline provides the opportune time to commence the screening process for hyperaccumulator plants. The benefits of integrating the screening in the flora surveys are as follows:

- Coverage: in general baseline biodiversity surveys endeavour to cover all habitats in the mining concession, including those areas that may contain deposits of sub-economic grade ore. These areas should also be recognised as a potential important source of hyperaccumulator species.
- Expertise: well-planned flora surveys usually include a botanist with local knowledge in order assist with field identification. Field identification of in situ hyperaccumulator species improves reliability and accuracy in the identification.
- In cases where EIAs and biodiversity baselines have already been conducted, screening for hyperaccumulator species can be conducted through targeted survey work. As with the flora surveys of the biodiversity baseline, it is recommended that the survey team include at least one botanist with local knowledge. Plant collections made during the flora survey (whether for the EIA or a targeted hyperaccumulator survey) should ensure that standard field records are maintained, including location of collections, geospatial coordinates, plant description, habitat description, notes on abundance and ecological role of target species, etc.

3.2 Screening

Field screening for candidate plant species to determine their metal content and hyperaccumulating potential is a relatively straightforward process. Concentrations of metals and other elements can be determined in the field or with collected leaves samples with a hand-held X-Ray Fluorescence (XRF) instrument (see Chapter "Tools for the Discovery of Hyperaccumulator Plant Species in the Field and in the Herbarium", Purwadi et al. in this book).

In the case of Ni, moistened samples of the identified plant, typically the leaf, but may include the roots or the phloem, are placed on indicator paper containing dimethylglyoxime (Reichen 1951) and a positive reaction (i.e. the appearance of pink coloration on the indicator paper) indicates Ni hyperaccumulation.

3.3 Prioritizing

Prioritizing hyperaccumulator species should be based on whether they occur locally, their metal hyperaccumulating properties, their habitat and their physiological properties. During the initial EIA a screening process should identify local native hyperaccumulator species and their habitats. Native species found on open exposed environments are preferred as they are more likely to match local exposed bioclimatic, geochemical and physiological conditions of post-mining surfaces. Species vary in their metal hyperaccumulating properties and priority should be given to species that have at least 1% foliar weight of either Ni or Mn (Losfeld et al. 2015a). Other physiological properties of priority are to have high biomass yield and be relatively straightforward to propagate by seeds, seedlings or cuttings. They should have ecological characteristics suitable for agromining, such as a tolerance to regular harvesting, so that foliage can be removed and reduce metal concentrations for rehabilitation. At the stage of prioritizing it is likely that only one or two of these criteria will be well enough understood for decision making on preferential candidate species. It is recommended that the list of species for the next stage, *Propagation and Conservation*, remain broad where uncertainty exists.

4 Conservation

The development of a list of priority hyperaccumulator species is the first step by the project owner in the recognition in the value of these species. The next phase in the integration of agromining in the *Mining Cycle* is to understand and assess the value of these species grown under exposed harvest conditions.

4.1 Conserving Priority Species

Looking broadly at hyperaccumulator species as components of the local biodiversity and assigning a conservation value for development planning is based on how the species is valued by people (local or elsewhere) or its particular functional importance. This can include rare or threatened species, species of cultural or social importance, species associated with important ecological processes or species of economic value (Gardner et al. 2013). In this context hyperaccumulator species have the potential to be recognized as having biodiversity value in at least one of the above-mentioned criteria.

Ni and Mn hyperaccumulator plant species are known to have a high degree of island endemism. In New Caledonia, 92 Ni hyperaccumulator species (previously 65) belonging to 21 families (Jaffré et al. 2013; Gei et al. 2020), also 70 Mn hyperaccumulator species belonging to 28 families (Losfeld et al. 2015a; Gei et al. 2020) have been reported. It appears likely that a similar order of magnitude of endemic species could be found through more consolidated survey work in the ultramafic geology of South East Asia. Given the economic potential for the exploitation of nickel ore from lateritic soils and the possibility of high levels of endemism from the 'strict' hyperaccumulator species for information on obligate versus facultative hyperaccumulators, it is necessary that the vulnerability and irreplaceability of the list of local hyperaccumulator species be assessed to determine their distribution and pinpoint micro-endemics found in future potential mine leaseholds (see Case Study on New Caledonia).

For project owners with biodiversity objectives such as *No Net Loss*, it will be important to develop specific actions for the conservation of hyperaccumulator species based on their assigned conservation value. Best practice in biodiversity management recommends the application of the mitigation hierarchy (i.e. Avoidance, Minimization, Restoration, Offsetting) as a basis for the development of these plans. Even where project owners have made no specific biodiversity objectives but recognize the value in hyperaccumulator plant species, the application of the mitigation hierarchy provides a sound basis for decision making for agromining development. Avoidance, minimization and offsetting for hyperaccumulator species all provide the benefit of preserving genetic diversity. By maintaining viable populations of hyperaccumulator species in situ, the project owner guarantees long-term stock (i.e. seeds, seedlings, cuttings) with greater resilience to pest and disease, and the capability to select specific individuals for propagation/restoration based on several traits deemed suitable for agromining including:

- Naturally high metal concentrations (>1%)
- Able to achieve rapid biomass growth (without a significant loss of target mineral content)
- The ability to grow in open conditions
- Easily propagated
- Can tolerate annual harvests
- Continue to produce the same amount of aerial biomass after agromining harvests

Conservation actions related to avoidance, minimization and offsetting are briefly described in the following, whilst *Recovery and Restoration* is covered after the section on *Propagation*.

4.2 Conservation Actions: Avoidance

Although it is unreasonable to assume that project owners will avoid clearing in the location of vicinity of economic ore reserves; site selection of supporting infrastructure, such as mine roads, laydown pads maintenance yards, fuel storage, can, with careful planning, avoid known stands of hyperaccumulator species in particular areas where other conservation needs are apparent (*ex:* rainforests and rivers). The key to careful planning is that information on hyperaccumulator plants is available at the time of decision-making. This fact reinforces the need to start the screening and prioritizing stage as early as possible.

Another form of avoidance is where a particular ore body may be only marginally feasible, either technically or financially, and the project owner decides to set aside this orebody, in order to assist in meeting biodiversity commitments. Setting aside such conservation areas provide a greater potential than other forms of avoidance by realizing the benefits of hyperaccumulator plant diversity which commonly occur in areas which contain high levels of the target minerals.

4.3 Conservation Actions: Minimization

Minimisation of the clearing of hyperaccumulator species typically occurs though implementation of operational activities, as opposed to avoidance during planning. Where avoidance is not feasible, or not 100% possible, and there will be a loss of hyperaccumulator species, ex situ conservation through collection of seeds and cuttings prior to clearing and supervision during land clearing activities is necessary to ensure losses are not greater than anticipated. It is recommended that land clearing procedures include management accountability for clearing, demarcation of areas to be cleared in the field and awareness for supervisors and equipment operators as to the clearing limits and the specific environmental sensitivities (i.e. the presence of hyperaccumulator species) beyond the limit.

Species salvaging, through the removal of seeds, seedlings and soils known to contain seed stocks of hyperaccumulator species, from areas designated for clearing provides valuable source material for propagation. Integrating the identification and collection of seedlings and parent trees of known hyperaccumulator species as part of the land clearing procedure, through the application of pre-clearing biodiversity surveys, is an efficient method for boosting seedling stocks. Where an abundance of one or more hyperaccumulator species is found in an area to be cleared, it may practical to delineate the area and remove the soil, as part of standard topsoil removal procedures, for direct return to areas to be trialled.

4.4 Conservation Actions: Offsetting

In the event that avoidance and minimisation have not adequately maintained viable populations of hyperaccumulator species with known agromining potential, project owners may commit to the development of an offset either based on hyperaccumulator species richness, or as part of a broader strategy for mitigating biodiversity losses and reinforcing existing populations and ex situ conservation. As the last step on the mitigation hierarchy, offsets are a risky strategy and are likely to only be applied where there is a regulatory and/or financial investment requirement to do so. However, offsets do present a significant opportunity to maintain genetic diversity of hyperaccumulator species, if a ‘like-for-like’ approach is taken in the selection of offset sites. Offsite conservation of hyperaccumulator species is particularly important for projects that propose agromining as a post-mine land use and where mines require large areas to be rehabilitated.

5 Propagation

The propagation stage provides further refinement of the prioritisation process and delivers the optimum basis from which recovery of the target mineral can take place. In this sense, propagation can be seen as the point in the agromining process, in which the potential of the hyperaccumulator species can start to be realized. A key outcome of the *Propagation* stage is that an accurate economic model can be developed for the feasibility of agromining for metals or eco-catalyst production.

5.1 Trials

Trials are a critical part of demonstrating that hyperaccumulating species are capable of growing in the modified environmental conditions following mining. The development of future rehabilitation planning depends upon accurate estimates of optimal period of seed collection (phenology), understanding seed dormancy, germination rates, seed storage and propagation potential, growth rates, canopy spread, site capture and root coverage (for erosion protection). Field trials need to clear the barriers to successful implementation of agromining production by delivering critical insights into the suitable combinations of soils, amendments and plants to obtain optimal growth conditions. Relevant parameters should be recorded and monitored e.g. growth, biomass production by unit surface, metal concentration, etc. For each type of trial conducted a full balance would need to be made (biomass production x nickel phytoextraction yield).

5.2 Economic Model

Scenarios of economic yield can be built on the data obtained from the field-based trials and be used as decision-making tools for the implementation of agromining at full-scale. Objectives for agromining, i.e. as a post-mining land use and/or recovery of minerals from uneconomic or technically constrained ore deposits (see Recovery section), will play an important role in assigning the assumptions on which the economic model is based. Results of grade control drilling to determine available minerals at the base of mined out pits or in uneconomic ore bodies can contribute important data (Robinson et al. 1999) to support the development of an economic model for agromining as part of a mine plan.

6 Recovery and Restoration

Recovery and restoration are the final stage in the agromining cycle and, if well implemented, can last beyond mine closure. Recovery involves the extraction of the target mineral from the source plant material and conversion to a saleable product. Restoration entails the integration of the use of hyperaccumulator species in the mine rehabilitation programme. Although these two processes are interrelated, they are not necessarily sequential.

Where results from floristic surveys (either through the biodiversity baseline development or during pre-clearing surveys) indicate an abundance of hyperaccumulator species at a particular location and test work on these plants suggest concentrations of the target mineral (e.g. nickel) suitable for extraction, the Project Owner may choose to recover this nickel. Alternatively, where mineral grades are found to be uneconomic to be mined in a conventional sense, or where mineral deposits lie in an area outsides the constraints set for safe mine pit development, i.e. such as steep topographic or the existence of watercourses, agromining presents an opportunity for additional mineral recovery. In the latter case, special attention will need to be paid to environmental impacts and safety risks that may arise. Broad scale removal of vegetation from slopes carries a significant risk of erosion and mitigation may prove costly. A phased approach in which hyperaccumulator species are initially planted in the form of ‘enrichment planting’ into existing native vegetation may ensure the stability of soils and eliminate the need for heavy equipment usage. As the initial plantings begin to stabilize/mature, further infill planting can be conducted.

The Halo

Agromining could be implemented both on the large ‘halo’ of mineralization around the mine leases, and as part of the progressive rehabilitation process after conventional resource extraction. A full demonstration of agromining at the mine site is required to provide evidence of real-life performance of economic viability and to evaluate the fundamental parameters (i.e. success of the crop, nickel yield per hectare).

7 Planning

Incorporation of hyperaccumulator species into mine rehabilitation and closure plans will help ensure that they species are managed during the life of the operation. Appropriate soil and plant management practices, based on insights from laboratory or field tests, need to be undertaken to produce high yields of the selected 'metal crop'. Optimizing nutrition in hyperaccumulator species is pre-requisite to large-scale agromining demonstration. Although opportunities exist in tropical regions for agromining operations, limited studies have been conducted in tropical regions to ascertain the type and quantity of fertilizer that may enhance biomass production while maintaining or improving annual mineral yields to-date. It is possible that N + P + K fertilization will increase biomass production but cause a 'dilution' of the target mineral in the biomass of tropical hyperaccumulator plants.

Agriculture on ultramafic soils often has a low productivity due to lack of Ca, K and P and excess Mg and Ni. Over time, agromining here will improve fertility and significantly decrease Ni availability; this can be regarded as a significant agronomic service.

Case Study 1: Halmahera Island, Indonesia

Prior to screening conducted at the Weda Bay project owned by Eramet, no hyperaccumulator plants were known from Halmahera Island, and just five hyperaccumulator species were known from Indonesia. The first stage in the research project was the identification of native hyperaccumulator plants occurring on the mine lease area. During the fieldwork a total of 21 non-permanent plots were made in which 824 herbarium vouchers and associated foliar samples, in addition to soil samples (totalling 90 samples), were collected for laboratory analysis. Plants were screened in the field (>1000 specimens) for Ni hyperaccumulation using dimethylglyoxime, and after positive reaction detailed samples were collected for these hyperaccumulator plants. Further screening of collected plants was conducted using both handheld XRF and elemental analysis using traditional wet chemical methods. This screening included samples of the rhizosphere soil, root, wood, branches and leaves. In total, 18 Ni hyperaccumulators species and one Mn hyperaccumulator were discovered (Fig. 2).

Case Study 2: New Caledonia

Ultramafic ranges cover one third of New Caledonia and possess substrate properties which virtually exclude agriculture and pastoral grasses, thus minimizing human impact. They provide rich sources of Ni, Cr and Fe ores and have been a focus of intensive mining activity for over a century (L'Huillier et al. 2010). Early revegetation trials in the 1970s focussed on fast growing exotic nurse trees, grasses, and native nitrogen-fixing *Casuarina collina* trees and *Acacia spirorbis* shrubs to stabilise slopes and generate a vegetation cover. These nitrogen-fixing species were to stimulate succession on mine overburden from surrounding native communities by improving the nutrient content of soils (Cherrier 1990; Sarrailh 2002; L'Huillier et al. 2010). However, most of these nurse species failed to provide these colonizing conditions on mine sites found on mountain tops as they were not adapted to high altitude environments. Certain ultramafic heathland species (of genera *Maytenus*, *Grevillea*, *Geissois*) were identified as Ni or Mn hyperaccumulators in the 1970s and 1980s and trialled on plantations without full knowledge of their potential in mine restoration (Jaffré and Latham 1976; Jaffré 1980). The first trial in New Caledonia on mine sites with hyperaccumulators included *Grevillea exul* (Fig. 3) and *Grevillea gillivrayi* (Fig. 4) in 1974 (Jaffré and Latham 1976). Later experiments were conducted with *Grevillea exul* (ssp. *exul* and ssp. *rubiginosa*), *G. gillivrayi, G. meisneri* (Proteaceae), *Geissois pruinosa* (Cunoniaceae) or *Cloezia artensis* (Myrtaceae). These were considered key revegetation species because of their good growth and their ability to adapt to different conditions (Jaffré et al. 1993; Rigault et al. 1996; Sarrailh 2002; Rabier et al. 2009). Results of these pioneering studies were expanded upon in the 1990s to evaluate the diverse endemic flora of these massifs and to identify species adaptations tolerant to lateritic substrates on mine sites in New Caledonia (Jaffré et al. 1994, 1997, 2009; Jaffré and Rigault 1991; Jaffré and Pelletier 1992; Lucon et al. 1997; Léon et al. 2006; L'Huillier et al. 2010). The objective of using this diverse species pool is to provide a range of

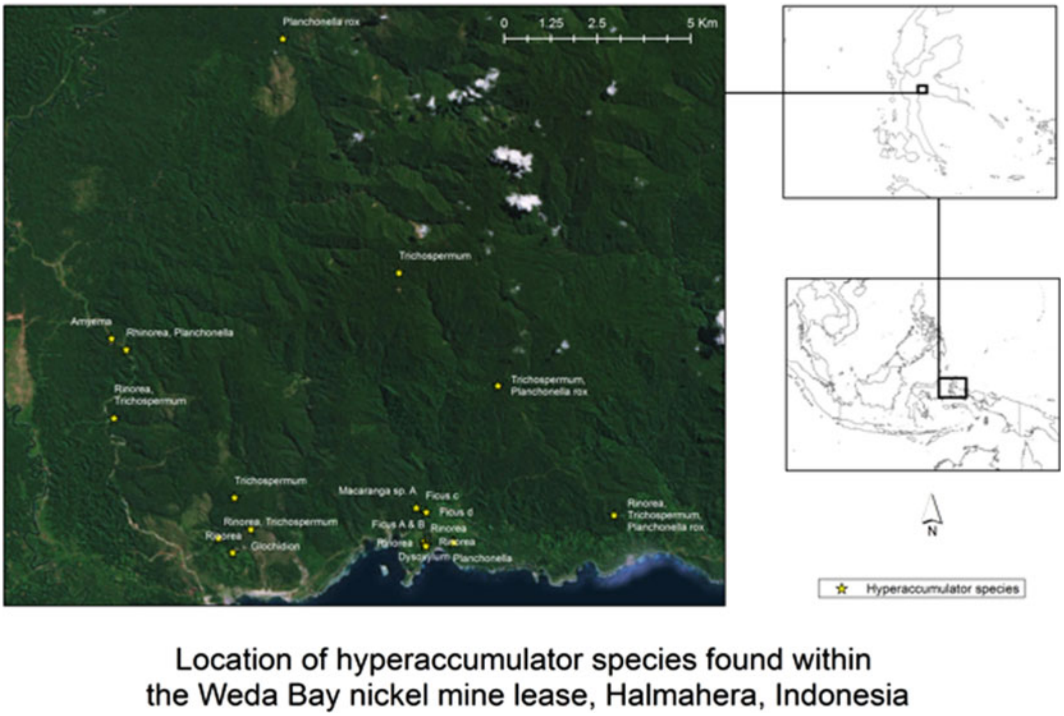

Fig. 2 Location of hyperaccumulators at Halmahera Island discovered after two weeks of field reconnaissance

rehabilitation solutions depending on the mine location and its final land use. Species screened included certain Ni or Mn hyperaccumulators (Jaffré 1980; Jaffré et al. 2013; Losfeld et al. 2015b) from maquis heathlands around mine sites (Jaffré and Pelletier 1992; L'Huillier et al. 2010) that have potential in agromining/phytomining on mine dumps and abandoned mine sites as they comply with survival and harvest criteria (Table 1).

More recently, mining companies such as SLN, Vale and KNS in collaboration with institutions such as L'Institut de Recherche pour le Développement (IRD), Institut Agronomique néo-Calédonien (IAC), Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) and University of New Caledonia (UNC) have expanded the knowledge base of heathland species in mine site revegetation to identify a suite of pioneer species that are easy to propagate and are adapted to exposed mine site environments (Fogliani et al. 2004; Wulff et al. 2012). Vale NC has an extensive propagation program that involves annual production of 120 endemic species from the Goro area for revegetation of its mine lease and surrounding areas in southern New Caledonia. Started in 1996, the revegetation programme also includes several Mn hyperaccumulators (*Grevillea exul* subsp. *rubiginosa*, *Grevillea gillivrayi*, *Polyscias pancheri*, *Pancheria billardierei*, *Denhamia fournieri*, *Peripterygia marginata*) and Ni hyperaccumulators (*Geissois pruinosa*, *Codia spatulata*, *Normandia neocaledonica*, *Cloezia artensis*) commonly found on laterites of southern New Caledonia (Jaffré 1980; McCoy et al. 1999). Most species resprout after fire which provides indirect evidence of regular aerial shoot production that is necessary for harvesting (McCoy et al. 1999). However, each species presents its own challenges. The most extensively produced species on an annual basis with more than 20,000 seedlings per year is *Grevillea exul* ssp. *rubiginosa*. This pioneer

Fig. 3 Wild growing *Grevillea exul* ssp. *exul* in New Caledonia

species, which can attain more than 20 cm of new growth in one year, is readily planted with other maquis species (*Gymnostoma deplancheanum, Tetraria* (*ex Costularia*) *comosa, Stenocarpus umbelliferus, Myodocarpus fraxinifolius*) to rehabilitate mine dumps and degraded laterites covering 10–20 ha. Since 2010 annual seedling production of pioneer shrub species such as *Grevillea exul* ssp. *rubiginosa* is subcontracted by Vale NC to satellite nurseries (Fig. 5) with coastal villages at Yaté, showing the potential socio-economic gains of such ventures with agromining projects. *Grevillea gillivrayi, Geissois pruinosa, Codia spatulata* and *Pancheria billardierei* also have good growth rates similar to *G. exul* ssp. *rubiginosa* but seed production in these species is irregular with seedlings produced when seeds are available. *Denhamia fournieri, Normandia neocaledonica, Cloezia artensis* are produced from cuttings, as seedlings are small or seed production low. *Polyscias pancheri* produces few seeds that germinate after 6 months suggesting dormancy issues. No harvest technology has been applied, thus the next stage of screening for agromining/phtyomining candidates would to evaluate growth and foliar contents of metals after harvesting on amended plots.

In spite of the renown of its metallophyte biodiversity (Jaffré 1993), agromining was never considered in New Caledonia until recently (Losfeld et al. 2012). With the development of ecocatalysis approaches that permit preparation of ecocatalysts from metal trace elements extracted from hyperaccumulator or accumulator (Losfeld et al. 2012), several New Caledonian endemic species were identified as candidates for plantations on degraded areas. The first trials were completed in 2012 in collaboration with Société Le Nickel (SLN) on two areas of mine spoils at Camp des Sapins mine, Thio (500 m asl.). The aim of the experiment was to assess the growth response of *Geissois pruinosa* and *Grevillea exul* ssp. *exul or* ssp. *rubiginosa* (1-year-old young plants produced from seeds) on mine spoils or on mine spoils with addition of topsoil.

Fig. 4 *Grevillea gilivrayi* found in New Caledonia has reasonable growth rates and is a noted Mn accumulator (8200 µg g^{-1})

The effects of the addition of several fertilizers were also evaluated (Losfeld et al. 2015b). A total surface of 3.5 ha of mine spoil was planted (1250 plant/ha) with *Grevillea* and *Geissois*. A plantation of *Geissois pruinosa* (≈1.5 ha) was established later in the year (Figs. 6 and 7) to investigate the effects of topsoil and fertilizers, along with the effect of endemic nitrogen-fixing plants *Gymnostoma deplancheanum* (Casuarinaceae), *Serianthes calycina* and *Storckiella pancheri* (Fabaceae). A similar protocol was applied in 2014 on the Kouaoua mine on an area of 2800 m^2 using different accompanying species (*Gymnostoma intermedium, Serianthes calycina* and *Storckiella pancheri*) in relation with both *Grevillea exul* subspecies. Other projects are currently underway near the massif of Tiebaghi, using the Mn hyperaccumulator *Grevillea meisneri* (Losfeld et al. 2015a) with different amendments of phosphorus. More recently trials have been developed by Koniambo Nickel SAS (KNS) on the Koniambo massif in the Northern Province of New Caledonia. For all experiments, mortality, growth and metal accumulation in young and old leaves have been annually evaluated. These data have helped guide the planting of metallophytes on mine spoils for reclamation and also to provide estimates of revenue from the ecocatalysis biomass production.

8 Conclusions

Successful mines and agromining projects require extensive planning and careful design before they start. Similarly, rehabilitation planning that includes hyperaccumulator plants needs to be detailed early in the mine planning process and integrated with the mining operation. There is a natural tendency for rehabilitation plans to reflect whatever appears to be the simplest land-use for an area, such as grazing or plantations, rather than attempt to use hyperaccumulator plants for which propagation and agronomic

Table 1 Nickel or manganese hyperaccumulators from New Caledonia maquis heathlands around mine sites with potential for mine rehabilitation

Family	Species	Leaf Ni ($\mu g\ g^{-1}$) range	Max. leaf Mn ($mg\ g^{-1}$)	References	Ecological distribution	Ecological role	Phenology/dispersal mode	Seed production/storage	Propagation history	Germination/survival %	Nursery seedling ht year[a] (cm)	Nursery seedling ht year[b] (cm)
Araliaceae	*Polyscias pancheri*	–	14.0	Losfeld et al. (2015a)	Localised common in south	Maquis shrub	Irregular, zoochore	Low, orthodox	Experimental scale germination (<100 seedlings)	<12 months, 10%	5	7
Celastraceae	*Denhamia fournieri* ssp. *fournieri*	68–1575	33.0	Jaffré (1977)	Widespread west coast massifs serpentinite	Small maquis shrub	Irregular, anemochore	Low, orthodox	Small-scale germination and cuttings (<1000 seedlings p.a.)	6 months, 10%	10	15
	Peripterygia marginata	23–1800	–	Jaffré (1980), Jaffré et al. (2013)	Widespread ultramafic	Small maquis shrub	Annual, anemochore	Low, orthodox	Small-scale germination and cuttings (<1000 seedlings p.a.)	Low, 10%	4	10
Cunoniaceae	*Pancheria billardierei*	–	6.5	Jaffré (1980)	Widespread main island	Small maquis shrub	Annual, anemochore	Moderate, orthodox	Small-scale germination (<1000 seedlings)	<2 months, 20%	12	20
	Codia spatulata	27–1420	–	Jaffré et al. (2013)	West and south coast massifs	Small maquis tree	Irregular, anemochore	Moderate, orthodox	Moderate scale germination (20,000 seedling p.a.)	Low, 20%	10	18
	Geissois pruinosa (all varieties)	3000–15,106	–	Jaffré et al. (1979, 2013)	Southern and west massifs	Small maquis tree	Annual, anemochore	Moderate, orthodox	Moderate scale germination (20,000 seedling p.a.)	Low, 10%	7	20
Mytaceae	*Cloezia artensis* ssp. *artensis*	35–1729	–	Jaffré et al. (2013)	Southern massif	Small maquis shrub	Annual, anemochore	Moderate, orthodox	Small scale germination and cuttings (<1000 seedlings p.a.)	Low, 20%	3	10
Proteaceae	*Grevillea exul* ssp. *rubiginosa*	–	6.2	Jaffré (1979)	Widespread common in south	Small maquis tree	Annual, anemochore	Moderate, orthodox	Large scale germination/seeding (20,000 seedlings p.a.)	<2 months 70%	8	18
	Grevillea gillivrayi	–	8.2	Jaffré (1979)	Widespread common in south	Small maquis tree	Irregular, anemochore	Low, orthodox	Moderate scale germination (5000 seedlings p.a.)	<2 months 50%	10	20
	Grevillea meisneri	–	11.0	Losfeld et al. (2015a)	Northern massifs	Small maquis tree	Annual, anemochore	Moderate, orthodox	Small scale germination (<1000 seedlings)	<2 months 50%	8	18
	Grevillea exul ssp. *exul*	–	3.9	Jaffré (1979)	Widespread common in south	Small maquis tree	Annual, anemochore	Moderate, orthodox	Moderate scale germination (5000 seedlings p.a.)	<2 months 70%	10	20
	Stenocarpus milnei	–	3.9	Jaffré (1979)	Widespread ultramafic	Small maquis shrub	Annual, anemochore	Moderate, orthodox	Small scale germination (<1000 seedlings)	<2 months 70%	8	18

(continued)

Table 1 (continued)

Family	Species	Leaf Ni ($\mu g\ g^{-1}$) range	Max. leaf Mn ($mg\ g^{-1}$)	References	Ecological distribution	Ecological role	Phenology/dispersal mode	Seed production/storage	Propagation history	Germination/survival %	Nursery seedling ht year[a] (cm)	Nursery seedling ht year[b] (cm)
Rubiaceae	*Normandia neocaledonica*	23–1000	–	Jaffré et al. (2013)	Southern and central massifs altitude	Small maquis shrub	Annual, anemochore	Moderate, orthodox	Small scale cuttings (1000 seedlings p.a.)	60% survival cuttings	3	10
Sapindaceae	*Guioa glauca*	–	4.5	Jaffré (1980)	Widespread	Small maquis shrub	Annual zoochore	Low, orthodox	Small scale germination (1000 seedlings p.a.)	<3 months 10%	3	10

[a] Germination (level or time)/end survival in the nursery (%)

[b] Growth in nursery

Fig. 5 *Grevillea exul* ssp. *rubiginosa* production at satellite nurseries at a satellite nursery in New Caledonia (Copyright Vale NC)

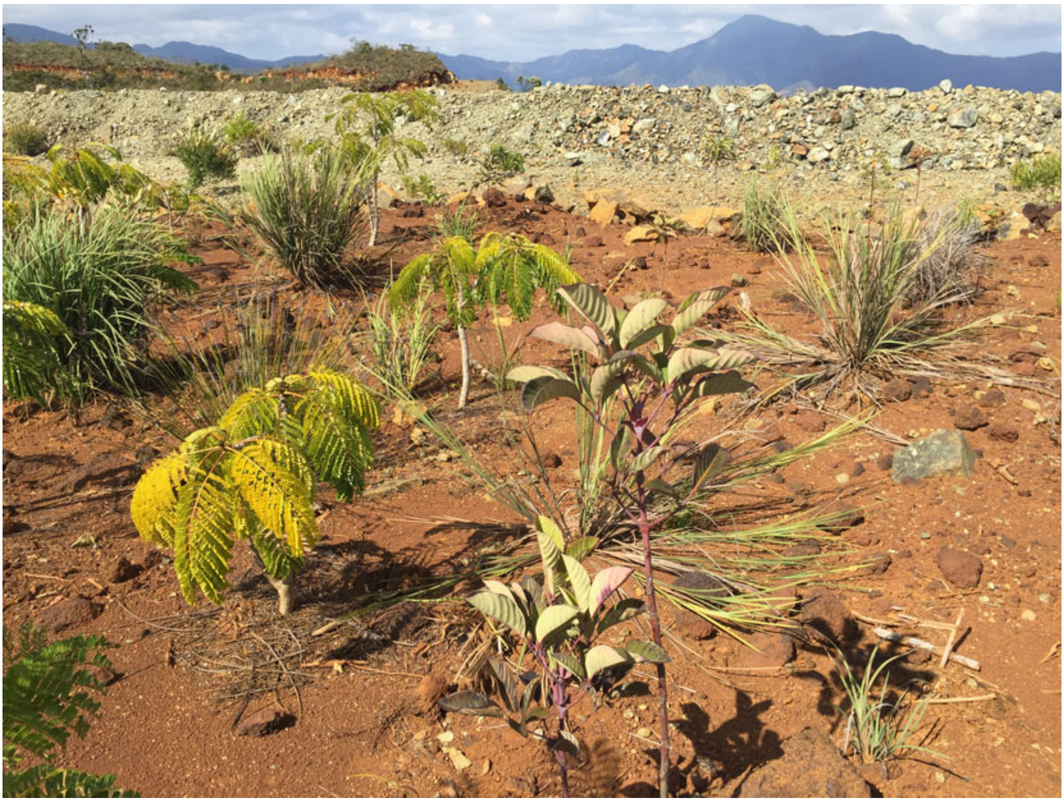

Fig. 6 A plantation of *Geissois pruinosa* developed to investigate topsoil, fertilizers and the effect of endemic nitrogen-fixing plants

techniques are less well known. While this predisposition is not surprising, the opportunity to maximise the biological resources of a site by discovering and utilising these unusual plant species, for either their local adaptations to difficult growing condition or potential for agromining, is an opportunity that should be taken so that unique biodiversity can be a valuable part of the mining process. Ultimately this type of approach can also facilitate multiple additional benefits including increased metal productivity per unit of biodiversity loss, improved soil fertility in mined out soils hence available for a wider range of metal sensitive plants, reduced

Fig. 7 A view across the mixed species plantation of *Geissois pruinosa* with endemic nitrogen-fixing plants *Gymnostoma deplancheanum*, *Serianthes calycina* and *Storckiella pancheri* at Thio, New Caledonia

erosion on mined out soils and wastes, and a more productive and viable post-mining land-use.

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Agromining from Secondary Resources: Recovery of Nickel and Other Valuable Elements from Waste Materials

Alice Tognacchini, Aurelien Buteri,
Gaylord Erwan Machinet, Jean
Louis Morel, Markus Puschenreiter,
Ramez F. Saad, and Marie-Odile Simonnot

Abstract

Industrial activities produce a variety of metal-rich waste, which are often classified as hazardous due to high concentrations of metals such as Ni, Zn, Cu, Cr, Pb and Cd. Metals recycling from waste materials is still limited, especially from those waste types characterized by complex matrices and multi-metal contamination, with large amounts of metal-rich waste materials being discharged into the environment. Although several pathways of metal recovery from waste have been developed, landfilling often remains the most convenient alternative in terms of costs. In recent years, a new approach to metals recycling from waste materials has been investigated: agromining with hyperaccumulator plants on waste-derived substrates. Hyperaccumulator plants can isolate specific metals from multi-metal waste matrices and bioconcentrate target metals in their biomass. Specific characteristics of industrial waste might limit plant establishment and uptake of target metals; thus, the addition of amendments is required prior to plant cultivations as well as the construction of waste-derived artificial substrates. Research conducted to date has shown limited effectiveness of agromining when applied on certain metal-rich industrial waste materials, while promising results were obtained from other types of waste. Upscaling trials are currently underway to demonstrate the applicability of waste agromining for metals recycling within waste generation industrial facilities.

1 Waste Materials as Secondary Resources

Metals such as Ni, Co, Zn, and Cu are essential for a variety of industrial processes. Moreover, the demand for valuable metals has increased in

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A. Tognacchini (✉) · M. Puschenreiter
Department of Forest and Soil Sciences, Institute of Soil Research, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria
e-mail: alice.tognacchini@boku.ac.at

A. Buteri
Aperam Isbergues—Research Center, Isbergues, France

G. E. Machinet
ENSAIA, Laboratoire Sols et Environnement, Microhumus, Université de Lorraine, Vandoeuvre-lès-Nancy, France

J. L. Morel
Laboratoire Sols et Environnement, UMR 1120, Université de Lorraine-INRAE, Vandoeuvre-lès-Nancy, France

R. F. Saad
Econick, Nancy Cedex, France

M.-O. Simonnot
Laboratoire Réactions et Génie des Procédés, UMR 7274, Université de Lorraine—CNRS, Nancy, France

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_14

recent years due to growing industrialization, followed by a decline in some primary resources. Nickel, for instance, is widely employed in the production of stainless steel ($\sim 58\%$), Ni-based alloys ($\sim 14\%$), casting and alloy steels ($\sim 9\%$), electroplating ($\sim 9\%$), and rechargeable battery ($\sim 5\%$) (Eramet 2009; Barnett 2010). Industrial activities that utilise metals, such as the metallurgical, electronic, electroplating, and automotive sectors, inevitably produce a variety of waste types. These wastes are often classified by national regulations as being 'hazardous' owing to high contents of Ni, Cu, Zn, Cd, Cr, Pb and other metals and their impact on water quality. It was estimated that about 2.5 billion t y^{-1} of waste are being discarded in the EU (data referring to 2012), of which close to 4% is classified as hazardous (EEA 2016). Total quantities of waste generated by industry have generally remained static in recent years (period 2010–2016; EEA 2019). Considering the large quantities of metal-rich hazardous waste produced worldwide and their relative contents of valuable elements such as Ni, it is evident that waste disposal represents a loss of non-renewable resources, besides the environmental concerns related to landfilling of hazardous substances. Toxicity of Ni and other metals commonly found in hazardous waste and problems associated with waste discharge have long since been recognized. However, metal recovery from waste still remains a marginal source of raw materials in many industrial chains and supply of metals as, for example, Ni still relies to a great extent on mining. Given that the mining and quarrying sectors generate the second largest quantities of hazardous waste (Eurostat 2019), it is unquestionable that industrial wastes should preferably be targeted as secondary metal resources. Ore bodies exploited by the mining industries are typically characterized by Ni concentrations of ≥ 30 g kg^{-1} (Li et al. 2003), with lower-grade deposits (about 10 g kg^{-1}) being mined in recent years due to global declines in ore grade (Mudd 2009). Meanwhile, considerable industrial waste materials, often richer in Ni and other elements than ore bodies exploited in mining activities, are being discarded. For instance, waste produced by galvanic industries, mostly discharged into landfills, can contain more than 100 g Ni kg^{-1}, which could represent 'higher grade' ores than ore bodies currently being mined.

Despite research efforts and the development of new technologies for metal recovery, no significant advancement has been achieved in implementing metal recycling from waste during the past years (EEA 2019). Unlike other materials, e.g. polymer plastics, metals can be recycled nearly indefinitely, and their properties can be restored fully, regardless of chemical or physical form (Jadhav and Hocheng 2012). Being a fully recyclable resource, Ni and Ni-containing alloys can be returned to their original state or converted into a different, but still valuable, form (Nickel Institute 2018). For example, stainless steel scrap is being turned into new stainless steel, and Ni from recycled batteries is being used for Ni-containing stainless steel (Nickel Institute 2018). According to the US Geological Survey (2019), recycled Ni in 2019 accounted for approximately 47% of US Ni consumption, mostly due to re-use of stainless steel. Despite the fact that heavy metals recycling is generally regarded as a preferred action compared to landfilling, a number of obstacles exist to increased collection and recycling (EC 2002). This is notably the case for those waste materials characterized by complex matrices and multi-metal contamination, where the recovery of Ni or other elements is more demanding in terms of separation processes. In general, separation is an option to consider where the substances are present in relatively high concentrations. However, if a substance occurs at a relatively low concentration, separation may be complicated and costly and large-scale arrangements and treatment facilities may be necessary (EC 2002).

Complex wastes are generally being generated by industry, a complexity due not only to the quantity of wastes, but also to their composition representing one of the main constraints to effective implementation of metal recycling (Wei and Huang 2001). The success of secondary metal markets depends on: (i) the cost of retrieving and processing metals embedded in abandoned structures, discarded products and other waste streams; and (ii) its relation to primary metal prices (Wernick and Themelis 1998). Several pathways have been developed for the recovery of valuable metals from waste. It remains, however, that in many cases a linear pathway based on landfilling represents the most ‘convenient’ solution for industry. With a cost of around 150 Euros per tonne of landfilled hazardous industrial waste, including transport and revalorization (i.e. landfill and storage), this option is still widely used. There is no doubt, however, that landfilling definitely represents the worst level of waste management options as defined by the ‘Lansink ladder’. This process will probably face strong limitations or constraints in coming years and decades, and should be progressively replaced by more sustainable ‘cradle-to-cradle’ pathways to ensure less waste generation and better landfills management, as evidenced by the recent action plan on circular economy published by the European Commission (2020).

Nickel recycling rates are at present low, as the Ni supplied from mining is the lowest supply cost option (Mudd 2010). Furthermore, it has to be considered that whereas industrial waste materials are often characterized by multi-element contamination, and in most cases represent complex matrices requiring specific recovery processes, ores have a more homogeneous composition and extraction processes are well established.

Studies are now underway aimed at the development of new or modified processes for the separation of metals, mainly from industrial waste by-products (Chmielewski et al. 1997; Bosecker 2001; Verstraete 2002). Several technologies may be used to recover metals from industrial waste: pyrometallurgy, electrowinning, hydrometallurgy and bio-hydrometallurgy are all possible (Syed 2006; Havlik et al. 2010; Hoque and Philip 2011; Vegliò et al. 2003; Silva et al. 2005; Vilarinho et al. 2012). In recent years, new technologies based on hyperaccumulator plant species have been developed for the recovery of metals from metal-rich substrates (phytomining, recently renamed agromining). Agromining technology employs hyperaccumulator plants to take up metals in harvestable plant biomass, with subsequent harvesting, drying, and incineration of the biomass generating a high-grade bio-ore (van der Ent et al. 2015). In particular, extensive research on Ni recovery (due to higher availability of Ni hyperaccumulator plants) from Ni-rich soils and contaminated matrices has raised the question as to whether these processes could also be applied for recovery of Ni and other valuable elements from waste materials. Whilst agromining experiments have been extensively implemented on ultramafic soils and marginally on contaminated soils and mine tailings, the application of this process to industrial waste represents a new and recent approach. For those types of waste where direct metal recovery is not economically feasible, hyperaccumulator plants could provide an ‘isolation and bioconcentration’ step, allowing the recovery of Ni and other elements. In fact, hyperaccumulator plants typically contain higher concentrations of the target element (e.g. Ni) than the substrate upon which they grow, being able to accumulate some trace elements to concentrations one order of magnitude greater than in metalliferous soils (van der Ent et al. 2013). To date, few studies have addressed agromining applicability on industrial waste-derived substrates. In this chapter, we illustrate the current knowledge on agromining from waste materials, including the development of artificial substrates derived from wastes (Technosols) and give an overview of current research on waste—agromining, highlighting the main outcomes and challenges.

2 Metal-Rich Industrial Waste Materials and Formulation of Artificial Substrates

A variety of metal-rich industrial waste materials is being produced, including battery and electronic wastes, and different sludges resulting from industrial activities such as automotive, stainless steel production, or electroplating processes. Whilst common characteristics can be identified, such as enrichment in certain metals at toxic levels, industrial waste materials differ in major characteristics such as pH, structure, humidity, salinity, or the relative abundance of other elements such as Ca, Mg, and Na, which are often not directly involved in the industrial process but added as waste 'post treatments'. Waste materials derived from industrial processes are typically not suitable for plant growth due to: (i) a lack of nutrients, (ii) excessive metal concentrations, (iii) very acidic or very alkaline pH, (iv) high salinity, (v) lack of organic matter, and (vi) unfavourable particle size distribution. Concerning metal concentrations, a differentiation is needed between target metals that will be accumulated by the plant, and non-target metals that might be toxic to the plant species employed. Metal hyperaccumulators are hypertolerant to metals that are being accumulated by the plant but can also be very sensitive to other metals. For example, the Ni hyperaccumulator *Odontarrhena serpyllifolia s.l.* (syn. *Alyssum serpyllifolium*) was found to be very sensitive to excessive Cu concentrations (Kidd and Monterroso 2005). Also, Rosenkranz et al. (2017) found that Cu toxicity was likely the main factor limiting the growth of several metal hyperaccumulators grown on incineration bottom ashes. Plant-growth limiting factors of waste materials can, to some extent, be corrected by adding amendments. Plant establishment in the extreme conditions of industrial wastes is rather challenging, as artificial substrates (Technosols) have to be constructed prior the cultivation of hyperaccumulator plants. Depending on characteristics of the waste material, different amendments will be necessary in order to allow satisfactory plant establishment and growth.

To meet the challenge of performing agromining on toxic industrial wastes, it is necessary to solve at least two essential problems related to the waste structure: (i) extreme pH and salinity, and (ii) the imbalance among elements. This is required in order to ensure the absence of phytotoxicity and a sufficient fertility to obtain satisfactory plant yields. It is also necessary to optimize phytoextraction of the metals of interest (e.g. Ni), which could be impaired by the presence of other metallic elements that are competitors for the accumulation (e.g. Zn). Two complementary approaches are possible. The first is to pre-treat the waste in order to reduce the phytotoxicity and to obtain a substrate favourable for plant growth. For example, several leaching steps may contribute to the reduction of salt concentrations. An example for improving the characteristics of waste incineration bottom ash to support plant growth is described by Rosenkranz et al. (2017). Furthermore, the addition of a mineral matrix might be necessary to provide structure to the substrate, especially when working with industrial sludges. The addition of organic matter may provide nutrients and binding sites for excessive salts and metals, correct the pH, and improve the physical structure of the material. Secondary materials of urban and/or industrial origin could be used as organic sources, as well as amendments that enhance the growth of hyperaccumulator plants, such as biochar (Rees et al. 2014, 2015, 2016, 2017). An alternative approach to limit phytotoxicity and allow uptake of the target metals is based on special arrangement of the materials in layers in order to reduce interactions between and among the elements, especially where other metals are present in the waste that might interact with uptake of the target metal (e.g. Zn and Ni); this approach is presented in more detail in Case Study 1 and in Sect. 6 on Upscaling. Further examples of the efficiency of different amendments in improving the waste material for supporting plant growth are given later in this chapter.

3 Agromining Experiments Conducted on Waste Materials

Agromining research and advancements have so far focused on field applications on ultramafic or Ni-contaminated soils, testing a variety of Ni hyperaccumulator plants in temperate (Robinson et al. 1997a, b; Chaney et al. 2007; Bani et al. 2015a, b; Echevarria et al. 2017; Pardo et al. 2018; Rosenkranz et al. 2019) and tropical (Chaney et al. 2018; Nkrumah et al. 2019) regions. In the last few years, experiments have been conducted to assess the feasibility of metals recovery from waste materials through agromining applications (Rosenkranz et al. 2017, 2018; Rue et al. 2019; Tognacchini et al. 2020). Despite the fact that research in this field is still at an early stage, important information can be discerned from the results obtained so far. Previous phytoextraction experiments on waste materials have been conducted targeting sewage sludges, with the aim of reducing metal loads from the sludge rather than metal recycling (Liu et al. 2005; Wu et al. 2007; Qiu et al. 2014; Nissim et al. 2018). The newly developed concept of 'waste agromining' has the additional aim of recovering metals from the waste materials for commercial use.

Agromining may in principle be undertaken to produce As, Se, Cd, Cu, Co, La, Mn, Ni, Pb, Tl and Zn, as hyperaccumulator plants are known for all of these elements (van der Ent et al. 2013). However, at present most studies on waste have targeted only Ni (and marginally Zn) since Ni hyperaccumulator plants were those primarily tested in previous agromining experiments, and a variety of Ni-rich waste materials are available. Furthermore, effective Ni recovery processes from plant biomass have already been developed (Barbaroux et al. 2012; Zhang et al. 2016; Houzelot et al. 2017). The works of Rosenkranz et al. (2017) and Rue (2017) represent the first attempts at extracting valuable elements from waste materials with hyperaccumulator plants for metals recycling. In the study by Rosenkranz et al. (2017), an agromining experiment was conducted on substrates derived from incineration bottom ashes, using the hyperaccumulator plant species *O. serpyllifolia s.l.* targeting Ni and *Sedum plumbizincicola* targeting Zn. Substrates were obtained from incineration bottom ashes mixed with residues from biologically treated municipal solid waste and biochar. Subsequently, Rue et al. (2019) tested the Ni extraction potential of the hyperaccumulator plant species *Odontarrhena* spp. on waste from automotive industry mixed with decontaminated soil and biochar; Tognacchini et al. (2020) cultivated the hyperaccumulator *O. chalcidica* on artificial substrates derived from Ni-rich galvanic sludges combined with a quarry waste and compost. Saad et al. (2019, unpublished results) have also tested the agromining potential of *O. chalcidica* on sludges derived from stainless steel production. Agromining trials conducted on incineration bottom ashes (Rosenkranz et al. 2017) showed that Ni extraction with hyperaccumulating plants was limited by low plant uptake and low biomass production. Nickel concentrations >1% in plant shoots are required for commercially valuable agromining applications (Chaney et al. 2007; van der Ent et al. 2013), but Rosenkranz et al. (2017) achieved a maximum shoot Ni accumulation of 0.025% (255 mg kg^{-1}) in *O. serpyllifolia s.l.* In the same experiment, 1694 mg kg^{-1} Zn in shoots of *S. plumbizincicola* were obtained, far below the hyperaccumulation threshold of >10,000 mg kg^{-1} as defined by van der Ent et al. (2013). Furthermore, a low plant biomass was obtained from incineration ash-derived Technosols due to unfavourable conditions, and the plants had severe problems of establishment on these substrates owing to high salinity and presumed Cu toxicity (Rosenkranz et al. 2017). In the same study, it was shown that the metal-resistant cultivars of *Brassica napus* and *B. juncea* were able to establish well on the bottom ash-derived substrates without showing any toxicity symptoms, thus demonstrating that certain species and cultivars are actually capable of adaptation to those extreme conditions and to tolerate salt and metal toxicity stresses. In a subsequent study, it was found that metal-accumulating high-biomass plants such as *Salix smithiana* and *Nicotiana tabacum* provided a higher Ni and Zn yield than the hyperaccumulating plants *O. serpyllifolia* and *S. plumbizincicola* (Rosenkranz et al. 2018).

Bottom ashes representing the remains of waste incineration processes are characterized by low metal availability due to the presence of metals in oxidized forms that are rather insoluble. Thus, a low availability of target elements was found by Rosenkranz et al. (2017) that may have limited plant uptake. *Odontarrhena* spp. growing on Technosols derived from Ni- and Zn-rich automotive sludge showed a good adaptation to the substrate and satisfactory growth rates, even higher than on ultramafic soils (Rue et al. 2019). Nickel extraction found by Rue et al. (2019) reached a maximum of 0.1% (1000 mg kg^{-1}), showing that Ni hyperaccumulation from sludge-derived substrates was, however, much lower than the 'economical' target of 1% (Chaney et al. 2007; van der Ent et al. 2013). Tognacchini et al. (2020) achieved a high Ni uptake (>1%) from Technosols constructed from galvanic sludges, due to high Ni availability and good plant establishment on the substrates. These results showed that it could be feasible to phytomine Ni from galvanic sludges. However, the Technosol formulations should be improved to obtain higher plant biomass. The main results obtained from the agromining trials conducted on industrial sludges (automotive, stainless steel, and galvanic) are presented in more detail in the following Case Studies 1, 2 and 3 in order to provide an overview of different agromining approaches conducted on three industrial sludges having different characteristics.

3.1 Case Study 1: Agromining Nickel on Toxic Acid Industrial Sludges Containing Zinc

An industrial sludge that is currently disposed in toxic waste landfill, thus with no metal recovery, was chosen to test agromining. The waste material was represented by a phosphate-rich sludge collected from an automotive company, a residue resulting after anti-corrosive treatment (cataphoresis) of metal parts using an acid bath composed of Ni and Zn phosphate. The sludge was characterized by a low pH (3.9) and high contents of metals and P, with a Zn:Ni ratio of 10 (6% Zn, 0.6% Ni).

Nickel was chosen as the target element and the Ni-hyperaccumulators *Bornmuellera emarginata* (syn. *Leptoplax emarginata*) and *O. chalcidica* (originating from an ultramafic area in Greece) were selected for the trial. Experiments were conducted using cultures in pots, in rhizotrons, and in lysimeters. The industrial sludge was either: (i) mixed with a filling material composed of treated soil and green waste compost to improve the physical and chemical fertility of the substrate, or (ii) arranged as a sole horizon in the soil profile (layer strategy). The treated soil was represented by a soil material excavated from a contaminated site, then treated by biopile (SITA France Waste). The compost was a mixture of green waste and sewage sludge (SUEZ, TERRALYS). The substrate so obtained was then amended with a biochar material (pyrolyzed wood chips, Maschinenring, Switzerland) at four rates (Rees et al. 2014; Rue et al. 2019): 0, 1, 3 and 5% (see Fig. 1). It was shown that the addition of biochar decreases metal mobility and increases the total root surface developed by the plant (Rees et al. 2016). Results from the agromining experiment showed that it is possible to grow hyperaccumulators on the constructed substrate, and that Ni could be recovered in the plant biomass. Amendment with biochar significantly improved plant growth and development and the extraction of Ni by the hyperaccumulators (Fig. 1). However, due to the very strong competition among metallic elements for root absorption, notably Zn and Ni (Deng et al. 2014), the accumulation of Ni remained only moderate. The alternative growing system where the agromining experiment was conducted consisted of a substrate made up of several superposed layers. The plants (previously cultivated in a nursery) were introduced into tubes where the roots were allowed to develop, avoiding any direct contact with the toxic waste and to ensure

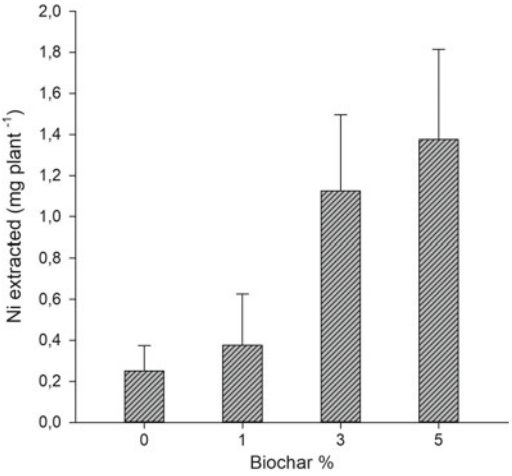

Fig. 1 Total uptake of Ni (mg plant^{-1}) by *Odontarrhena chalcidica* from a substrate made of industrial sludge amended with different percentages of biochar

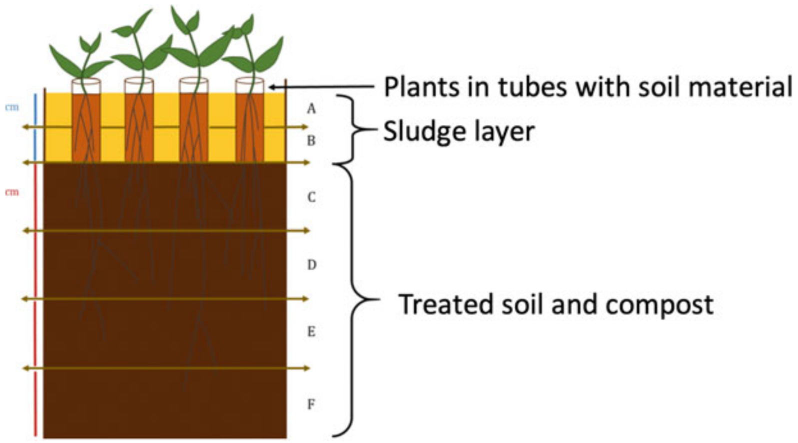

Fig. 2 Arrangement of materials in layers in rhizotrons to limit the root-sludge contact (layers A and B) and to decrease the Zn/Ni ratio in the vicinity of roots (layers C, D, E, F) (Rue 2017)

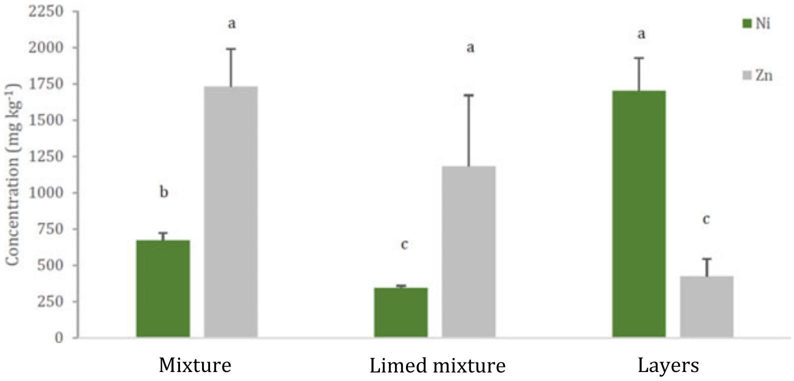

Fig. 3 Concentrations of Ni and Zn in the hyperaccumulator *Bornmuellera emarginata* after three months of culture on three types of composition and organization of the substrate (Rue 2017)

the availability of the target metal (Figs. 2 and 3; Rue 2017). Indeed, there was a differential leaching of Zn and Ni from the sludge to the underlaying substrate where the roots were guided and where they developed. Nickel was more easily transported from the sludge layer to the underlaying substrate than was Zn. The absorption of Ni was then increased due to less competition between Zn and Ni. This work, which was the subject of M. Rue's Ph.D. thesis (2017), as part of the LORVER project (2012–2018), was supported by the Grand Est Region and the European Union (ERDF), and by the Agromine project (2014–2018) funded by the French National Agency for Research (ANR). It has demonstrated the technical feasibility of creating the conditions for effective agromining on toxic industrial materials. Tests in lysimeters (not presented here) confirmed the performances of the layered devices obtained in both pots and rhizotrons. There is now a need to scale-up this technology. Required technical conditions for doing so are met with the availability of large lysimetric plots (50 m^2) built on the GISFI Experimental Station (http://www.gisfi.univ-lorraine.fr). The legal conditions are also favourable with the promulgation of the Law 2020–105 on the circular economy in France (10 February 2020), which explicitly considers agromining technology and develops its legal framework. The design of the process chain is now being developed by the Econick company.

3.2 Case Study 2: Agromining Ni-Rich Alkaline Galvanic Sludges

Three Ni-rich waste sludges (C1, W1, and W2, see Table 1) were collected from galvanic industries and used as substrates for Ni agromining experiments. The waste materials were characterized by Ni concentrations between 8.4 and 15% and alkaline to very alkaline pH. The main characteristics of the sludges are given in Table 1. Artificial substrates were formulated from the three galvanic sludges using a serpentinite sand discarded from quarry activities as a mineral matrix, and compost obtained from municipal organic waste as a source of organic matter. Five artificial substrates (Technosols) were prepared mixing the serpentinite sand with different proportions of galvanic sludge (on a wet weight basis) and compost, as illustrated in Table 2. All of the substrates were homogenized, lixiviated with deionized water in order to remove excess salts, air-dried, stored in closed plastic containers, and left to equilibrate for two months. An agromining pot experiment was conducted on the five Technosols using the Ni hyperaccumulator species *O. chalcidica*. Fertilized replicates (indicated with -*F*) and a reference ultramafic soil (S) were included in the experiment. After three months, the plant biomass was harvested and Ni concentrations in the plants were measured. Results of this agromining pot experiment showed that on the fertilized Technosols, the plant biomass of *O. chalcidica* obtained was comparable with that obtained from the control ultramafic soil S (Fig. 4).

The Ni uptake from most of the waste-derived Technosols was comparable or even higher than the Ni accumulation by *O. chalcidica* obtained from the ultramafic soil S (Fig. 5), showing that agromining on substrates derived from galvanic sludges can produce similar Ni yields as those from natural ultramafic soils. Reduced biomass production and Ni uptake were observed for the Technosols W2H (Figs. 4 and 5), presumably due to, respectively, Cu toxicity and inhibition of Ni uptake related to higher Zn concentrations in the substrate. This work was conducted as part of the LIFE Agromine project (LIFE15 ENV/FR/000512).

3.3 Case Study 3: Agromining Stainless Steel Industrial Sludge

In the frame of the LIFE Agromine project (LIFE15 ENV/FR/000512), agromining tests on industrial waste are currently being carried out in France at a pilot scale within the facilities of the APERAM company, one of the major European producers of stainless steels and high-added value alloys (Ni-based). The stainless-steel production processes and more specifically the chemical pickling operation, which is required after thermal annealing, are responsible for the generation of industrial sludges whose main characteristics are presented in Table 3. These sludges, resulting from a flocculation process, present a mean dryness of about 40%, obtained owing to a post-treatment filter press. Table 3 provides a characterization overview of the sludges used in the experiments, which are detailed later in this chapter. About 37% of the sludges were composed by Ca and Fe due, respectively, to the neutralization post-treatment of the sludges (using calcium oxide) and the origin of ore (iron). Among trace elements, the amounts of Cr reached up to 3.19% of dry matter (DM), whereas Ni had a mean of 1.36% of DM. Sludges were characterized by contents of Al, Cu, Zn and Mo <1% DM (Table 1a), and negligible amounts of Pb (50.1 mg kg^{-1}), Cd (1.1 mg kg^{-1}), As (13.9 mg kg^{-1}), and Hg (0.5 mg kg^{-1}).

The results given in Table 3b show that the pH was slightly alkaline (7.8), which is compatible with agromining. P_2O_5 concentrations had high values (8 180 mg kg^{-1}), whereas the amounts of total organic carbon (1.29%), total nitrogen (1%), and K_2O (705 mg kg^{-1}) were low, that implies potential lack of fertility for the hyperaccumulator plants. Moreover, the sludges had a high conductivity value of 2490 µS cm^{-1}, due to soluble sulphate and chloride contents (data not shown), which limited the first germination tests of seeds conducted with the hyperaccumulator plant *O. chalcidica*. Alkaline pH conditions limited the availability of phytotoxic elements and reduced Ni concentrations in

Table 1 Pseudo-total concentration, DTPA extractable metals (average values ± relative standard deviation %, n = 2), water content and pH of the three galvanic sludges C1, W1 and W2

(a) Pseudo-total		Fe	Cr	Ni			Zn	Cu	Pb	Mn
LOQ	mg kg^{-1}	0.206	0.706	0.827		mg kg^{-1}	0.534	0.140	0.065	0.005
C1	g kg^{-1}	2.56 ± 15	<LOQ	150 ± 0.5		mg kg^{-1}	<LOQ	932 ± 7.6	106 ± 27	38.9 ± 1.7
W1	g kg^{-1}	31.3 ± 2.7	9.23 ± 3.2	84.9 ± 1.3		mg kg^{-1}	1100 ± 5.3	367 ± 0.4	44.3 ± 4.4	687 ± 0.2
W2	g kg^{-1}	105 ± 3.2	2.73 ± 9.0	104 ± 1.0		mg kg^{-1}	1580 ± 2.6	648 ± 4.2	32.3 ± 1.7	1024 ± 3.8
(b) DTPA		Mn	Fe	Ni	Cu	Zn		Water % fresh sludge	pH initial	pH after two months
LOQ	μg kg^{-1}	0.109	11.4	0.368	0.050	1.02				
C1	mg kg^{-1}	<LOQ	<LOQ	3405 ± 6.2	1.33 ± 46	<LOQ		45.4	10.1	8.9
W1	mg kg^{-1}	3.90 ± 15	116 ± 18	228 ± 20	6.89 ± 6.2	41.5 ± 14		64.9	8.2	8.2
W2	mg kg^{-1}	1.08 ± 4.7	<LOQ	290 ± 7.6	6.81 ± 60	14.2 ± 15		64.4	8.8	8.6

Values refer to dry weight. <LOQ = below the limit of quantification. Table modified from Tognacchini et al. (2020)

Table 2 Formulation of five technosols from the galvanic sludges C1, W1 and W2

Technosols composition	C1D	C1F	W1B	W2F	W2H
Sludge (%)	1	10	5	10	50
Compost (%)	2	2	1	2	2

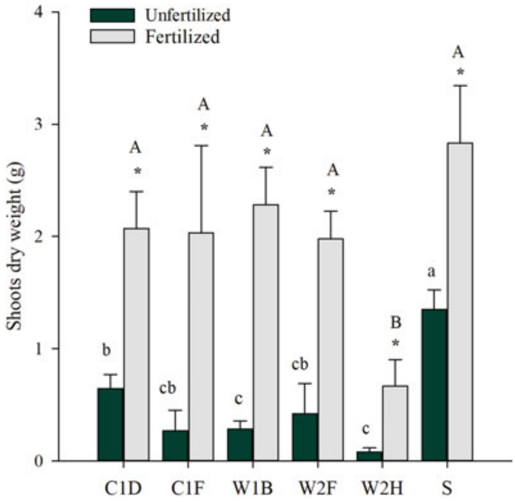

Fig. 4 Shoot dry weight of *Odontarrhena chalcidica* per pot. Average values ± standard deviation (n = 4). Statistical difference ($p < 0.05$) among substrates is indicated with different letters, lowercase for unfertilized, uppercase for fertilized replicates. *indicates statistical difference ($p < 0.05$) between fertilized and unfertilized treatments. C1D, C1F, W1B, W2F and W2H = Technosols, S = control ultramafic soil. Figure from Tognacchini et al. (2020)

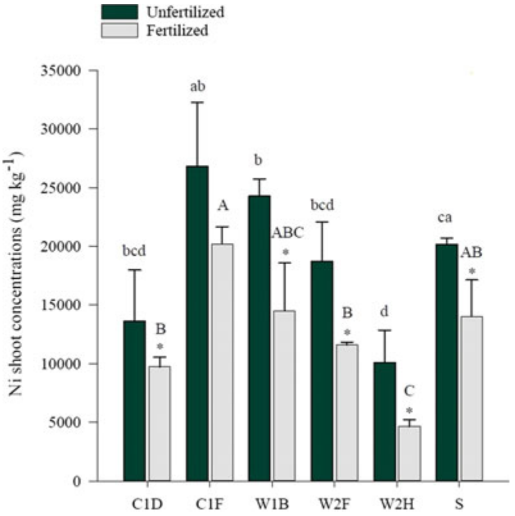

Fig. 5 Nickel shoots concentrations (mg kg^{-1}) of *Odontarrhena chalcidica*. Average values ± standard deviation (n = 4). Statistical difference ($p < 0.05$) among substrates is indicated with different letters, lowercase for unfertilized replicates, uppercase for fertilized replicates. *indicates statistical difference ($p < 0.05$) between fertilized and unfertilized treatments. C1D, C1F, W1B, W2F and W2H = Technosols, S = control ultramafic soil. Figure from Tognacchini et al. (2020)

Table 3 Main characteristics of APERAM sludges used in agromining experimentations: (a) pseudo-total amounts of Ca, Fe, Cr, Ni, Al, Cu, Zn and Mo in mg kg^{-1}, (b) agronomic parameters, and (c) quality of leachates

(a) Pseudo-total (mg kg^{-1} DM)

	Ca	Fe	Cr	Ni	Al	Cu	Zn	Mo
Sludges	192,000	179,000	31,900	13,600	2990	739	222	403

(b) Agronomic properties

	pH	Total organic carbon	Total nitrogen	P_2O_5	K_2O	Conductivity
Units	–	% DM	g kg^{-1} DM	mg kg^{-1} DM	mg kg^{-1} DM	µS cm^{-1}
Sludges	7.8	1.29	1	8180	705	2490

(c) Leachates (mg kg^{-1} DM)

	As	Ba	Cr	Cu	Mo	Ni	Pb	Zn	Cd
Sludges	<0.200	<0.100	1.810	0.380	15.700	0.520	<0.100	<0.200	0.018

leachates (0.52 mg kg^{-1}) (Table 3c). These results showed a possible reduced availability of Ni that might negatively impact Ni uptake by the hyperaccumulator plants. These sludges were classified as hazardous materials mainly because of the amount of Mo in leachate (15.7 mg kg^{-1}), which is higher than the authorized European limit (<10 mg kg^{-1} to be considered as non-hazardous; EU Directive 2003/33/CE), but also due to the H14 criterion relative to the ecotoxicity of the waste (EU Directive 2008/98/CE—Annex III). As an indicator, the regulatory limit for the Ni content in leachate to be considered as non-hazardous waste is 10 mg kg^{-1} (in dry matter). In order to compensate for the lack of sludge fertility as well as to optimize the availability of Ni, setting up agromining on APERAM's sludges required engineering formulation. Used as a source of Ni, sludges should be first amended in order to formulate functional Technosols for cultivating hyperaccumulator plants and for agromining.

The first analyses indicated a fertility lack for APERAM's sludges (low organic matter content and low nutrients concentrations). In order to improve the agronomic characteristics of these sludges with an aim to formulate a functional Technosol for agromining, some local amendments were identified that limited additional costs (transportation, etc.). A mix of pickling soil, waste rock and sand enriched with local compost, named Subster®, was produced by a local operator. Before upscaling (see Sect. 6 of this chapter), different concentrations of Subster® were tested in mixture with the sludges. The test concentrations of Subster® amendments were 10, 50, 90 and 95% v/v. Two seeds of the hyperaccumulator plant *O. chalcidica* were sown in 1-L pots under controlled conditions (growth chamber). Sludges and Subster® were well mixed and homogenized before filling the pots. Each mix of sludges and Subster® had three replicates and the plants were harvested and analyzed after three months (Fig. 6). Figure 6 shows the clear effect of Subster® addition on plant growth and biomass production. Fresh and dry plant aerial biomass were measured at the harvest. The results are summarized in Fig. 7 (Saad et al. 2019, unpublished). A clear augmentation of fresh and dry biomass was shown when the concentration of Subster® was increased. However, no difference of plant biomass was evident between the levels of 10 and 50% of Subster® and between 90 and 95% Subster®.

Figure 8a, b shows the effect of Subster® addition on the concentration of Ni and quantity of Ni extracted by *Odontarrhena chalcidica*. Surprisingly, the concentration of Ni in the plant aerial part was not positively correlated with the Subster® gradient in the Technosol. On the one hand, the highest values of Ni concentration and Ni extracted were obtained in the 50% mixture of APERAM's sludges and Subster® amendment. On the other hand, the lowest values were observed in the case of the 95% Subster® amendment. The last observation could be explained by the low presence of sludges in the mixture and therefore available Ni for the hyperaccumulator plants. According to these results and the goal to optimize the quantity of sludges treated (90 or 95% Subster® amendments are not both industrially viable and environmentally acceptable), two different mixture solutions were finally used for the upscaled experimental tests in the APERAM facilities:

Fig. 6 Pot experiment where the gradient of Subster® concentrations was tested. From left to right 10, 50, 90 and 95% of Subster® v/v (three pots for each concentration) (Saad et al. 2019, unpublished results)

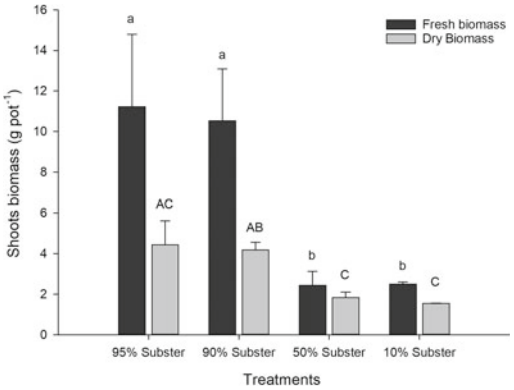

Fig. 7 Fresh and dry plant aerial biomass of *Odontarrhena chalcidica* at the harvest time (g pot^{-1}). Average values ± standard deviation (n = 3). Statistical difference ($p < 0.05$) among substrates is indicated with different letters, uppercase for dry biomass and lowercase for fresh biomass (Saad et al. 2019, unpublished results)

50%/50% (Sludges/Subster®) and 70%/30% (Sludges/Subster®), as presented in Sect. 6. Of course, in the case of future industrialization of the approach, full compliance with the EU Directive 2008/98/CE (and the EU Communication 2018/C124/01), relative to the waste qualification and management, enforcing a mixture ban of the waste (including other non-dangerous waste or product) will have to be respected. With this perspective, as detailed in the following Upscaling Sect. 6, only the extreme upper layer of the testing facilities will be composed of the stated mixture of sludges/Subster®, in order to ensure the sustainability of the seedlings in the early stages of the experiment.

4 Effects of Different Amendments

In agromining experiments conducted on Technosols derived from incineration bottom ashes (Rosenkranz et al. 2017), automotive sludge (Rue et al. 2019), and galvanic sludges (Tognacchini et al. 2020), organic amendments added to the waste materials were essential to improve the physical structure and provide macro- and micronutrients. Preferably, organic waste or by-products should be used as organic amendments on the metal-rich waste (being quite easy to find

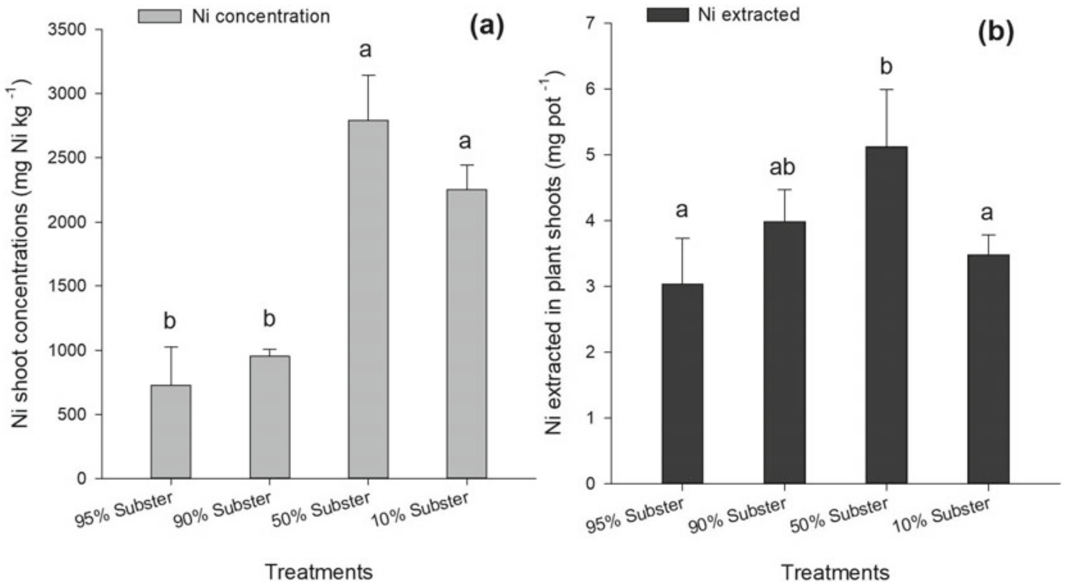

Fig. 8 **a** Nickel concentrations (mg Ni kg^{-1} DM) in *Odontarrhena chalcidica* and **b** Ni extracted (mg pot^{-1}) in the plant aerial part of *Odontarrhena chalcidica* at the harvest time. Average values ± standard deviation (n = 3). Statistical difference ($p < 0.05$) among substrates is indicated with different letters (Saad et al. 2019, unpublished results)

local compost sources from green wastes or water treatment works near the production site of such wastes), rather than more valuable compounds such as manure or biochar. Local compost represents an inexpensive solution to improve the Technosol fertility as well as the water-holding capacity. Composts are commonly rich in microorganisms (fungi and bacteria), which biodegrade organic matter and provide long-term plant nutrition. Rosenkranz et al. (2017) added 20% residues from biological treatments of municipal solid waste and 10% biochar to the incineration bottom ashes, whereas Tognacchini et al. (2020) added 1 or 2 wt% of compost obtained from municipal waste to the waste mixtures. Biochar amendments (up to 5 wt %) resulted in an increase of biomass production in *Odontarrhena* spp. (Rue et al. 2019) with no effect on plant Ni uptake.

As well as the addition of organic compounds, it was observed that NPK fertilization was essential for achieving a reasonable plant growth on galvanic sludge-derived substrates (Tognacchini et al. 2020), seemingly due to the poor N and P contents in the sludges and an unbalanced K supply. Even if organic fertilizers are preferable under field conditions in terms of environmental impact, for artificial matrices derived from waste and agromining applications in controlled conditions, the use of chemical fertilizers or industrial by-products with similar characteristics are suggested to provide easily available and balanced nutrient supplies. In this regard, an advantage of working with waste materials could be the possibility to combine nutrient-rich compounds discarded from other industrial processes that are not applicable on field conditions to fertilize waste-derived Technosols. Besides the addition of organic matter, mineral compounds might be necessary to improve the structure of Technosols. Quarry by-products can be a source of local mineral matter (for e.g. sand, pickling soil, waste rock) useful to enhance the agronomical properties of Technosols (texture, porosity, pH, carbonate, macro- and microelements). Those materials are often discarded by quarrying activities and could find a use in Technosol formulations.

Due to the extreme pH values often found in waste materials, pH corrections can be necessary prior to agromining applications. A galvanic sludge having very alkaline pH (10.1) resulted in a natural decrease in pH after two months of ageing (pH after two months, 8.9), which further decreased after Technosol formulations (Tognacchini et al. 2020). This result shows that for certain waste materials the ageing process is sufficient to achieve pH corrections without further amendments. On incineration bottom ashes, incubation with diluted nitric acid (0.814 mol L^{-1}) before the formulation of artificial substrates lowered the pH to 7.6 on materials with original pH values of 11.2 and 12 (Rosenkranz et al. 2017). This approach, even if effective, could present some limitations in large-scale applications, especially due to the costs of nitric acid. Other approaches of pH adjustments commonly used in agriculture, such as sulphur addition, have not been tested so far to balance the extreme pH values in waste materials for phytomining experiments. Only liming treatments were tested for pH correction on substrates derived from acid automotive sludge in Rue (2017), resulting in a lower Ni uptake in shoots of *B. emarginata* compared to the untreated substrate (Fig. 1). Again, by-products or discarded materials from industrial production with highly acidic or alkaline pH could be employed in pH correction treatments on the metal-rich waste targeted by agromining. Leaching treatments might also be necessary in order to reduce soluble salts of waste materials, paying attention that target elements and essential nutrients are not being leached out. Water leaching, for example, might provide an effective reduction of soluble salts without loss of Ni or other target elements, as previously tested on incineration bottom ashes (Rosenkranz et al. 2017). Pre-tests are suggested for defining the effectiveness of leaching treatments, especially on those waste materials where the solubility of target metals might be high.

Whilst pH adjustments, structure improvements, removal of excessive salts, and supply of basic nutrients might be essential steps to allow plant establishment on waste-derived substrates, other types of treatments could be applied to improve plant growth, metal resistance, and uptake of target metals. An important factor to consider when working with waste-derived artificial substrates is their poor microbiome compared to natural soils, especially if no incubation time was left after substrate formulations. Seemingly, the addition of organic amendments represents an important source of microbiota in waste materials, assuming that microbial communities in industrial waste materials are rather poor. Because hazardous waste materials are to the greater extent being discharged, no interest has yet emerged in characterizing microbial communities found on those substrates. However, a number of studies have recently investigated bacterial strains isolated from heavy metals-contaminated industrial wastewater for bioremediation purposes (Bharagava and Mishra 2018; Nokman et al. 2019), showing that several bacterial strains could be found despite the unfavourable conditions of industrial streams. Positive effects of microorganisms on plant growth and resistance to stress factors are commonly recognized, and beneficial effects of introducing microbial communities on waste-derived substrates might be even greater than on soil. Considering the different waste characteristics that could cause stress response in plants, such as salinity or presence of toxic metals, the use of microorganisms could be notably advantageous to support plant establishment. Extensive research is available on plant-microbe associations, due to the recognition of positive effects on plant growth as well as on metal uptake and resistance.

Bacterial strains have been demonstrated to enhance resistance to metal toxicity as well as promote plant growth on contaminated substrates (Álvarez-López et al. 2016). Plant-associated bacteria can potentially improve phytoextraction by altering the solubility, availability and transport of heavy metals and nutrients by reducing soil pH, release of chelators, P solubilization or redox changes (Ma et al. 2011). Plant growth promoting bacteria (PGPB) can support their host plants by stimulating growth and biomass production, nutrient acquisition, and increasing resistance to various stress factors as, for example, heavy-metal toxicity (Mendes et al. 2013), and can alter the uptake of heavy metals through their effects on plant growth dynamics or through acidification, chelation, precipitation, immobilization and oxidation-reduction reactions in the rhizosphere (Ma et al. 2011). Recently, the concept of 'MAP' (microbial assisted phytoextraction or phytomining/agromining) was introduced (Thijs et al. 2016), referring to soils for improving metal extraction through increases in biomass (yields) production and metal availability (plant uptake). This concept could be extended to microbially assisted agromining from waste materials (MAPW).

Several plant-associated bacteria have been reported to significantly accelerate phytoremediation in metal-contaminated soils by promoting plant growth and health (Grandlic et al. 2008; Kuffner et al. 2008; Kidd et al. 2009; Ma et al. 2009; Glick 2010). For example, increased Ni mobilization and uptake in *Odontarrhena* spp. when inoculated with rhizobacteria isolated from rhizosphere of *Odontarrhena* spp. was reported (Abou-Shanab et al. 2006). Rhizobacteria inoculation also enhanced the growth and phytoextraction capacity of *N. tabacum* (Álvarez-López et al. 2016). In *B. juncea*, higher biomass and Ni, Zn, and Cr were obtained with metal-resistant PGPB isolated from fly ash-contaminated soil (Kumar et al. 2008), and higher biomass and Cu uptake with inoculation of Cu-resistant PGPB (Ma et al. 2009). Endophytic fungi also showed positive effects on plant growth under metal stress on *Arabidopsis arenosa* (Domka et al. 2019). Considering the various constraints that limit plant establishment on substrates derived from waste materials, advantages of microbial inoculations appear to be especially beneficial. At present, microbial inoculations on waste materials were only investigated on a substrate formulated from incineration bottom ash with PGB-bacteria isolated from contaminated substrates (Rosenkranz et al. 2018). Results reported by Rosenkranz et al. (2018) showed an enhanced biomass production on *N. tabacum* and *S. smithiana* growing on incineration ash-derived Technosols, but no effect on metal uptake.

Bacterial strains known for PGP effects could be tested on waste materials to investigate resistance to those specific conditions and if similar effects are found, then tested with hyperaccumulator plants. The same approach is valid for endophytic fungi or other microorganisms exhibiting positive effects on growth and/or metal uptake. Strains could also be isolated directly from the waste materials and then tested for their PGP effects, with the advantage that these strains would already be adapted to the conditions of the substrate. In this regard, further studies should be conducted to test efficiency of endophytic fungi/bacteria or rhizobacteria in enhancing biomass production, and resistance to toxic metals and salinity.

5 Main Findings and Limitations

As for Ni-rich soils, a high Ni shoot uptake as well as high plant biomass production are essential for Ni agromining applications on waste. Current research shows that the main limitations on use of waste-derived Technosols resulted in either minimal plant uptake and/or low biomass production. A very low plant biomass of *O. serpyllifolia s.l.* was reported by Rosenkranz et al. (2017), plausibly as a symptom of high contents of soluble salts and Cu toxicity. As mentioned above, excessive salt contents in waste could be addressed with leaching treatments on the waste materials; the presence of bioavailable potentially toxic elements such as Cu should be addressed during the waste selection stage and eventually monitored after substrates formulations. Several studies report plant biomass reduction as a consequence of Cu phytotoxicity (Ebbs and Kochian 1997; Pietrzak and Uren 2011; Lange 2016), and also on the Ni hyperaccumulator *O. serpyllifolia* (Kidd and Monterroso 2005). Tognacchini et al. (2020) reported significant plant growth inhibition in *O. chalcidica* on a galvanic sludge-derived substrate with higher Cu availability (DTPA-extractable Cu 33.3 mg kg^{-1}), with the biomass reduction being strongly correlated with increasing Cu concentrations in shoots. Therefore, it is suggested that agromining operations utilising *Odontarrhena* spp. should be avoided on substrates having high Cu availability due to a strong phytotoxic effect.

A critical step prior to agromining operations is therefore the selection of suitable waste substrates, taking into account the presence of potentially phytotoxic elements depending on the plant species that will be used. Tognacchini et al. (2020) applied a waste selection criterion based on the Zn/Ni and Cu/Ni concentration ratios of either total and plant-available (DTPA-extractable) fractions: only waste materials with a Zn/Ni or Cu/Ni ratio <0.25 were considered suitable for agromining applications. The chosen Zn/Ni ratio was effective as no Zn interferences with Ni uptake were detected. However, for Cu absolute values of total, or more especially, plant-available concentrations, should be considered rather than the ratios. After substrate formulation from the raw waste materials, plant-availability of target and potentially interfering/phytotoxic elements should be re-evaluated, as changes in pH or increased amounts of organic matter might affect the available fractions of different metals. Tognacchini et al. (2020) showed that Cu availability (DTPA-extractable) of a galvanic sludge had increased approximately 4-fold after substrate formulations were prepared. In selecting appropriate amendments, it is crucial to find a balance between increasing availability of target and potentially toxic metals, e.g. through pH corrections. Rosenkranz et al. (2017) showed that in the incineration bottom ashes tested, the total and NH_4NO_3-extractable Cu in the substrates were higher than those of the target elements Ni and Zn. Other factors that might constrain agromining from waste is the presence of other metals potentially interfering with Ni uptake into shoots.

As mentioned above in Case Study 1, Rue et al. (2019) demonstrated that the main limitation to successful agromining was represented by high Zn concentrations in the substrate, which caused interference with Ni uptake in shoots of *Odontarrhena* spp. Even if *Odontarrhena* spp. showed resistance to high Zn availability (DTPA-extractable Zn 90.8 mg kg^{-1}; Rue et al.

2019), interferences with Ni uptake occurred, perhaps due to common transporters. In *Noccaea* (syn. *Thlaspi*) *caerulescens*), a Ni-, Zn-, and Cd-hyperaccumulator plant, Zn ± Ni interference was also reported in several experiments (Escarré et al. 2013). Thus, when Ni extraction is targeted, waste materials having relatively high Zn concentrations (total and plant-available) compared to Ni should be avoided, unless special experimental 'layered' set-ups are being developed to allow Ni ± Zn separation in the substrate (see Case Study 1). Known hyperaccumulator plants should be tested for salinity resistance and resistance to toxicity of other elements possibly present in waste matrices, in order to assess suitable applications on specific waste types. So far, the salinity resistance of the Ni hyperaccumulators *O. chalcidica* and the Ni, Zn, and Cd hyperaccumulator *Noccaea* (syn. *Thlaspi*) *caerulescens* have been investigated (Comino et al. 2005); a moderate salinity resistance (NaCl) in *Odontarrhena* spp. was observed, whereas *N. caerulescens* proved to be more sensitive to salinity (Comino et al. 2005).

Assessments of plant-available metals in soil are commonly used in agromining application on Ni-rich soils and are considered essential for achieving reasonable uptake into plant shoots. Different extractions with DTPA, NH_4NO_3, or water have been applied on waste and waste-derived artificial substrates to assess metal availability prior to agromining experiments. However, it has to be considered that metal availabilities in waste materials are regulated by different factors than in soil. Rosenkranz et al. (2017) hypothesized that in bottom ashes, Ni availability was mostly controlled by silicate-glass compounds present in the ashes rather than by amorphous Fe-oxides as reported for ultramafic soils (Massoura et al. 2006). For example, acid incubation did not have the effect of increasing Ni availability, as would be expected in soil. Moreover, the ability of hyperaccumulator plants to potentially mobilize metals from the substrate needs to be considered. In an experiment conducted on waste from a serpentine quarry (Puschenreiter, unpublished), it was observed that *O. chalcidica* was capable of accumulating 3380 mg Ni kg^{-1} from a substrate containing serpentine rock mixed with commercial potting substrate and characterized by moderate Ni availability (770 mg kg^{-1} total Ni; 0.4 mg kg^{-1} $Sr(NO_3)_2$-extractable Ni; 0.15 μg Ni L^{-1} in soil solution). Thus, waste materials with low to moderate Ni availabilities should not be excluded a priori, since mobilization processes in the rhizosphere could still allow sufficient metal concentrations in shoots. This aspect is particularly important since few studies on plant-availabilities of target elements in waste have been conducted, and no interest in this parameter had arisen before the introduction of waste-agromining. Furthermore, it has to be considered that methods to estimate plant availability have been developed for soils and do not necessarily have the same effectiveness when applied to different matrices such as waste materials. In this context, it is always advisable to use various extractants (e.g. $CaCl_2$ for measuring soluble and easily exchangeable metals; DTPA or EDTA for determining metals that can be mobilized by complexation, etc.) for assessing different operationally-defined pools, and to conduct pilot tests for evaluating the capacity of plants to accumulate Ni or other metals from a certain matrix.

Another factor to be considered and which can limit plant growth is the imbalanced nutrient supply in waste-derived Technosols. Even if sources of essential nutrients are provided (e.g. by NPK fertilization), plant uptake of certain nutrients might be limited by excessive concentrations of other elements. In some galvanic sludges, for instance, Ca concentrations are much higher than Mg, contrarily to natural ultramafic soils that are characterized by low Ca/Mg ratios. In general, germination tests with the plant species to be used in waste-agromining are recommended, in order to define the adaptability of hyperaccumulator plants on specific waste types. Concerning the low biomass production obtained with waste-agromining trials, it has to be considered that all experiments were conducted in small pots under controlled greenhouse conditions. With further upscaling of agromining trials, it will be clearer whether the biomass production rates are comparable with those of field applications on ultramafic soils.

Certain characteristics of waste materials which could represent a limiting factor for agromining could be addressed at the industry level. For some types of waste materials, measures could be applied aiming at facilitating metal recovery and agromining. For example, plant toxicity and salinity issues of the waste could be addressed by avoiding the mixing of industrial waste streams containing potentially phytotoxic metals (e.g. Cu), as well as finding alternative ways of sludge precipitation employing less Na-containing compounds. Nevertheless, for some waste types as, for example, incineration ashes, it would be very challenging to facilitate metal recovery by modification of waste composition at the waste generation level.

6 Upscaling

To date, agromining experiments on waste-derived substrates have been conducted only on a trial scale through greenhouse experiments. In this Section, we illustrate how agromining applications can be upscaled from greenhouse trials to larger containers and how this technology could be implemented within waste-generation facilities. Pioneering trials of upscaling agromining on industrial waste, currently underway at APERAM facilities as part of the LIFE Agromine project (LIFE15 ENV/FR/000512) are presented.

After the successful formulation of Technosols and positive results obtained with growth of the hyperaccumulator plant *O. chalcidica* on APERAM sludges at the laboratory scale (in pots—cf. Section 3, Case Study 3), the agromining process was upscaled to be set up in large containers within APERAM facilities at Isbergues, France. In order to conform to the national regulation for the use of polluted substrates, the Technosols were built in mobile and waterproof containers with the aim to avoid any soil or water contamination (Fig. 9a). Four specific containers, having double walls and a 400 µm filtering tarp, were used, integrating two specific Technosols within each, only differing from one to another by the top layer, as illustrated in Fig. 9b. The bottom and middle layers were the same for all four containers, but the 10-cm-thick top layer was different depending on the Technosol used. Slags coming from Electric Arc Furnaces (produced by APERAM—Recyco) and sieved between 10 to 30 mm, were used as the bottom layer (50 cm height) in order to drain rainwater (representing about 10 t in each container). Above the slags, a 50-cm-thick middle layer composed of APERAM pure sludges (representing about 4 t in each container—composition described in Table 3) was placed. Finally, the top layer was formulated from APERAM's pure sludges and SubsTer® amendment (mixture of sand, pickling soil, waste rock, and compost—this amendment was already tested in controlled conditions). As explained in Sect. 3 Case Study 3, two different mixes were selected as a compromise for plant growth, Ni extraction capacities, and industrial viability: 50%/50% v/v of APERAM's sludges and SubsTer®, respectively and 70%/30% v/v of APERAM's sludges and SubsTer®, respectively. Two hyperaccumulator plants were cultivated for Ni agromining: *O. chalcidica* and *B. emarginata*. The two Technosols were tested with each one of these hyperaccumulator plants, resulting in the creation of the four different containers. Hyperaccumulator plants were grown beforehand under controlled conditions, with exactly the same soil properties and mixtures as in the containers, before being transplanted in November 2019, according to the defined grid shown in Fig. 9c.

As a result of the integrated water filtration process of the containers, the drained water was collected weekly and analyzed in order to ensure a strict compliance of environmental legislations. Table 4 lists the mean values for the different metallic elements present in the drained water as filtered by the Technosols, compared to the imposed upper legal limits for a direct water rejection in the environment: all of the values are below these environmental legal limits combined to a pH mean value of 7.4 (±0.4), respecting the legal authorized interval for a direct water rejection in the environment (pH 6.5 to 9).

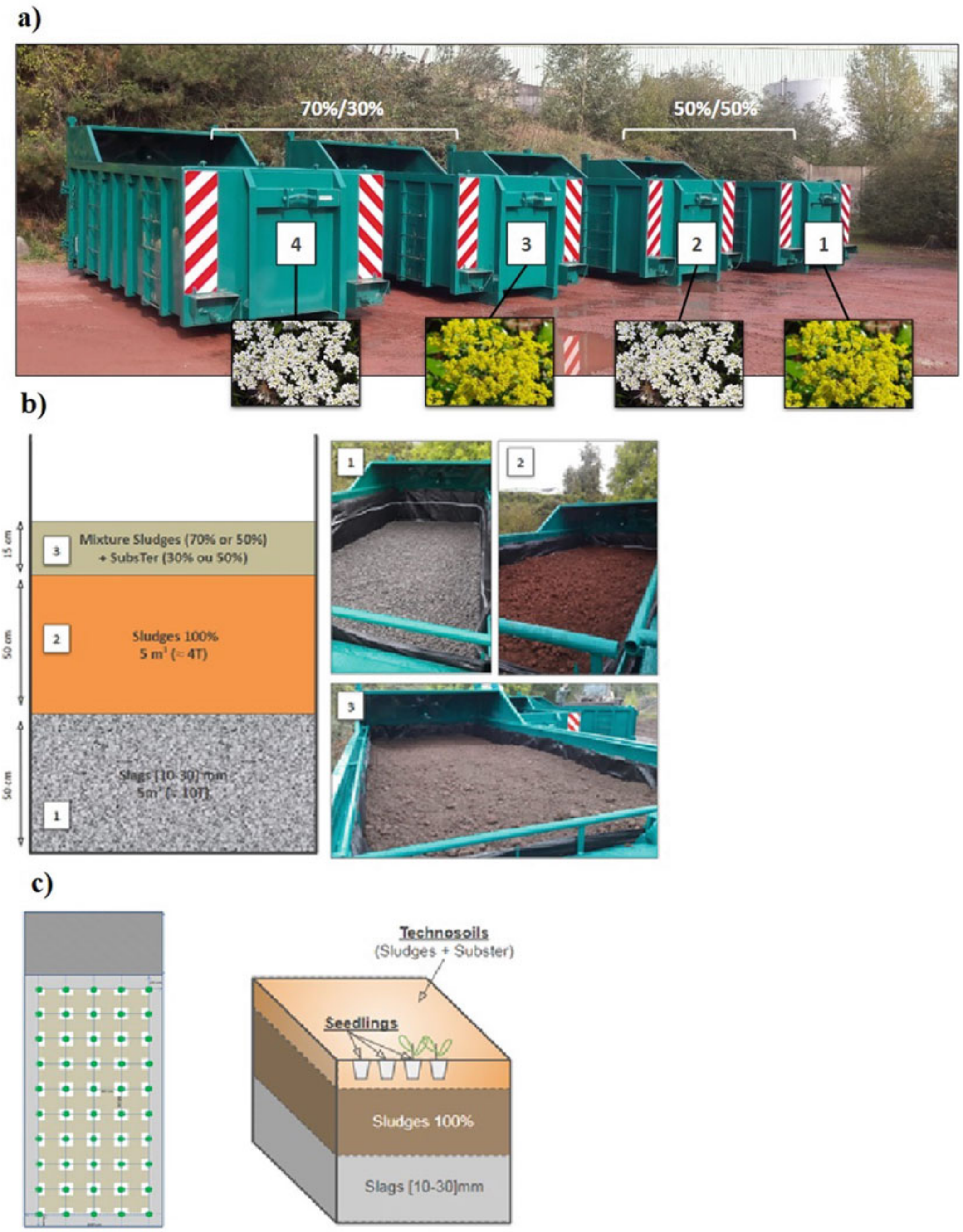

Fig. 9 **a** Four containers for Ni-agromining set-up within the APERAM's site at Isbergues, France, **b** figures showing the two formulated Technosols in the container, **c** hyperaccumulator plants transplantation in each container according to the defined grid

Table 4 Mean values (mg l^{-1}) of the different metallic elements present in the drained water of the Ni-agromining upscaled set-up and legal limits for a direct water rejection in the environment (*French legislation)

Drained water analyses versus legal limit (mg l^{-1})

	As	Cr	Cu	Mo	Ni	Pb	Zn	Cd
Drained water	0.02	0.07	0.05	2.87	0.05	0.07	0.39	0.002
Legal limit*	0.10	2.10	2.00	N/A	2.00	0.50	3.00	0.20

Experiments are still in progress and the hyperaccumulator plants will be harvested during Spring 2020. Plants and Technosols will be analysed in order to estimate the efficiency of Ni-agromining on APERAM's sludges.

7 Metal Recovery from Plants Versus Waste: Advantages and Limitations

Agromining on waste materials is still at a development stage and experiments conducted so far have shown several limitations in addition to some promising results. Regardless of this, the successfulness of Ni recovery from waste-derived substrates using hyperaccumulator plants leaves an open question: why not recover Ni or other valuable elements directly from the waste materials? As discussed above, wastes often contain target elements in concentrations that are too low to allow an economically convenient direct recovery, thus hyperaccumulator plants could provide an efficient bioaccumulation step that further concentrates metals before extractions. In practice, agromining trials conducted so far on waste materials have shown: (i) low Ni plant uptake due to low Ni availability in the waste, (ii) low Ni plant uptake due to Zn interference, or (iii) very high Ni plant uptake from waste already rich in Ni and with high Ni availability. From a galvanic sludge containing ~80 g Ni kg^{-1}, ~1 g Zn kg^{-1}, ~0.4 g Cu kg^{-1}, 4 g K kg^{-1}, 30 g Fe kg^{-1}, and 50 g Ca kg^{-1}, a dry biomass of *O. chalcidica* was obtained containing ~15 g Ni kg^{-1} and ~0.08 g Zn kg^{-1}, ~0.005 g Cu kg^{-1}, ~17 g K kg^{-1}, ~0.2 g Fe kg^{-1}, and ~37 g Ca kg^{-1} (Tognacchini et al. 2020), corresponding approximately to 150 g Ni kg^{-1}, 0.8 g Zn kg^{-1}, 0.05 g Cu kg^{-1}, 170 g K kg^{-1}, 2 g Fe kg^{-1}, and 370 g Ca kg^{-1} assuming a concentration factor of 10 after biomass incineration. This result suggests that cultivation of *O. chalcidica* on a substrate derived from a galvanic sludge results in a plant-derived bio-ore material with significantly higher Ni/Zn, Ni/Cu and Ni/Fe ratios, and significantly lower Ni/K and Ni/Ca ratios, than in the original sludge.

Lower Fe concentrations in the 'ore' are especially beneficial in terms of Ni recovery, thus an advantage of bio-ores compared to waste-ores could be the relative reduction of Fe compared to Ni. Hyperaccumulator plants can efficiently separate Ni from Fe, with the Ni/Fe mass ratio upgraded from 1:100 in the soil to 40:1 in the plant dry matter; the hyperaccumulator plant is thus able to achieve a 4000-fold relative enrichment of Ni (van der Ent et al. 2015). Furthermore, a bioconcentration of Ni by almost a factor of 2 was obtained in Tognacchini et al. (2020) from a galvanic sludge to the biomass of *O. chalcidica*, thus using hyperaccumulator plants a 'bio-ore' with double the Ni concentration was obtained. Bioconcentration factors might be much higher when targeting lower-grade waste materials. Potassium and Ca were higher in plant biomass; however, in the same experiment, K and Ca concentrations in shoots were comparable when *O. chalcidica* was cultivated on ultramafic soil (Tognacchini et al. 2020). It is worth mentioning that within greenhouse pot experiments involving short growth periods and low plant biomass, the relative amounts of different elements in the biomass might not be representative and that with technology ameliorations and upscaling, the ratios between elements in plant biomass might change.

Efficient Ni recovery from plant biomass largely depends on the composition and relative abundance of Ni and other elements. In industrial waste materials, other metals such as Cd, Cr, Zn and Cu can be present at very high concentrations. Plant ashes can have higher concentrations than the waste itself and allow energy recovery during the incineration process. Nickel concentrations of up to 100–200 g kg^{-1} were found in ashes of the hyperaccumulator *Odontarrhena* spp. grown from soils with about 0.2 wt% Ni, which corresponds to concentration increases from soil to ash of factors of 50–100. Thus, an advantage of an agromining approach is the possibility of targeting those waste materials where insufficient Ni concentrations do not allow direct recovery. Also, from plant ashes, high-purity Ni compounds such as Ni salts could be obtained, which have a higher market price that metallic Ni. In addition, new techniques are being developed to extract Ni compounds directly from the plant biomass before incineration. Common characteristics of industrial wastes are the variability and lack of homogeneous compositions among waste stocks produced at different times. Although a certain variation can be found in plant composition depending on the substrate, with use of plants a more homogeneous product can be obtained from different materials, allowing standardizations of the Ni extraction methods. Results of Tognacchini et al. (2020) show that a certain degree of variability in element composition resulted in *O.chalcidica* when growing on different waste-derived Technosols or on ultramafic soils; nevertheless, the shoots of *O. chalcidica* growing on the different waste-derived substrates presented a much more homogeneous composition than the wastes themselves.

A potential disadvantage of agromining from waste is the fact that waste materials in most cases cannot be directly phytomined but require an intermediate step of substrate formulation. Although this constraint might in some cases be demanding, further research aimed at formulating substrates, optimally combining different industrial by-products as organic materials, could provide effective and standardized processes. With the development of established and effective formulations and process chains, and by defining which specific waste materials may be targeted, agromining could certainly represent an advantageous approach for exploiting secondary resources. Agromining could also reduce contamination levels in some waste materials before landfilling, hence reducing metal loads (hazardous level) and thus the discharge costs of the wastes.

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Phytoextraction of Cadmium: Feasibility in Field Applications and Potential Use of Harvested Biomass

Thibault Sterckeman and Markus Puschenreiter

Abstract

Cadmium is one of the most threatening soil contaminants because of its high toxicity and widespread anthropogenic distribution. Therefore, remediation of Cd-polluted soils is urgently required. Cropping Cd-accumulating plants appears to be the most relevant approach for removing this pollutant from large soil surfaces. Several field studies have shown the potential of phytoextraction to clean up moderately Cd-contaminated sites, but this process still has important limitations. In some cases, hyperaccumulating plants such as *Noccaea caerulescens* and *Sedum plumbizincicola* showed considerable Cd removal rates due to their extraordinary accumulation capacities, but often suffer from strongly limited biomass production, especially *N. caerulescens*. *Sedum plumbizincicola* appears as a promising species for the remediation of low-Cd contaminated soils in subtropical climates. Interesting results were also found for other plants, e.g. some cultivars of 'indica' rice (*Oryza sativa*) or *Solanum nigrum*, but in these cases, further studies are required for confirmation. Some fast-growing willow clones, cultivated in short rotation coppice, offer a possibility to produce energy biomass on contaminated soils more than a means to decontaminate them, because of their low extraction rate. Relatively little is yet known about use of the harvested biomass produced through phytoextraction. A few studies showed that combustion is a feasible option, since Cd is mainly retained in the fly ash, whereas the bottom ash contains relatively low amounts of Cd and could even be used safely as fertilizer. Further investment is required in order to evaluate the possibility of producing high-performance cultivars of the best Cd hyperaccumulators. If this work yields positive results, the complete process chain will need to be tested on a large scale, including the biomass valorization.

1 Introduction

Cadmium toxicity was first recognized in the middle of the 19th century. Several cases of acute poisoning by the metal, resulting from intense exposure of workers, were described in the first half of the 20th century. The toxicological effects of Cd were confirmed through animal

T. Sterckeman (✉)
Laboratoire Sols et Environnement, Université de Lorraine, INRAE, BP 20163, Vandœuvre-lès-Nancy Cedex 54505, France
e-mail: thibault.sterckeman@univ-lorraine.fr

M. Puschenreiter
Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences Vienna (BOKU), Konrad-Lorenz-Straße 24, 3430 Tulln, Austria

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_15

experiments in the early part of the 20th century. Subsequently, Cd and its compounds were classified as carcinogenic to humans (Nordberg 2009). Furthermore, Cd was demonstrated to be strongly retained in the human body, particularly in the kidneys (Järup and Åkesson 2009). This fact makes low-level chronic exposure a serious threat for human health, through accumulation of the metal that can cause kidney failure and increase the risk of cancer (EFSA 2012). As regulations have reduced atmospheric Cd emissions, food is now the primary source of human exposure in the non-smoking population. Itai-Itai disease, described in Japan in the 1950s, was the first recognized case of acute Cd intoxication of humans through food intake. This painful bone disease was caused by the consumption of Cd-contaminated rice. A mine had released the metal in the Jinzu River from which water was used to irrigate the paddy fields (Nordberg 2009). Subsequently, various epidemiological studies were carried out, revealing that numerous people were affected by Cd exposure (Nawrot et al. 2010). The consequence of these investigations is that the US Agency for Toxic Substances and Disease Registry (ATSDR) and the European Food Safety Authority (EFSA) (ATSDR 2012; EFSA 2011) set recommended provisional safe intake limits for Cd. If the EFSA limit is considered (2.5 μg Cd per kg of body weight per week), the populations of Europe, USA, and China are on average just at the limit, whereas those of Japan and much of the remaining world would be above (according to FAO/WHO). Referring to the US limit (0.7 μg Cd per kg of body weight per week), the health of nearly all populations in the world would be threatened by Cd in food (Clemens et al. 2013).

In Europe, EFSA estimated that grains and grain products (26.9%), vegetables and vegetable products (16.0%), and starchy roots and tubers (13.2%) had the greatest impact on dietary exposure to Cd (EFSA 2012). The Cd contamination of food is due to widespread, low-level contamination of soils and to transfer of the metal towards the harvested plant organs through root absorption and subsequent translocation. Soil contamination originates from human activities that release Cd into the environment, as the metal is an impurity in various raw materials (fuels, phosphate ores, lead and zinc ores, etc.) or is used in products such as batteries or pigments (Clemens et al. 2013). Despite its toxicity, the industrial Cd usage in the world has increased steadily from 18,400 tonnes in 2003 to 20,400 tonnes in 2007 (Moulis and Thévenod 2010). In agricultural soils, Cd inputs are mainly derived from atmospheric deposition, phosphate fertilizer, sewage sludge, farm manure, and amendments to soils (Six and Smolders 2014). In Europe, the median Cd content in topsoils is 0.145 mg kg^{-1}; 90% of the topsoils contain <0.48 mg kg^{-1}, although concentrations up to 14.1 mg kg^{-1} have been measured (Salminen et al. 2005). Much higher concentrations of several tenths to several hundreds of mg kg^{-1} can be found in some industrial or mining regions.

In the densely populated and industrialized Nord-Pas de Calais region of France, Bourennane et al. (2010) found that the Cd concentration in the agricultural topsoil was up to 10 times higher than the pedogeochemical background in unaffected rural areas, and up to >30 times the background around industrial or urban areas. Although the use of Cd tends to be more and more restricted, for instance in Europe by the REACH regulation, Cd in polluted soil will not significantly disappear as it is strongly retained by the soil constituents, particularly organic matter. Moreover, the increasing need for phosphate fertilizers, in the face of limited phosphate resources (Gilbert 2009), will probably enhance the use of low-grade phosphate ores as well as the recycling of urban waste composts or sewage sludge, all containing Cd as impurities. The cultivated soil surfaces affected by Cd contamination are vast and two solutions seem currently possible for reducing the concentrations in plant production: selecting low Cd-accumulating cultivars and cultivating plants that extract the soil metal (Clemens et al. 2013). The feasibility of the latter approach, i.e. Cd phytoextraction (which does not exclude the first one), is examined in this chapter.

The ability of some plants—the hyperaccumulators—to accumulate high concentrations of metallic elements in their above-ground biomass has been known for more than 100 years, but for decades was only considered a botanical curiosity. In the early 1980s, it was proposed that hyperaccumulators could be used for phytoextraction (Chaney 1983). The 'metal yield' of this process is the product of harvested shoot biomass and its metal concentration. However, especially in temperate regions, these plants are normally relatively small, thus the metal yield remains unsatisfactory. As an alternative to the use of hyperaccumulators, two approaches were developed: (1) cultivation of high biomass and fast-growing plants moderately accumulating Cd, and (2) chemically assisted cultivation introducing acidifying amendments or using metal chelators (Vangronsveld et al. 2009). Because the second strategy revealed poor efficiency and threats to water resources through leaching of chelated contaminants (Lin et al. 2014; Nowack et al. 2006), it will not be considered in this chapter.

The phytoextraction approach has been tested for various metal contaminants including Cd, Zn, As, Ni, Pb and Cu. However, only Cd phytoextraction is considered here as this element is likely the most critical one regarding contamination of the food chain, as indicated above, and also because, in the context of soil clean-up, its phytoextraction seems more feasible compared to other metal contaminants. Indeed, Cd usually occurs in relatively low concentrations in soils (<10 mg kg^{-1}), and sufficient extraction rates were reported for several plant species. This is the reason why the feasibility of Cd phytoextraction is evaluated here, in which we propose species and traits for the definition of an ideotype. This evaluation is exclusively based on the results of field trials aiming at soil decontamination. An important issue concerning phytoextraction is the fate of the harvested parts, containing elevated amounts of the toxic metal. Several processes of treating this biomass in a way that will reduce cost of the clean-up have been proposed and are also examined.

2 Cadmium Phytoextraction Using *Noccaea caerulescens*

About 16 field trials testing *N. caerulescens* (Alpine pennycress, formerly *Thlaspi caerulescens*) cultivation for soil metal extraction were carried out in the last 25 years at several places in the world. The first trials of this type were conducted from the beginning of the 1990s in Great Britain, at Woburn (Fig. 1), under the supervision of the then Rothamsted Experimental Station (now Rothamsted Research) (Baker et al. 1994; McGrath et al. 1993, 2000, 2006). At that time, the US Department of Agriculture also carried out a field experiment, in Beltsville. Maryland (Brown et al. 1995). In the UK, another trial was conducted in 2000, in the area of Nottingham (Maxted et al. 2007b). In France, Schwartz (1997; Schwartz et al. 2003) first cultivated this hyperaccumulator in the field over two growth seasons at a site in the vicinity of Nancy (La Bouzule, Lorraine region) (Fig. 2). This was followed 12 years later by Lovy (2012), who grew *N. caerulescens* for three successive cultivations (2008–2011) in large trays under natural climatic conditions (in Vandoeuvre, Lorraine region). Also in Lorraine at the Homécourt Experimental Station, Rees (2014, Rees et al. 2020) cultivated this species in two 2 m^3 lysimeters to test the effect of biochar application on Cd and Zn phytoextraction. Several trials were also carried out in Switzerland from the end of the 1990s to the beginning of 2000, mainly in the Jura region, and particularly in Dornach (Felix 1997; Hammer and Keller 2003; Kayser et al. 2000; Keller and Hammer 2005; Keller et al. 2003). In Brussels, Belgium, metallicolous and non-metallicolous populations of *N. caerulescens* were tested for the extraction of Cd and Zn from moderately contaminated urban soils (Jacobs et al. 2017, 2018a, b). All of these trials were done in a temperate climate but there was also one attempt to phytoextract Cd using *N. caerulescens* under tropical climatic conditions in Thailand (Mae Sot, Tak Province) (Simmons et al. 2014).

Fig. 1 A view of the original Woburn site (Rothamsted Research, UK) with the early horticultural experiment (top) and sub-plots of *Noccaea caerulescens* (right-hand corner) and various metallophytes (images supplied by Prof. Alan J. M. Baker)

The soils used in all of the above trials were mostly agricultural and not from industrial sites, and were contaminated from amendments such as sewage sludges, composts, and farmyard manure (Woburn, Nottingham, Beltsville, La Bouzule), or by emissions from metallurgical plants (Dornach, Homécourt) or mining activities (Mae Sot). The soils contained variable amounts of Cd, from *ca.* 0.2–50 mg kg^{-1}. They also often contained other excess metals, such as Zn, Cu and Pb and had pH values from slightly acid to neutral.

2.1 Plant Genotypes Used

The plant accessions (i.e. populations) used in these trials were all wild types, i.e. not ameliorated through breeding. The calamine population from Prayon (Belgium), which is now known to poorly accumulate Cd, was used in the first UK trials at Woburn (McGrath et al. 1993, 2000; Baker et al. 1994). It was also used in the Dornach trial (Hammer and Keller 2003), in Beltsville (Brown et al. 1995), La Bouzule (Schwartz 1997), and probably also at Ziefen (Felix 1997).

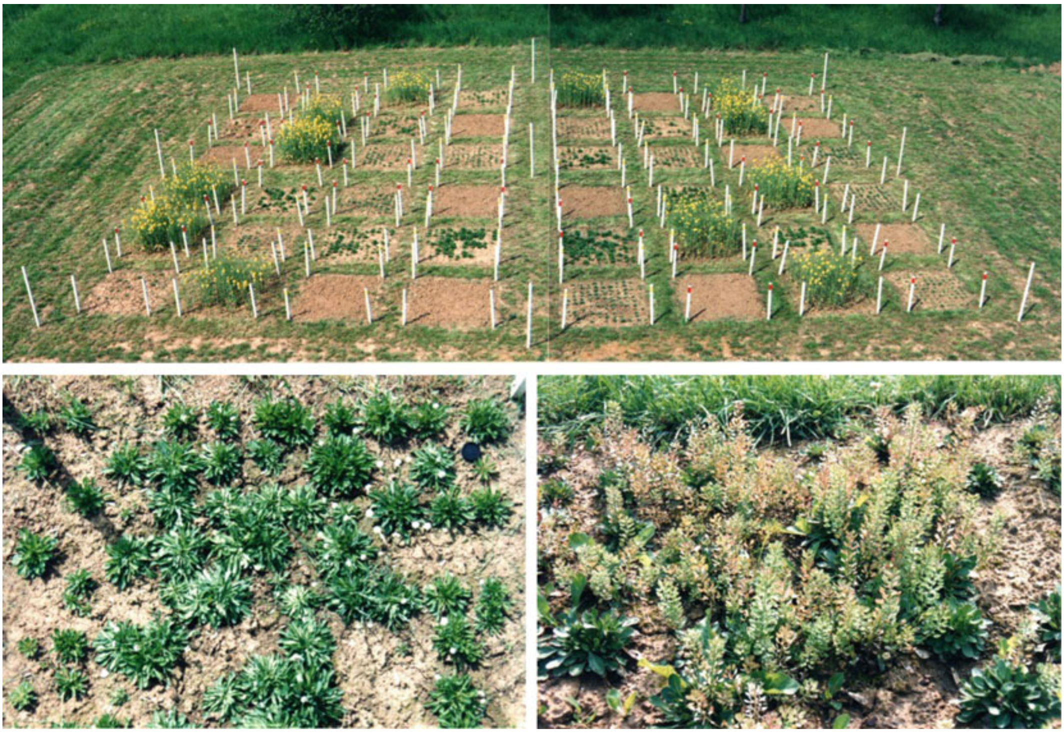

Fig. 2 A general view of the La Bouzule experiment (ENSAIA, France) with sub-plots planted with *Noccaea caerulescens* and *Brassica napus* (top). The hyperaccumulator at the beginning of the flowering stage (bottom left) and at the end of the fructification stage (bottom right) (images supplied by Prof. Christophe Schwartz)

The Ganges population, coming from a mining site in the Cévennes and well known to be among the more Cd-accumulating populations, was used later in the last Woburn trials (McGrath et al. 2006), in those at Dornach and Caslano (Hammer and Keller 2003), Nottingham (Maxted et al. 2007b), Brussels (Jacobs et al. 2017), and Homécourt (Rees 2014, Rees et al. 2020).

In the first Woburn trials, the Whitesike mine (UK) population was also used. It comes from a mining site located on Alston Moor (Cumbria) but was no longer used after the trials of Baker et al. (1994). The Viviez population was used in the trials at La Bouzule (Schwartz 1997), at Vandoeuvre (Lovy 2012), and at Mae Sot (Simmons et al. 2014). In this last Thai trial, other Cévennes populations were tested for the first and only time in the field: Saint-Félix-de-Pallières, Les Malines, and La Sanguinède. The number of populations (*ca.*7) tested is rather small compared to the number of inventoried populations in Western Europe, on the order of a hundred (Gonneau 2014; Gonneau et al. 2017). Populations used were nearly all from calamine sites and in most trials, generally the older ones, they were not those that strongly accumulate Cd (Prayon, Viviez, and Whitesike). It is only recently that non-metallicolous populations (Wilwerwiltz and Goebelsmühle from Luxemburg) were used for the first time, in order to evaluate the advantage of their higher biomass production and Zn accumulation (Jacobs et al. 2017).

2.2 Planting and Harvesting

As far as can be ascertained from the published technical details, in most of the cases the crops were planted as 4–12-week-old seedlings, at densities of 16–200 plants m^{-2}, more often 100 plants m^{-2}. The great majority of the crops started in April or May and were harvested 2–4 months later. Most of the crops were not vernalized so they probably did not flower. However, variable cultivation calendars were also used to conduct the crop trials. Hammer and Keller (2003) harvested the shoots in November, seven months after sowing, whereas Baker et al. (1994) harvested plants in July or August that had been transplanted six months earlier (in January or February). Lovy (2012) grew *N. caerulescens* for 10 months, basing on the natural cycle of the species, i.e. sowing by the end of August and harvesting in spring or at the beginning of summer the following year.

Hammer and Keller (2003) carried out three successive croppings of about two months each, between April and November, with transplanting each time, or two crops of about three months, after sowing. Keller et al. (2003) transplanted *N. caerulescens* in late July for harvest in mid-October. McGrath et al. (2000) observed a regrowth of the plants after harvest of the rosette, from crowns left in the soil during winter. McGrath et al. (2006) also conducted a lengthy cultivation (8 months) with vernalisation, the plants being planted in October for harvesting in June of the following year, when they fruited.

Jacobs et al. (2017, 2018a) cultivated the plants for 6.0–6.5 months between May (seedling transplantation) and October/November. They also tested 12-month cultivations, from September or November later on, starting either from transplantations or from sowings (Jacobs et al. 2018b).

2.3 Biomass Production

In the field trials, the dry biomass production varied from 0.28–7.8 t ha^{-1}. It is difficult to explain this wide range, since factors such as population, soil type, and cropping practices can strongly influence the plant growth. Many crops produced less than 1 t ha^{-1}. A yield of 2 t ha^{-1} can be considered productive and 4 t ha^{-1} as exceptionally high. Yields >7 t ha^{-1} were obtained in Woburn by McGrath et al. (2000) in a trial whose duration was about 10–14 months, for which half of the plants had been harvested the previous year and then used to provide regrowth. These authors obtained a production on the order of 4 t ha^{-1} after four months cultivation on the same site. It should be noted that the production obtained in the same test plot a few years later was more modest, from 0.08–3.6 t ha^{-1}, with an average of 2.14 t ha^{-1} for a 14-month-old crop (McGrath et al. 2006). However, individual plants of 28 g dw (Prayon) biomass and 31 g (Whitesike) were also achieved in the Woburn trials during 1991–1992 (Baker et al. 1994; McGrath et al. 2000). Planted at a density of 35 plants m^{-2}, such individual plants would enable a biomass production of *ca.* 10 t ha^{-1}. The experiments in Brussels produced from *ca.* 1.2–4.8 t ha^{-1}, depending on the site, the transplantation mode, the ecotype, or the cultivation duration, among other factors (Jacobs et al. 2017, 2018a, b). They showed that biomass, whatever the seeding season and population, was two times greater with transplantation than with direct sowing. Moreover, fall establishment yielded a greater biomass production than spring establishment due to a longer growing season.

Crops in tropical soils (Mae Sot, Thailand) provided among the lowest yields, 0.28–0.65 t ha^{-1}, likely due to incompatible climatic conditions and severe pest attacks. Copper toxicity (>500 mg Cu kg^{-1} in soils), but also the more clayey texture, could explain the low yields obtained in the Dornach trials. Schwartz (1997) lost plants sown in the autumn because of the winter cold and insufficient Zn supply, which could also have caused the observed low plant growth.

2.4 Effectiveness of Phytoextraction

In the field trials cited above, Cd concentrations in the shoot varied three orders of magnitude (from one to thousand mg kg^{-1}), as well as quantities harvested (in g ha^{-1}), although the latter metric has not always been published by other authors. Again, it is difficult to explain this variability. The concentrations in the plants depend in particular on soil concentrations, its properties (especially pH), and on the plant population. Additionally, differences in dry matter yield contribute to the variability of the exported quantities. Maxted et al. (2007b) clearly demonstrated the great variability of Cd concentrations that accumulated among individuals from the same population of *N. caerulescens* ('Ganges') grown on a single plot, with a factor of 10 existing between the most and least concentrated plants. However, Cd concentrations in the shoots have been shown to be generally much lower for plants of the Prayon population. The Cd concentrations in plants from Viviez and Whitesike were also relatively low, whereas those found in the 'Ganges' population can be extremely high, such as in the Homécourt trial where over 3000 mg kg^{-1} were achieved. The effectiveness of phytoextraction was not always assessed, and when it was, was quite variable. The amounts of exported Cd were in the range of 10–700 g ha^{-1} (McGrath et al. 2000, 2006; Keller et al. 2003; Keller and Hammer 2005). Unfortunately, no estimate of the exported Cd quantity or of the biomass was given in their Beltsville trial (Brown et al. 1995). The concentrations in the *N. caerulescens* plants (Prayon) were very low (2–30 mg kg^{-1}) and it is likely that the remediation potential for this species is therefore very low.

Clean-up of the Dornach site (achieving a soil Cd concentration of 0.8 mg kg^{-1}) would require 33–49 years depending on whether *N. caerulescens* was transplanted or sown (Hammer and Keller 2003). For the Caslano soil, the remediation period was somewhat shorter, on the order of 9–26 years. It should be noted that these authors considered a constant Cd uptake by each successive crop, which assumes a constant availability of soil Cd during phytoextraction. Importantly, this assumption is incorrect, because the extractable amount decreases as the clean-up progresses (the so-called 'Law of Diminishing Returns'). It seems that transplanting that enables a greater number of short-duration crops and a higher biomass production would be more favourable than sowing. Kayser et al. (2000) did not give any time required for clean-up of the Dornach site using the Prayon *N. caerulescens* population. However, they estimated that a plant containing 45 mg kg^{-1} and producing 10 t ha^{-1} biomass could remove half of the soil Cd within 10 years. No estimate for the proposed clean-up time was given from the results of the Woburn trials. McGrath et al. (2000) calculated that a crop producing 10 t DM ha^{-1} and containing 100 mg Cd kg^{-1} would decrease Cd soil content from 1 to 0.2 mg kg^{-1} in two years, and from 10–3 mg kg^{-1} in 18 years. These time periods would be raised to five years and 46 years, respectively, if the plant contained only 40 mg kg^{-1}. A 14-month-old crop extracted on average 8.7% of the Cd from variously contaminated soils; in one of those, containing 7.5 mg kg^{-1} Cd, one crop of the 'Ganges' population extracted 21.6% of the metal from the top 20 cm layer. This extraction record corresponded to the most concentrated plants that produced 2.69 t dw ha^{-1} (McGrath et al. 2006). The Cd concentration in the plants did correlate weakly with that of soil ($r^2 = 0.46$); the change in biomass strongly related to differences in phytoextraction performance. Plants from the Viviez population cultivated in Vandœuvre-lès-Nancy extracted 5–10% of the total Cd present in the upper 10 cm layer containing 5 mg Cd kg^{-1} (Lovy 2012). The clean-up duration was estimated to be 20 years, assuming a constant uptake each year. Using the 'Ganges' population, Jacobs et al. (2018b) extracted 6.4–12.3% of the soil total Cd, and 12.2–23.4% of the exchangeable metal, depending on the cultivation cycle. However, they did not estimate the time needed to decontaminate the site.

The aim of the Maxted et al. (2007b) trials was to evaluate the time needed for a phytoextraction crop to extract the Cd provided by sewage sludge regularly spread onto agricultural plots. Thus, a *N. caerulescens* crop producing 4 t DM ha^{-1} every 14 years would be sufficient to eliminate an annual application of 10 tons of sludge containing 4.0 mg Cd kg^{-1}. These values are reasonable based on realistic contents and modelling of the bioavailability of soil Cd and its concentration in the plant. The La Bouzule cultivations (Schwartz et al. 2003) allowed the Viviez plants to extract only 4–9% of the Cd provided by the sludge and compost amendments. The trial that showed the highest Cd phytoextraction efficiency is that of Homécourt (Rees et al. 2020). On both lysimeters, one a 'Ganges' crop of 4.2 months, extracted 25% of the metal from the upper 30 cm layer which had a Cd content of 17 mg kg^{-1}. However, because of the logarithmic decrease in phytoextraction efficiency with time, the amount extracted after four years represented about 40% of the initial Cd content, and the time necessary to remove the excess of metals in the topsoil was estimated at 111 years.

2.5 Appraisal of the Cadmium Phytoextraction Efficiency When Using *N. caerulescens*

Results of most of the field trials with *N. caerulescens* in terms of efficiency or duration of phytoextraction are very disappointing. The extraction rates were low, estimated clean-up duration was long, often exceeding one or several decades, and usually was also underestimated because the authors considered a constant Cd concentration in the aerial tissues of the successive crops. The main reasons for the low efficiency were the reduced production of biomass and insufficient Cd concentration in the shoots. Whilst several authors agree on the need to produce at least 10 t ha^{-1} of dry matter, the trials have often led to yields 5–10 times lower. Moreover, in many trials, low-accumulating Cd populations were used (at Prayon and Viviez), or when a more effective population such as the 'Ganges' was used, it suffered from soil toxicity as at Dornach (Cu toxicity). Some results show the great variability of Cd contents in plants and the existence of very high levels in some populations and individuals, although few different populations were used. Similarly, plants having significantly greater biomass were observed. They indicate a genetic variability that could serve as basis for improving biomass production and tissue concentrations. Sterckeman et al. (2019) showed that breeding *N. caerulescens* through pure-line selection could be a means to improve biomass production and metal accumulation of the plant. However, their results obtained in greenhouse still need to be confirmed under field conditions. Phytoextraction of Cd by the hyperaccumulator will only be possible if varieties with high production of biomass are selected, and also those presenting traits allowing for their multiplication and cultivation in an economically acceptable manner. The results obtained to date indicate that we are far from this goal and that achieving it will require constant investment in plant breeding, including biotechnological techniques such as interspecific hybridization (Brewer et al. 1999). Finally, Rees et al. (2020) presented results (among many others) showing that even with a successful cultivar, it is the availability of trace metal in the soil that largely controls its removal by the plant. For this reason, some authors have proposed that phytoextraction consists in eliminating only the phytoavailable fraction of the pollutant. However, simply exhausting the available pool would be risking its gradual replenishment once phytoextraction is suspended.

3 Cadmium Phytoextraction Using Other Hyperaccumulator Plants

Among the Zn and Cd hyperaccumulators, *Sedum alfredii* (Yang et al. 2004) and *S. plumbizincicola* (Wu et al. 2013) were recently discovered at mining sites in China. These Crassulaceae are adapted to growing in subtropical areas. Although their hyperaccumulation characteristics and physiology have now been studied extensively at the laboratory scale, relatively few published data exist on their performance for Cd phytoextraction at field scale.

Wu et al. (2007) cultivated *S. alfredii* in comparison with and co-cropping with maize on a paddy field soil containing 2.0 mg Cd kg^{-1}. After six months of cultivation, the hyperaccumulator extracted about 5% of the total soil Cd (*ca.* 40 g Cd ha^{-1}), similar to that extracted by a maize crop, whereas co-cropping the two plants enabled removal of *ca.* 20% of the metal. Another experiment was carried out in the same area by Zhuang et al. (2007), comparing *S. alfredii* to 7 other species on a soil containing 7.2 mg Cd kg^{-1}. The hyperaccumulator extracted 0.25% of the soil Cd after three months of cultivation, producing 5.5 t ha^{-1} of dry matter and about 50 g Cd ha^{-1}. This extraction rate was the lowest after that of *Dianthus chinensis* (0.10%). *S. alfredii* extracted less Cd than the hyperaccumulating *Viola baoshanensis* (0.88%) and the metal-tolerant *Rumex crispus* (0.86%), *Rumex* 'K1' (0.34%), and *Vetiveria zizanioides* (0.50%) that produced much higher biomass (*ca.* 30 t ha^{-1}) than *S. alfredii*.

Sedum plumbizincicola (Crassulaceae) (Fig. 3) has been tested for the phytoextraction of Cd (and Zn) in a long-term field experiment over eight years (Deng et al. 2016). The soil initially contained 3.0–4.6 mg Cd kg^{-1} in the upper 15 cm. The total soil Cd content decreased by 85.5% after seven years of *Sedum*/maize intercropping and by 87.8% after *Sedum* monocropping. The peak removal rate was observed in the fourth year with 1.03 kg Cd ha^{-1} y^{-1}. Cadmium concentrations in the grains of maize and sorghum intercropped with *S. plumbizincicola* did not decrease together with phytoextraction but remained below the Chinese National Food Quality Standard (0.2 mg Cd kg^{-1}). Hu et al. (2019) confirmed the interest of using *S. plumbizincicola* for Cd phytoextraction, in this case in a slightly contaminated acid soil containing 0.60 mg Cd kg^{-1}, used for rice production. The hyperaccumulator produced 3.5 t DM ha^{-1} and after one cultivation, the soil Cd content declined by 22% on average. After two successive phytoextractions, the Cd removal rate varied from 40–55% depending on the plot. Cadmium in grains of rice cultivated after the phytoextractions also consistently declined. The soil Cd concentration mainly declined in the upper 0–15 cm layer, due to the shallow rooting of *S. plumbizincicola*, suggesting that a tilling of the topsoil is necessary between each phytoextraction cultivation, in order to bring to the surface, the polluted soil located more than 15 cm deep. Another difficulty of phytoextraction with *S. plumbizincicola* is the cost of the transplantation of the cuttings at a high density (*ca.* 445,000 cuttings ha^{-1}).

Solanum nigrum (Solanaceae) is also a recently discovered Cd hyperaccumulator (Wei et al. 2005). Niu et al. (2015) grew it for three months before cultivation of Chinese cabbage in a soil containing 0.53–0.97 mg Cd kg^{-1}. They observed a decrease of 10.7% of soil Cd content and also in the cabbage shoot, the latter of which was 0.15 mg kg^{-1} without previous phytoextraction and 0.06 mg Cd kg^{-1}, i.e. below the Chinese National Food Quality Standard, after remediation. Growing *S. nigrum* for three months also, alone or together with Welsh onion, Wang et al. (2015) observed a decrease of nearly 8% in soil Cd concentration, which initially ranged from 0.45–0.62 mg Cd kg^{-1}. However, the Cd phytoextraction had no effect on Cd content of the onion, which was always below 0.1 mg Cd kg^{-1}. Ji et al. (2011) applied *S. nigrum* in a field experiment conducted on a moderately polluted arable soil in China. The maximum Cd concentrations reached 9.9 mg Cd kg^{-1} on a site containing 1.96 mg Cd kg^{-1} in the topsoil (0–10 cm). In this work, different agronomic practices were tested to increase the phytoextraction efficiency, i.e. fertilization, variable plant density, double cropping, and double harvesting. Double cropping at a planting density of 11 plants m^{-2} (30 × 30 cm) was the most successful approach, resulting in a total biomass of 42.1 t ha^{-1} y^{-1} and a Cd removal of 0.4 kg Cd ha^{-1} y^{-1}.

4 Cadmium Phytoextraction with Non-hyperaccumulator Species

4.1 Trials Using Willow (*Salix* Spp.)

Agroforestry practices such as short rotation coppicing (SRC) for the cropping of *Salix* are well-established, since chipped willow stems have been used for decades as biomass fuel for energy production (Pulford and Dickinson 2005). Although *Salix* spp. typically have lower Cd concentrations in their above-ground tissues

Fig. 3 A field experiment to evaluate the ability of *Sedum plumbizincicola* to remediate a Cd contaminated soil in Xiangtan county, Hu’nan Province, China (top). Co-cropping of *S. plumbizincicola* and maize (*Zea mays*) in Zhejiang Province, China (bottom) (images supplied by Prof. Longhua Wu)

(5–70 mg kg^{-1}) compared to hyperaccumulators, the efficiency of SRC for Cd phytoextraction has been tested because of the large biomass production and fast growth that allow a consistent metal yield together with energy production. This type of evaluation has been made in several field trials on agricultural soils that were slightly or moderately contaminated, as in Belgium, the UK, Sweden, and Switzerland.

Eriksson and Ledin (1999) studied the changes in soil Cd concentrations in slightly contaminated soil (maximum concentration *ca.* 0.25 mg Cd kg^{-1} in topsoil) in 8–30-year-old *S. viminalis* plantations used to produce stem wood at various cutting frequencies. They did not find a significant change in total Cd in the soil. However, it seems that these trees decreased the plant-available Cd up to a depth of 65 cm, although this diminution was not always significant. Hammer et al. (2003) cultivated a Swedish clone of *S. viminalis* in two moderately contaminated soils. The total metal uptake was 170 and 197 g Cd ha^{-1} over five years in the control and S-treated plots, respectively. They estimated that 31 years of cropping were necessary to decrease the Cd concentration in the upper 20 cm soil layer from 3 to 0.8 mg kg^{-1}, and 142 years if a 60-cm-soil depth was considered.

Maxted et al. (2007a) cultivated six willow genotypes at a site near Nottingham, UK, on a sewage sludge-contaminated soil containing 41.6 mg Cd kg^{-1}. The bioavailable Cd was determined as isotopically exchangeable Cd and was shown to be 32% of the total content. Their experiments indicated that willows achieved approximately 15–20% of the uptake rate required to extract the bioavailable Cd in 25 years. Willow clearly appeared unsuitable for decontaminating such highly Cd-contaminated soils, even if only the phytoavailable fraction is targeted.

Using eight accessions of different willow species and hybrids (extracting 17–241 g Cd ha^{-1}), van Slycken et al. (2013) estimated from 101–1303 years as the time needed to reduce soil contamination from 6.5–3.0 mg Cd kg^{-1}, harvesting the leaves together with the stems, which is not the usual practice for SRC. This is still a high contamination level for the sandy soil from Campine, where the background concentration was originally *ca.* 0.2 mg Cd kg^{-1}. This time span is on the same magnitude as that found by Vangronsveld et al. (2009) and Ruttens et al. (2011) in Lommel (Belgium) in order to reach a total soil Cd concentration of 2.0 mg kg^{-1}, starting from 5.7 mg kg^{-1}. By removing the shoot together with leaves, the amount of extracted metal ranged from 113–244 g Cd ha^{-1} (Ruttens et al. 2011). Furthermore, Kubátová et al. (2018) found that a ‘summer harvest’ (carried out in September), including of the leaves, resulted in a much higher Cd phytoextraction efficiency compared to a ‘winter harvest’. They calculated that 70 years would be required for reducing the Cd concentration in soil from 7.3–0.5 mg kg^{-1} when applying the ‘summer harvesting’ approach using a clone of *S.* x *smithiana*. Michels et al. (2018) observed that neglecting the leaves from harvesting of poplars for Cd phytoextraction may even lead to an increase of Cd concentrations in topsoil due to litter fall.

Greger and Landberg (2015) recently reported the results of Cd phytoextraction using *S. viminalis* in two moderately Cd-contaminated soils, one clayey with 0.32 mg Cd kg^{-1} and one sandy containing 0.45 mg Cd kg^{-1}. In their experiment, the Cd phytoextraction efficiency was not only assessed by the reduction of total Cd concentrations in the soil but also by the change in Cd availability for the subsequent crop, wheat. After four years of Cd phytoextraction with willow, the total Cd in soil was reduced by up to 27%, but the Cd concentration in wheat grain decreased by up to 33%. However, there was a strong deficit in the Cd mass balance of the remediation crop. In the clayey soil, there was a decrease of 475 g Cd ha^{-1} after the four years of willow cultivation, whereas analyses of the plant parts showed a Cd removal of only 22 g Cd ha^{-1}. This result made the authors suggest that the difference of 453 g Cd ha^{-1} could have disappeared from the system with (1) the dispersed withered leaves during the three autumns before harvest, but this is estimated to only 22.6 g Cd ha^{-1}; or (2) by leaching below the rooting zone at a rate of 430.4 g Cd ha^{-1}. This suggests that in this case, phytoextraction was in fact a ‘phytoleaching’ of the contaminant, which would have led to dilution of the metal in a higher volume of substrate and to potential contamination of groundwaters. Moreover, the authors calculated that reducing the concentration at the measured level by a real plant removal would take 87 croppings, harvesting both shoot and roots each time.

In summary, it seems that for willow, clean-up times calculated based on the reduction of total Cd concentrations in soil, reveal that several decades are needed for achieving the remediation target. Thus, Cd phytoextraction with *Salix* spp. should preferably be combined with other biomass use options such as energy production. This approach has been suggested as a phytomanagement strategy for a contaminated site, rather than being primarily a remediation option (Robinson et al. 2009).

4.2 Feasibility of Cadmium Phytoextraction with Other Non-hyperaccumulating Species

The Lommel trial in Belgium, mentioned above, was also used to test other high-biomass and fast-growing species such as poplar, maize (*Zea mays*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and tobacco (*Nicotiana tabacum*) (Vangronsveld et al. 2009). The time needed to decrease the total soil Cd concentration from *ca.* 5–2 mg kg^{-1} ranged from 58–255 years. The lowest time was obtained using tobacco, all other plants requiring a clean-up time greater than a century. In a later experiment conducted at the same site, the species mentioned above were compared with various poplar and willow clones during 2011–2014. Results showed that the shortest remediation time (i.e. 60 years) for reducing the total soil Cd from 4–2 mg kg^{-1} would be achieved using the commercial *Salix viminalis* clone 'Zwarte Driebast' (Thijs et al. 2018). Time spans on the order of one to several centuries were also found by Felix (1997) with the same species tested in a Jura soil containing 6.6 mg Cd kg^{-1} and requiring remediation to the Swiss regulatory limit (0.8 mg Cd kg^{-1}). In this situation, phytoextracting Cd with Indian mustard (*B. juncea*) would take 214 years. Maize, sunflower, Indian mustard, and tobacco were also used in the Dornach site trials and compared to *N. caerulescens* (Kayser et al. 2000). Here, the hyperaccumulator only produced 0.49 t Cd DM ha^{-1} because of soil compaction and heat, but probably also due to very high concentrations of Cu in the soils. Cadmium concentrations in tobacco were below those of the hyperaccumulator but clearly above those of Indian mustard, sunflower, and maize. The amount extracted by the different species ranged from 2–30 g Cd ha^{-1}. As in the Lommel trial, tobacco appeared as the crop with the highest Cd content in its above-ground biomass, which was even more than in *N. caerulescens* in this case, because of its high biomass production. The authors did not give values for the clean-up time but they estimated that even the highest metal removal rates achieved in this study were still far from what would be required to make phytoextraction practicable for the remediation of the Dornach site, containing only 2.5 mg Cd kg^{-1}.

Interesting results were obtained by Murakami et al. (2009) for the phytoextraction of Cd from contaminated paddy fields. They cultivated the 'indica' rice cv. 'Chokoukoku' for two years without irrigation in soil containing 1.6 mg Cd kg^{-1}. This process extracted 883 g Cd ha^{-1}, i.e. 38% of the total soil Cd, and reduced by 47% the Cd grain content of the subsequently grown 'japonica' food rice, without decreasing its yield. The above results suggest that adapted cultivars of tobacco and 'indica' rice could be used to decontaminate moderately Cd-polluted soils, mainly agricultural soils. More research is needed to select the most appropriate cultivars and test them in the field in order to evaluate their impact on the amount of bioavailable and total soil Cd and on the metal concentrations acquired in subsequent food crops.

5 Biomass Valorization Options for Cadmium Phytoextraction Crops

The biomass obtained from harvested phytoextraction plants could be used for various purposes similar to other agricultural non-food crops, i.e. combustion or gasification for energy production or the production of fibre, oil, or biofuels. Due to the high concentration of metals and/or metalloids, the biomass could also serve as a source for recovery of these elements, either directly from the biomass or from the ash after incineration. However, very little information is available concerning the potential options for the use of biomass. A survey conducted by Bert et al. (2017) revealed that people working on platforms on combustion or anaerobic digestion in different European countries showed that there is still little awareness of phytotechnologies in general and phytoextraction in particular, but these people also agreed that biomass obtained from phytoremediation sites could be used under certain conditions. However, biomass obtained from phytoextraction plots was considered more of a risk than those derived from phytostabilization approaches.

5.1 Hyperaccumulator Plants

Keller et al. (2005) reported on the potential use of harvested *N. caerulescens* leaves obtained from a phytoextraction field trial, where it was shown that gasification under reducing conditions (pyrolysis) is more suitable for the potential recovery of Cd from fly ash and concomitant use of the bottom ash as a fertilizer. The fate of metals during the combustion process was investigated for *S. plumbizincicola* (Zhong et al. 2015). In this case, the metal distribution between bottom and fly ash was largely temperature-dependent and the percentage of metals recovered from the fly ash increased with increasing temperature. Use of kaolin or activated carbon can further reduce the emission of Cd and other potential pollutants (e.g. PAHs, NOx, CO) (Wu et al. 2013). The recovery of Cd (and of Zn) from harvested biomass of *N. caerulescens* has recently been investigated and seems possible using a cementation process (Hazotte et al. 2017), which could be used also for *S. plumbizincicola*.

5.2 Willow

Salix and *Populus* species have been grown for decades in short rotation coppice (SRC), using the harvested biomass for energy production (Pulford and Dickinson 2005). Consequently, this option has also been discussed and tested when using these woody species as phytoextraction crops (Keller et al. 2005; Delplanque et al. 2013). For SRC phytoextraction plants, work on harvested biomass has focused on either leaves or stems, or leaves and stems. Keller et al. (2005) investigated the combustion of *Salix* leaves and found in general the same results as for the incineration of foliar *N. caerulescens* biomass (see above). They reported that *S. viminalis* leaves from a Cd ± Zn phytoextraction field trial required treatment with gasification under reducing conditions (pyrolysis) for a greater potential recovery of Cd and a concomitant use of the bottom ash as fertilizer. Similarly, Delplanque et al. (2013) reported that the highest concentrations of Zn and Cd were in the fly ash, which thus may serve as a potential source for Cd recovery.

6 Conclusions

Cadmium is one of, if not the most, threatening soil contaminants as nearly all plant populations in the world are, or may soon be, over-exposed to this trace metal through food consumption. The metal arrives in the diet through root absorption by crops, the Cd contamination being at relatively low levels in most cultivated soils. Reducing soil Cd contamination is a direct method needed to improve food quality, but this supposes the decontamination of vast surface areas of cultivated land. In principle, phytoextraction is the more appropriate technique to reduce soil Cd concentrations in such a situation, which allows preservation of the soil properties. There are plants that can concentrate Cd in their harvestable parts and cropping them at a reasonable cost would not need a 'know-how' nor tools very different from those required for other specialist crops. The main problems remaining to be solved in order to develop a successful Cd phytoextraction are the creation of high-metal yielding cultivars and efficient processes for treatment of the contaminated harvested biomass.

The field trials discussed in this chapter most frequently have yielded disappointing results as the phytoextraction rate is generally low, and hence the time needed to reach an acceptable Cd concentration in soils is too long, whichever species has been tested. This is the consequence of an inadequate metal yield, the plant with a high biomass production being often insufficiently concentrated in Cd, and vice versa. In some cases, the low availability of Cd in soils, particularly those highly contaminated, could also be a limit for successful phytoextraction of this metal.

However, there are still a few options which could lead to success, as some plant species present a potential for reaching a high metal yield. This is the case for *N. caerulescens*, which showed very high decontamination rates in some experiments in temperate climate. This plant also has high phenotypic variability both in terms of biomass and of Cd accumulation, neither of which have not been sufficiently exploited yet to select high yielding varieties. An investment in plant breeding should be made in the coming years to create a cultivar of *N. caerulescens* with a regular biomass production around 10 t DM ha^{-1}, together with a Cd concentration factor at least equal to that of the 'Ganges' population. Such a cultivar would enable the decontamination of soil containing up to 10 mg total Cd kg^{-1} but would be more suitable for soils containing <1 mg total Cd kg^{-1}.

Our review indicates that in (sub)tropical areas *S. plumbizincicola* is a species with an interesting potential for decontamination of slightly Cd-contaminated soils. More field experiments should be carried out to confirm its ability for Cd phytoextraction, but also to mechanize planting of the cuttings. This species should also be investigated with the aim of cultivar creation and multiplication, as well as treatment of the harvested biomass.

An 'indica' rice cultivar has demonstrated an ability to efficiently decontaminate moderately polluted paddy fields. However, few results are published about this possibility as a technique for Cd phytoextraction, thus further field trials are required for confirmation. This is also the case for *Solanum nigrum,* which showed promising results suggesting that this species could be helpful in decontaminating some contaminated soils used for food production.

The amounts of Cd extracted in the context of soil clean-up are on the order of few 100 s g ha^{-1}, making the metal recovery from the biomass poorly profitable. Valorization of the harvested biomass from Cd phytoextraction crops has been insufficiently investigated. A few studies have shown that combustion is a feasible option that would potentially allow the use of bottom ash as a fertilizer, since most of the Cd is retained in the fly ash.

Acknowledgements Région Lorraine and European Union (FEDER) funded the research carried out by F. Rees in the frame of the LORVER programme.

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Element Case Studies in the Temperate/Mediterranean Regions of Europe: Nickel

Aida Bani, Dolja Pavlova, Beatriz Garrido-Rodríguez,
Petra S. Kidd, Maria Konstantinou,
Dimitrios Kyrkas, Jean Louis Morel,
Angeles Prieto-Fernandez, Markus Puschenreiter,
and Guillaume Echevarria

Abstract

Initial experiments using Mediterranean Ni-hyperaccumulator plants for the purpose of phytomining were carried out in the 1990s. In order to meet commercial phytoextraction requirements, a technology has been developed using hyperaccumulator species with adapted intensive agronomic practices on naturally Ni-rich soils. Ultramafic soils in the Balkans and other parts of Europe display a great variability in Ni concentrations and available Ni levels. In Albania, Vertisols are currently being used for low-productivity agriculture (pasture or arable land) on which phytomining could be included in cropping practices. In northwestern Greece (Pindus and Vourinos mountain regions), agricultural soils may occur on ultramafic Cambisols. In Spain and Austria, these soils are much more erratically distributed and are seldom used for crops and pastures. In the Balkans, *Odontarrhena chalcidica* (synonym *Alyssum murale*) occurs widely on these ultramafic soils and is a spontaneous weed that grows among other crops. Field studies across Europe have now been carried out outside Mediterranean areas and have evaluated the Ni-hyperaccumulator *O. chalcidica*, as well as two other species native to northwestern Greece (*Bornmuellera emarginata* and *B. tymphaea*). At each site, local hyperaccumulator plants were tested for comparison (*Noccaea goesingense* in Austria and *Odontarrhena serpyllifolia s.l.* in Spain), in the context of two recent EU-funded projects (by Agronickel and LIFE-Agromine). Soil and crop management practices are being developed in order to optimize the Ni agromining process. Field studies have evaluated the potential benefits of fertilization

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A. Bani (✉)
Agricultural University of Tirana, Kodër-Kamëz, 1100 Tirana, Albania
e-mail: aida_alushi@hotmail.com

Agro-Environmental and Economic Management Center, Rr. Zef Jubani. Nr 5, Tirane, Albania

D. Pavlova
Department of Botany, Faculty of Biology, University of Sofia, Sofia 1504, Bulgaria

B. Garrido-Rodríguez · P. S. Kidd · A. Prieto-Fernandez
Instituto de Investigaciones Agrobiológicas de Galicia, CSIC, Avda de Vigo, Santiago de Compostela 15705, Spain

M. Konstantinou
Department of Landscape Architecture, International Hellenic University, Kavala 654 04, Greece

D. Kyrkas
University of Ioannina, Arta 451 10, Greece

J. L. Morel · G. Echevarria
Laboratoire Sols et Environnement, Université de Lorraine, INRAE, 54000 Nancy, France

M. Puschenreiter
Department of Forest and Soil Sciences, Institute of Soil Research, University of Natural Resources and Life Sciences, Vienna, Austria

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_16

regimes, crop selection, and cropping patterns (introducing agroecological practices), and bioaugmentation using plant-associated microorganisms.

1 Introduction

Nickel-rich soils, such as ultramafic or serpentine soils, have a high potential for metal recovery with possible applications in metallurgical processes. Ultramafic outcrops in Europe cover >10,000 km^2 and soils derived from this bedrock are generally characterized by low fertility (low total N and available K and P contents) and productivity, making them unattractive for agriculture (Bani et al. 2015a, b). As a result, many of these areas are slowly being abandoned by local farmers, leading to declines in rural population and land abandonment. Such outcrops present stressful environments for plant growth, and the plant communities in these areas often present a high number of endemic species that have evolved both morphological and physiological adaptations, differentiating them from the flora of adjacent geological substrates (Proctor 1971). The ultramafic flora includes unusual plant groups (hyperaccumulators), which are able to accumulate extremely high concentrations of Ni in their aerial biomass (van der Ent et al. 2013). Ultramafic soils cover large areas in the Balkans, more than in any other part of Europe. Therefore, this region is a potential target for agromining activities (van der Ent et al. 2015). Europe has about 40 endemic taxa of Ni hyperaccumulators in two families (Brassicaceae and Asteraceae). Moreover, the Balkans also have the highest diversity in Ni hyperaccumulator plants in Europe and one of the highest globally together with Anatolia in Turkey. This region is home to the widespread plant *Odontarrhena chalcidica* (synonym *Alyssum murale*), one of the most studied species worldwide for use in phytomining (e.g. Nkrumah et al. 2016). Initial experiments using Mediterranean Ni-hyperaccumulators for the purpose of phytomining were carried out in the 1990s. In order to meet commercial phytoextraction requirements, technology has been developed using hyperaccumulator plant species with adapted intensive agronomic practices on natural Ni-rich soils.

In the early stages of investigation, several studies were carried out in order to identify the best soil conditions for establishing field trials. The native species were then evaluated for their potential in phytomining. Finally, trials were set up to optimize cropping conditions in the Temperate/ Mediterranean European context. Nickel phytomining research is presently being carried out in the tropics and on several ultramafic sites in Europe (Kidd et al. 2018). Within a European research network (AGROMINE, LIFE15 ENV/ FR/000512), the main tested crop is *Odontarrhena chalcidica* belonging to the *A. muralis s.l.* complex (Cecchi et al. 2018). Other plant species being tested include *Bornmuellera tymphaea* and *B. emarginata* (synonym *Leptoplax emarginata*). At the moment, the most profitable product gained from the phytomining process is a Ni salt (ANSH, ammonium nickel sulfate hexahydrate) (Zhang et al. 2016). LIFE-AGROMINE offers a fully integrated and new phytomining agriculture that could cover thousands of km^2 in Europe, and contribute to the restoration of degraded areas and to recycling of waste products. Phytomining-based agricultural systems will be designed as a trade-off between improved traditional crops (already used in cultivated ultramafic areas) and new 'green' production of raw materials (especially Ni).

2 Agromining Nickel in the Temperate/Mediterranean Europe Regions

2.1 Properties of Ultramafic Soils of the Temperate/ Mediterranean European Regions and Suitability for Agromining

2.1.1 Balkans

Ultramafic outcrops in Europe are distributed from northwestern Portugal and Galicia (ES) to Thrace (GR and BU) and Asian Turkey. Across

Europe and Turkey, serpentine areas are biodiversity hotspots, especially due to the presence of large ultramafic areas in the Balkans (Stevanović et al. 2003). In some countries, ultramafic outcrops can represent up to 11% of the total surface including large agricultural areas.

Outcrops of ultramafic rocks in the Balkans (Dilek and Furnes 2009) extend towards central Bosnia, western and central Serbia, southern Bulgaria, and north-central and southeastern Albania, to Epirus and Thessalia in Greece (Stevanović et al. 2003). Mediterranean Europe has been recognized as one of the major areas of metallophyte diversity worldwide (Whiting et al. 2004; Bergmeier et al. 2009). The important Balkan centre of diversity is well known chiefly due to its large serpentine areas (Ritter-Studnička 1970; Horvat et al. 1974; Brooks 1987). According to Stevanović et al. (2003), approximately 335 taxa (including species and subspecies) of Balkan endemics grow on ultramafic soils of which 123 are obligate to this substrate. Most floristically rich areas are situated in northwestern Greece (Pindus Mountain range), the island of Evvia, and northern Albania, together with southwestern Serbia and northern Greece (Mount Vourinos area).

The Pindus Mountains are located in northwestern Greece and extend about 160 km from the southern border of Albania. Ultramafic terranes in Albania and the Pindus Mountains constitute the largest blocks of ultramafic outcrops in the Balkans. The outcrops also host similar floristic assemblages: northeastern and southeastern Albania to northwestern Greece (Reeves et al. 1983; Shallari et al. 1998; Tan et al. 1999; Stevanović et al. 2003), located in similar climate zones (a Mediterranean climate with montane influences). The ultramafic soils of Albania and the Pindus Mountains (Greece) contain elevated levels of metals such as Ni, Cr, Co and Fe. Most soil cover on these ultramafic materials falls into the Cambisol type (refer to Chapter "Genesis and Behaviour of Ultramafic Soils and Consequences for Nickel Biogeochemistry" in this book). Surface horizons of the soils are characterized by extremely high Mg/Ca quotients of up to 30 in Albania and up to 17.7 in Greece (Bani et al. 2009, 2010), typical of ultramafic soils worldwide (Reeves et al. 1997).

The ultramafic surface area in Albania with 10–43% MgO covers 313,300 ha or 11.05% of the total surface of the country (Lekaj et al. 2019). Agricultural areas, an important land cover category of the ultramafics in Albania, were estimated to be 20,907 ha and included Mg-rich arable Vertisols covering about 10,000 ha in 2018 (Zdruli 1997; Lekaj et al. 2019). Vertisols were selected among all candidates as the target soil type for agromining in these regions, namely in Albania, because of their high Ni phytoavailability and position in the landscape (downslope soils and alluvial valleys) and easy access to agricultural machinery (Bani et al. 2009). These Vertisols are currently being used for low-productivity agriculture by which agromining could be included in management practices (Bani et al. 2007, 2015a, b; Bani and Echevarria 2019).

2.1.2 Austria

Major areas covered by ultramafic soils in Austria occur in the eastern part of the country, close to the Hungarian border (Bernstein, Redlschlag; Wenzel and Jockwer 1999) and in Styria near Bruck an der Mur (Wenzel and Jockwer 1999). Total soil Ni concentrations range from 2000 to 2500 mg kg^{-1}. On both sites, *N. goesingense* has been identified as the only Ni-hyperaccumulating species. LIFE-AGROMINE will establish field plots within the area of Redlschlag (E Austria; Map 3). The predominant vegetation type is an oak-pine forest, and the main crops grown within the agricultural areas are maize, wheat and sunflower, as well as grass forage. The elevation varies from 300–800 m, with a humid continental climate and a mean annual temperature of 8.4 °C and precipitation of 838 mm.

2.1.3 Spain

The main peridotite outcrops on the Iberian Peninsula are found in the regions of Galicia (NW Spain), Andalusia (S Spain) and Trás-os-Montes (NE Portugal). LIFE-AGROMINE field plots are established in the ultramafic areas of Galicia. The ultramafic area of Melide covers a surface area of approximately 66 km^2 where the main agricultural crops are grass forage and maize (for fodder). In recent years, there has been a trend towards the establishment of *Eucalyptus* plantations, leading to important habitat loss for serpentine flora. The area is characterized by a European humid-temperate climate with a mean annual temperature of 12.9 °C and mean annual precipitation of 1381 mm. Total soil Ni concentration is approximately 2200 mg kg^{-1}. Two Iberian variants of *Odontarrhena serpyllifolia s.l.* are serpentine-endemics and also hyperaccumulators of Ni: one from Galicia and Trás-os-Montes and another from Andalusia. An extensive cover by the former is a feature of fallow fields at these sites.

2.2 Nickel Hyperaccumulation by European Plant Species

The Mediterranean region is considered a global centre of distribution for Ni hyperaccumulator plants, mainly involving species in the genus *Alyssum* (Reeves 2003). The hyperaccumulation phenomenon was first discovered in *A. bertolonii* (Brassicaceae, now named *O. bertolonii*) in Italy (Minguzzi and Vergnano 1948). Subsequently, similar reports have been given for *A. murale* in Armenia (Doksopulo 1961) and *A. serpyllifolium* ssp. *lusitanicum* in Portugal (Menezes de Sequeira 1969), the latter now considered to be a variant of *O. serpyllifolia s.l* Later investigations revealed that Ni hyperaccumulation is widespread in the genus *Alyssum* on the ultramafic soils of Mediterranean Europe, Turkey, and adjacent countries (Brooks et al. 1979; Reeves and Adigüzel 2008; Reeves et al. 2018). According to the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/), the genus *Alyssum* comprises 62 taxa, for which their number in the Mediterranean area is at least 22, all in Section *Odontarrhena* that is now accepted as a separate genus (Španiel et al. 2015; Cecchi et al. 2018). According to the Alybase data (Španiel et al. 2015; http://www.alysseae.sav.sk/ accessed 10 January 2020), *Odontarrhena* includes 87 species mainly distributed in Mediterranean Europe and Irano-Turanian regions. Almost the same number of accepted species (88) come from the genus included in the BrassiBase checklist version 1.3 (https://brassibase.cos.uni-heidelberg.de/). New taxonomic data presented for the genus *Odontarrhena* in Ala (Cecchi et al. 2018) changed the number of species, but an exact number for Mediterranean Europe is difficult to report for several reasons. Firstly, there are taxonomic problems related to the nomenclature, identification, and status of some taxa. Although a taxonomic basis for *Alyssum* and *Odontarrhena* has been given (Španiel et al. 2015), there are still unresolved names for a great number of taxa that need more revision in the context of the whole genera, especially for those from the eastern Mediterranean. Secondly, there is still a lack of precise data on biogeography. For example, some taxa are known as being endemic to more-or-less restricted areas. Most of these taxa are poorly understood and, in some cases, only known from the original description and type collections (Cecchi et al. 2018). Thirdly, a problem exists in the lack of sufficient phylogenetical analyses including for all species from *Odontarrhena*. Moreover, molecular data presented in some phylogenetic papers are considered of limited utility because they reveal a low rate of sequence divergence among different genomic regions and provide limited resolution of species-level relationships (Cecchi et al. 2018).

Some Ni hyperaccumulator plants described as ‘obligate’ hyperaccumulators (Pollard et al. 2002) are restricted in their distribution to metalliferous soils and always exhibit hyperaccumulation. However, other so-called ‘facultative’ hyperaccumulators (Pollard et al. 2014) are more widespread both on metalliferous and non-metalliferous soils and demonstrate unusual Ni accumulation only on metalliferous (ultramafic and polluted) soils. The precise number of obligate and facultative species in Mediterranean Europe is also difficult to determine because of problematic issues of taxonomy, biogeography and chemical analyses of leaves, as discussed in detail by Pollard et al. (2014). In any case, *Odontarrhena* is a large and critical group that includes at least 33 native species (about 20 distributed in Mediterranean Europe) of obligate hyperaccumulators from serpentine soils (Brooks et al. 1979; Reeves and Adigüzel 2008). These data suggest a clear phylogenetic trend toward species-wide hyperaccumulation (Pollard et al. 2014; Cecchi et al. 2018), and species having scientific and practical applications.

A great number of Ni hyperaccumulators are concentrated in the eastern part of Mediterranean Europe, mainly the Balkans that represents the largest area of ultramafic bedrock in Europe (Stevanović et al. 2003). The number of Ni hyperaccumulators decreases from east to west; Corsica and the Iberian Peninsula host only one or two native species. The naturalized species in Corsica, *O. corsica*, native to Anatolia in Turkey, is also a Ni hyperaccumulator (3350–13,500 µg g^{-1}, according to Brooks and Radford 1978). The Balkan region is a major diversity centre for *Odontarrhena*, with 15–25 species, most of which are reported as endemic to more-or-less restricted areas, and in some cases are known only from the original description and type collection (Cecchi et al. 2018). This number is a significant proportion of the *Odontarrhena* hyperaccumulators recorded in Europe (Cecchi et al. 2010, 2018), suggesting that the Balkan Peninsula is a 'hotspot' for ultramafic flora of the continent. Although this number of Ni hyperaccumulators could be reduced after acquisition of new molecular evidence and an accepted taxonomy of the genus (Cecchi et al. 2010, 2013, 2018; Španiel et al. 2015), it is higher than in other parts of Europe. The majority of Ni hyperaccumulators distributed in Mediterranean Europe constitute ultramafic endemics. Greece has the largest number of local endemics followed by Albania, a small country with vast serpentine outcrops (Stevanović et al. 2003).

The data for Ni accumulation carried out on a wide range of herbarium and field-collected specimens of *Odontarrhena* species in Mediterranean Europe and those in the Global Hyperaccumulator Database (with some new records) are listed in Table 1. Precise comparisons of the hyperaccumulation abilities of the species are not possible owing to the uncertain identity of plant material and lack of revisions of the voucher specimens deposited in the Herbaria. Nickel hyperaccumulators show a wide variation in accumulating ability depending on the species and soils on which they are growing. The highest Ni concentrations were previously recorded in *O. chalcidica* (as *A. murale*) and *O. heldreichii* (as *A. heldreichii*) as 21,000 µg g^{-1} and 11,800 µg g^{-1}, respectively (Bani et al. 2010, 2013). More recent analyses of Ni accumulation in a critical group of metallophytes from a major serpentine hotspot in Albania nominate the tetraploids *O. chalcidica* and *O. decipiens* with maximal Ni levels of 23,000 µg g^{-1} and 17,300 µg g^{-1}, respectively, as the most promising candidates for phytoextraction of Ni from soil (Cecchi et al. 2018). Other taxa delimited by the same authors for the flora of Albania, such as *O. moravensis*, *O. rigida*, and *O. smolikana* ssp. *glabra* are also hyperaccumulators with maximum values of 14,300 µg g^{-1}, 17,100 µg g^{-1}, and 14,000 µg g^{-1}, respectively. Only the species *O. albiflora* has low Ni concentrations (2700 µg g^{-1}). The species *O. serpyllifolia s.l.* and *O. chalcidica*, widely distributed in the West and East Mediterranean, respectively, are both effective Ni hyperaccumulators (Bani et al. 2010, 2013; Tumi et al. 2012; Konstantinou and Tsiripidis 2015; Morais et al. 2015, Kidd et al. 2018; Xhaferri et al. 2018). Currently, *O. chalcidica* is considered one of the most promising candidate taxa for Ni agromining because of its capacity for biomass production and growth under various field conditions (Bani et al. 2015a, b). Being one of about 15 species belonging to the genus *Odontarrhena* that has been shown to have some Ni values above 2 wt% Ni, the Greek serpentine endemic *O. lesbiaca* is also promising for agromining practice (Kazakou et al. 2010). Experiments carried out in Italy on potential use of the Ni hyperaccumulator *O. bertolonii* for phytomining of ultramafic soils have demonstrated good results for Ni content (0.8 wt% in dry matter, 11 wt% in ash), and Ni yield (72 kg ha^{-1}). This species was previously suggested as being suitable for phytomining not only in Italy but

Table 1 Nickel-hyperaccumulator species from family Brassicaceae native to temperate/Mediterranean Europe (including Cyprus) and range of Ni concentration (mg kg^{-1}); nomenclature follows BrassiBase species checklist version 1.3 (https://brassibase.cos.uni-heidelberg.de/), Španiel et al. (2015) and Cecchi et al. (2018)

Taxa	Range of Ni concentration	References	Origin of the studied material
Alyssoides utriculata (L.) Medik. subsp. *utriculata*	Mean 1065	18	Italy
Bornmuellera baldaccii (Degen) Heywood	6480–27,300	1, 8, 13, 16	Greece, Albania
B. emarginata[a](Boiss.) Rešetnik	2040–34,400	1, 5, 13, 17	Greece
B. × *petri* Greuter, Charpin and Dittrich	3420–11,400	1, 8, 13	Greece
B. tymphaea (Hausskn.) Hausskn	1590–31,200	1, 8, 13, 17	Greece
Cardamine resedifolia L.	1050	1, 4, 8	Italy
Noccaea aptera (Velen.) F. K. Mey.	1360–21,500	1, 8	Bulgaria, ex-Jugoslavia
N. boeotica F. K. Mey.	23,400	1	Greece
N. bulbosa (Spruner ex Boiss.) Al-Shehbaz	2000	1	Greece
N. caerulescens (J. Presl and C. Presl) F. K. Mey.	Unknown	1	
N. cypria (Bornm.) F. K. Mey.	42–5120	9	Cyprus
N. epirota (Halácsy) F. K. Mey.	3000	1	Greece
N. firmiensis F. K. Mey.	16,200	1	Greece
N. goesingensis[b] (Halácsy) F. K. Mey.	Unknown	1, 7	Austria
N. graeca (Jord.) F. K. Mey.	<14–16,840	1, 7	Greece
N. kovatsii (Heuff.) F. K. Mey.	<3–21,550	7, 16, 22	Bulgaria, Serbia
N. ochroleuca (Boiss. and Heldr.) F. K. Mey.	499–23,400	1, 7, 14, 16	Albania, Bulgaria, Greece
N. praecox (Wulfen) F. K. Mey.	6.0–14,700	7, 16, 21	Bulgaria, Serbia
N. tymphaea (Hausskn.) F. K. Mey.	3430–16,540	1, 7, 13	Greece
Odontarrhena akamasica (B. L. Burtt) Španiel & al	3660–9090	1, 3	Cyprus
O. albiflora (F. K. Mey) Španiel & al.	2700	20	Albania
O. alpestris (L.) Ledeb.	3640–4480	1, 3	S. Europe
O. argentea (All.) Ledeb.	(3)-1150–29,400	1, 3, 6, 8	NW Italy
O. bertolonii (Desv.) Jord. & Fourr. subsp. *bertolonii*	Unknown	1	Italy
O. chalcidica[d] (Janka) Španiel & al.	4600–23,000	2, 13, 20	Albania, Greece
O. chondrogyna (B.L. Burtt) Španiel & al.	3980–16,250	1, 3	Cyprus
O. cyprica[e](Nyár.) Španiel & al.	7670–23,640	1	Cyprus
O. decipiens (Nyár.) L. Cechi and Selvi	7900–17,000	20	Albania
O. diffusa[f] Jord. and Fourr.	730–9460	1, 2, 3, 11	Greece
O. euboea (Halácsy) Španiel & al.	26–14,000	1, 2, 3, 8, 13	Greece
O. fallacina (Hausskn.) Španiel & al.	3960	1, 2	Greece (Crete)

(continued)

Table 1 (continued)

Taxa	Range of Ni concentration	References	Origin of the studied material
O. heldreichii (Hausskn.) Španiel & al.	1440–32,040	1, 2, 13, 16	Greece
O. lesbiaca P. Candargy	1820–29,560	1, 13, 14	Greece
O. markgrafii (O.E. Schulz ex Markgr.) Španiel & al. (=*O. chalcidica*)	<3.0–19,100	1, 2, 8, 10, 13, 19	Albania, Kosovo, Serbia
O. chalcidica[g] sensu lato (Waldst. and Kit.) Endl.	7–34,690	2, 8, 11, 13, 15,16	Albania, Bulgaria, Greece, Serbia
O. rigida (Nyár.) L. Cechi and Selvi	7500–17,000	20	Albania
O. robertiana (Bernard ex Gren. & Godr.) Španiel & al.	2730–12,500	1, 2	France
O. serpyllifolia[h] (Desf.) Jord. & Fourr.	1880–10,000	3, 12	Spain, Portugal
O. smolikana subsp. *glabra* (Nyár.) L. Cecchi & Selvi	7700–14,000	20	Albania
O. smolikana subsp. *smolikana* (Nyár.) Španiel & al.	1700–6600	1, 2, 13	Greece
O. troodi (Boiss.) Španiel & al.	5120–17,100	1, 2, 3, 8	Cyprus

References 1: Global Hyperaccumulator Database; 2: Brooks and Radford (1978); 3: Brooks et al. (1979); 4: Vergnano Gambi and Gabbrielli (1979); 5: Reeves et al. (1980); 6: Vergnano Gambi et al. (1982);7 Reeves and Brooks (1983); 8: Reeves et al. (1983); 9: Reeves (1988); 10: Obratov-Petković et al. (1997); 11: Reeves et al. (1997); 12: Asensi et al. (2004); 13: Bani et al. (2010); 14: Kazakou et al. (2010); 15: Tumi et al. (2012); 16: Bani et al. (2013); 17: Zhang et al. (2014); 18: Roccotiello et al. (2015); 19: Salihaj et al. (2016); 20: Cecchi et al. (2018); 21: Mišljenović et al. (2018); 22: Mišljenović et al. (2020)

[a] Originally in Reference 5 as *Peltaria emarginata* (Boiss.) Hausskn

[b] Some Greek specimens were earlier incorrectly identified as *T. goesingense* Halácsy in Herbaria and in Reference 7

[c] Originally in Reference 1 as A. *janchenii* Nyár. ex Novák

[d] Originally included in Reference 1 as *A. murale*

[e] Only data from Cyprus are included here

[f] Originally in Reference 1 as *A. tenium* Halácsy

[g] Only specimens from Albania, Bulgaria, Greece and Serbia are included here. Data for *A. murale* subsp. *pichleri* from References 13 and 16 are also included

[h] Data for *Alyssum pintodasilvae* T. R. Dudley, *Alyssum malacitanum* (Rivas Goday) T. R., *A. serpyllifolium* subsp. *malacitanum* and *A. serpyllifolium* subsp. *lusitanicum* Dudley originally in References 3 and 12 are included here

elsewhere, particularly in the Mediterranean region (Robinson et al. 1997).

Nickel hyperaccumulation in the Brassicaceae family of Mediterranean Europe occurs not only in the *Odontarrhena* (*Alyssum*) species, but also in species of the genera *Thlaspi s.l.* (*Noccaea*), *Bornmuellera*, *Alyssoides* and *Cardamine* (Table 1).

The taxonomically complex genus *Thlaspi s.l.* (divided into many separate genera, *Noccaea* being the largest) contains numerous Ni-hyperaccumulating species. Several *Noccaea* species have been reported by Reeves et al. (2001), Reeves (1988, 2006), Reeves and Brooks (1983) and Krämer (2010). About 10 species are distributed in Mediterranean Europe and are included in the Global Hyperaccumulator Database. Importantly, hyperaccumulation of Ni has been recorded in most of the European species occurring on serpentine soils. The highest Ni concentrations have been measured in *N. ochroleuca* (15–23,400 µg g^{-1}) followed by *N. kovatsii* (21,550 µg g^{-1} according to Bani et al. 2010). Facultative Ni hyperaccumulators (*N. ochroleuca, N. praecox* and *N. kovatsii*) have Ni-accumulating potential only where growing on ultramafic soils (Mišljenović et al. 2018, 2020). Measured Ni concentrations in leaves of these species from the Balkans are approximately four times higher than those reported for Turkey (Reeves and Adigüzel 2008). The number of *Noccaea* species locally endemic to the ultramafic soils is highest in Greece. The Greek endemic species *N. bulbosum*, *N. epirota*, *N. tymphaea* and *N. graeca* demonstrate different concentrations of Ni, the highest measured occurring in the last species (16,840 µg g^{-1}; Reeves and Brooks 1983).

Noccaea species are known to hyperaccumulate more than one metal (Cd, Ni, Pb, Zn). Reeves and Brooks (1983) and Reeves (1988, 2006) have shown that at least 30 species of *Noccaea* distributed in the Mediterranean region and Turkey are capable of accumulating Ni or Zn, or both, depending on nature of the soils on which they grow. The most extensively studied among them, *N. caerulescens*, has an extraordinary ability to accumulate Zn above 10,000 µg g^{-1} not only from Zn-rich soils but even from a variety of soil types with normal Zn concentrations. For the Austrian species *N. goesingense*, Reeves and Baker (1984) demonstrated that the ability to accumulate Ni and Zn is an innate property, unrelated to soil geochemistry. *Noccaea ochroleuca* grown on various substrates shows a wide range of concentrations of both Ni and Zn, each element exceeding 1000 µg g^{-1} (Reeves and Adigüzel 2008). Recently, new data on the natural variation of hyperaccumulation of Ni, Zn and Cd in *N. kovatsii* and *N. praecox* were reported, suggesting that Ni accumulation and translocation are restricted to ultramafic populations of both species, whereas these are species-wide traits for Zn (Mišljenović et al. 2018, 2020).

The genus *Bornmuellera* is restricted to Greece, Kosovo, Serbia, south Albania, and Turkey (Marhold 2011). Some of its species are ultramafic endemics. The highest Ni concentrations are reported for *B. tymphaea* from 1590–31,200 µg g^{-1} (Bani et al. 2010). Recently, in the light of available molecular data (Rešetnik et al. 2013) and re-evaluation of morphological characteristics, the monotypic genus *Leptoplax* (previously considered closely related to and even included in *Peltaria*) was merged with *Bornmuellera* (Rešetnik et al. 2014). A local ultramafic-endemic species, *B. emarginata* (*L. emarginata*), occurring mainly in northern Greece and on the island of Evvia, is reported to have widely varying Ni concentrations of 2040–34,400 µg g^{-1} (Bani et al. 2010; Zhang et al. 2014). When compared to *O. chalcidica* and *B. tymphaea*, *B. emarginata* was evaluated as the most efficient species for Ni phytoextraction and for decreasing the available Ni pool in soils (Chardot et al. 2005). The Ni yield from *B. emarginata* received from experimental plots in Greece (151 kg ha^{-1}) is considered exceptionally high and very promising in phytomining according to Kyrkas et al. (2019a, b). Although other taxa of *Bornmuellera*, including the hybrid *B. baldaccii* × *tymphaea* (*B.* × *petri*), are Ni hyperaccumulators also restricted to serpentine soils, their use in phytomining practice is limited because of small size, limited biomass production, and preferential high-montane distribution.

Alyssoides utriculata ssp. *utriculata* is the only taxon from the genus reported to be a facultative Ni hyperaccumulator (leaf Ni concentration 1065 µg g^{-1}) and a plant having good phytoremediation potential (Roccotiello et al. 2015). The data for the other European species (Reeves et al. 1983) do not demonstrate very high values of Ni and most of specimens are probably from limestone terranes or other low-Ni substrates. Recently, after much reconsideration of the species names, partly as a result of DNA work, the BrassiBase checklist now includes only one species, *A. utriculata*, (https://brassibase.cos.uni-heidelberg.de); other species names have been transferred or reduced to synonymy.

The Global Hyperaccumulator Database (Reeves et al. 2018) includes one more representative of the Brassicaceae, *Cardamine resedifolia*, collected from Italy and reported as a Ni hyperaccumulator (1050 μg g^{-1}) by Vergnano Gambi and Gabbrielli (1979). The hyperaccumulation abilities of this species have not been confirmed. Two additional Ni hyperaccumulators are reported from other families: the Asteraceae (*Centaurea thracica*, with 3830 μg g^{-1}) and the Violaceae (*Viola vourinensis*, 1023 μg g^{-1}) (Psaras and Constantinidis 2009), and both species are representatives of the species-rich flora of Greece. Recently, foliar Ni concentrations in leaves of *Centaurea thracica* collected in the Pindus Mountains and on the island of Evvia showed much higher values that exceed 11,100 mg kg^{-1}.

Nickel concentrations in different hyperaccumulator plants are quite different. Several taxa collected from a large variety of soil types correspondingly show a wide range of Ni concentrations. Such variation has even been reported at the population level. Some hyperaccumulators have occasionally lower Ni contents in the 100–1000 μg g^{-1} range, which might indicate that such a plant also exists on soils with lower Ni availability, possibly as a result of the soil being only partly of serpentinite origin. However, many hyperaccumulator species invariably have high Ni concentrations and are almost certainly true serpentine endemics (Bani et al. 2010).

It is noteworthy that all species recognized to date as Ni hyperaccumulators are from regions that were beyond the maximum advances of the main northern and southern icecaps during the Pleistocene era. These plants may therefore represent a type of behaviour that evolved prior to the most recent glacial episodes (Reeves et al. 1983).

Hyperaccumulator plants are of substantial fundamental interest and practical importance especially for phytoremediation and phytomining. Many of the known hyperaccumulators are both small and grow slowly, and often are rare species of limited population size and very restricted distribution. The ideal phytoremediation crop may combine rapid growth and high biomass with high metal accumulation within shoot tissues (Lasat 2002). Leaves remain the most important plant part to harvest for phytomining purposes, although the stems have intermediate Ni concentrations and potential for high biomass production (Zhang et al. 2014). The percentage of leaves in the biomass of different hyperaccumulator species is highly variable, even within a given species, thus being crucial for defining the potential phytoextraction yield. The distribution of Ni among the different organs of hyperaccumulator plants depends on the biology of the species and can be influenced by climate and edaphic factors such as soil pH, etc. A significant variation in Ni phytoextraction across different phenological stages has also been demonstrated (Adamidis et al. 2017; Xhaferri et al. 2018). However, agronomic practices have not been applied to the majority of hyperaccumulator species and, therefore, the maximum amount of plant biomass that can be achieved under favourable conditions (climate, nutrient levels, etc.) remains unknown (Reeves 2006; Kazakou et al. 2010). It is, however, becoming apparent that several efficient populations of *O. chalcidica*, *B. emarginata*, and *B. tymphaea* (Greece) display the highest efficiency for Ni phytomining (Chardot et al. 2005; Bani et al. 2009, 2014; Zhang et al. 2014; Kidd et al. 2018; Xhaferri et al. 2018).

3 Case Study in the Temperate/Mediterranean Europe Regions

The Life-Agromine project established a network of pilot-scale field sites in ultramafic regions across western, central, and southern Europe in order to cover a range of edaphic conditions and to apply the agromining of Ni as an agricultural alternative for rural ultramafic landscapes. *Odontarrhena chalcidica* occurs widely and spontaneously on ultramafic Vertisols and is a spontaneous weed among other crops. Both *Bornmuellera tymphaea* and *B. emarginata* are native to the Pindus Mountains, the first being endemic to ultramafic soils of northwestern Greece. The Ni-hyperaccumulator *O. serpyllifolia s.l.* is endemic to Iberian ultramafic soils and is common on fallow fields in the Melide ultramafic complex close to the village of Eidián in northwestern Spain.

The four sites across Europe that were studied for field demonstration of agromining plots cover different climatic conditions from humid-temperate (NW Spain) to sub-Mediterranean (Albania and Greece). Edaphic conditions are also quite similar with most being Cambisolic, Eutric, and Magnesic in all soils (refer to Chapter "Genesis and Behaviour of Ultramafic Soils and Consequences for Nickel Biogeochemistry"). Total Ni concentrations often exceed those of K; Ni availability ranges from 37 mg kg^{-1} in the most acidic soils to >100 mg kg^{-1} in the Balkans site (drier climate). Although experiments in Albania have been ongoing since 2005, the Life-Agromine project since 2016 has provided a complete set of reference sites across Europe to test the influence of climatic conditions, plant species, and soil conditions on the yield of Ni agromining, as well as on cropping options (e.g. co-cropping, fertilization pattern).

3.1 Albania: 8-Year Agromining Field Experiments with *Odontarrhena chalcidica*

Ultramafic areas in Albania occupy 313,300 ha, i.e. 11.1% of the total surface of the country (Fig. 1). Geologically, they vary from partly serpentinized peridotite (harzburgite) to serpentinite (Bani et al. 2014; Estrade et al. 2015). Long-term agromining studies have been carried out intermittently over the period 2005–2018 in the Pogradec district (Pojskë) (Bani et al. 2007, 2015a, b). Consequently, investigations of the performance of a phytoextraction system using varied agronomic practices were undertaken in order to evaluate whether semi-extensive phytoextraction of Ni was feasible on Mg-rich ultramafic Vertisols. *Odontarrhena chalcidica* was the easiest crop to plant, especially because of its high rate of seeding and high frequency of occurrence in ultramafic agricultural lands of Albania. Experiments were undertaken from 2005 to 2009 and from 2012 to 2014 on in situ experimental plots. Field experiments (Bani et al. 2015a, b; Bani and Echevarria 2019) were aimed at studying (i) the distribution of Ni and other elements in plant organs, (ii) plant nutrition and fertilization practices, (iii) weed control, (iv) the crop establishment technique (natural cover *vs.* sown crop) and (v) planting density. Firstly, the cropping of native stands of *O. chalcidica* was studied for three years. After this period, the *O. chalcidica* cover was renewed by sowing native seeds without density control. At another stage in the set of experiments, the effect of density was studied from data on 1–6 plants m^{-2}. In the last field experiments, the effectiveness of different fertilization practices in the Ni phytoextraction yield were compared (three main sources of nutrients were compared from the following amendments: inorganic NPK fertilizer, pig manure, and chicken manure). The final objective was to propose a cropping system for *O. chalcidica* that would optimize agromining crops.

3.1.1 Fertilization Regime and Nutrient Requirements of Native Stands

Natural stands of *O. chalcidica* were cropped in Pojskë from 2005 until 2007 (Bani et al. 2015a). In March of 2005, six 36 m^2 plots were designed, three of which were fertilized in April with 120 kg ha^{-1} of N, P and K and 77 kg ha^{-1} of Ca (ammonium nitrate, potassium sulfate, and calcium phosphate). The other three plots were not fertilized (Bani et al. 2007). In 2006, the same fertilization pattern was used, but three plots were also treated with anti-monocot herbicide (FocusTM ultra, 33 mL applied in 3 L water sprayed on toto 108 m^2) (FH); a further three were not treated (NFNH). The optimal fertilization pattern for *O. chalcidica* had been carefully determined using the same soil in glasshouse pot experiments (Bani et al. 2007; Nkrumah et al.

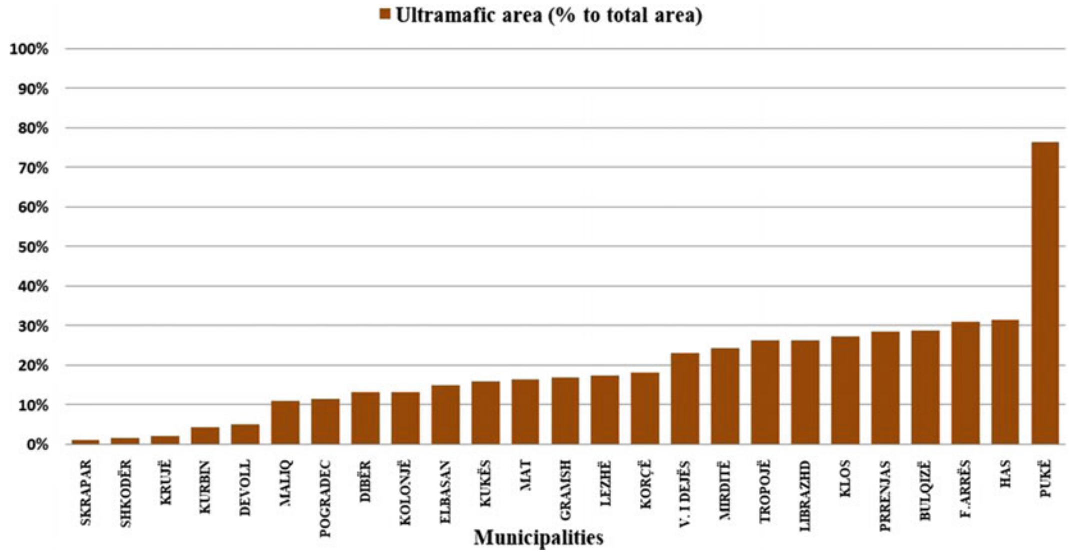

Fig. 1 Area of ultramafic substrates (10–43% MgO) in Albanian municipalities (% to the total area of municipalities (Lekaj et al. 2019)

2016). Plants were harvested for the first and second year at the end of June (before studies on Ni distribution in plant parts according to the phenological stage). In 2007, the fertilization protocol involved 100 kg ha^{-1} P and K; for N there was a split application of 120 kg ha^{-1}. In early April, 60 kg ha^{-1} were brought into early vegetation and a further 60 kg ha^{-1} were fertilized at the end of April, during the stem elongation stage. Following phenological studies (Bani et al. 2015a; Estrade et al. 2015), plants were harvested in mid-June for the last three years of the experiment. The overall vegetation cover responded dramatically to fertilization, by doubling the biomass and phytoextraction yield. For the first year (2005) in the fertilized plots, a total biomass of 6.3 t ha^{-1} (dry weight) for all species was obtained and a Ni phytoextraction yield of 22.6 kg Ni ha^{-1}; in unfertilized plots, the total biomass was only 3.2 t ha^{-1} (Figs. 2 and 3) with a Ni phytoextraction yield of 1.7 kg Ni ha^{-1}. These data thus show a highly significant difference ($p < 0.01$) between fertilized and unfertilized plots (Fig. 3). In total phytoextraction yield, the main contributory species was *O. chalcidica*. In the third year (2007) after the fertilization protocol had been changed and the date of harvest, biomass yield in the fertilized plots was 6.0 t ha^{-1}, whereas in unfertilized plots it was only 0.5 t ha^{-1} (Fig. 2) (Bani et al. 2015a).

3.1.2 Sowing *O. chalcidica*

During 2007–2008, the soils of the six initial 36 m^2 plots were tilled to prepare appropriate seedbeds (Bani et al. 2015a). Seeds had previously been collected in the immediate vicinity of the experimental plots. All plots were hand-sown in September of 2007. Fertilized plots received 100 kg ha^{-1} P and K, 65 kg ha^{-1} Ca, and 50 kg ha^{-1} N in April and were treated with antimonocot herbicide. A second N fertilization was performed (50 kg ha^{-1}) two weeks later. The N addition was lowered in order to take into account the mineralization of plant residues. The other three plots were not treated (neither with fertilizer nor herbicide). During the growth period, the treated experimental plots were irrigated with water from a natural stream in the area. The harvesting date was early July of 2008, when the biomass was considered high enough (little flowering had occurred in the newly planted plots). In 2009, the protocol of fertilization and irrigation was repeated exactly as carried out in 2008, and the plots were harvested on June 12 at

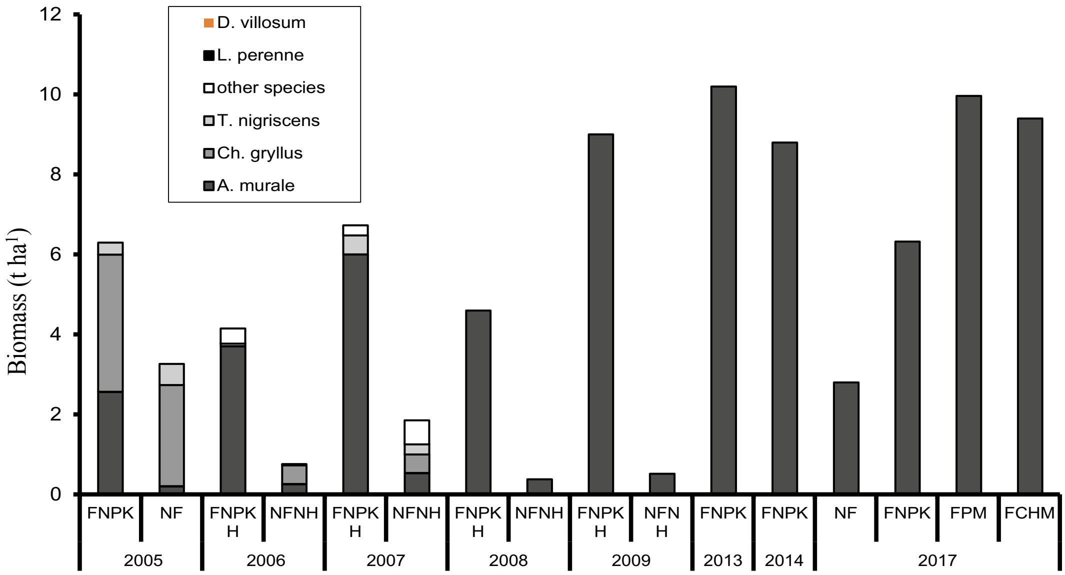

Fig. 2 Evolution of biomass-production of *Odontarrhena chalcidica* during 8 years of field study at Pojskë, Pogradec, Albania

the mid-flowering stage. In 2008, with split N-fertilization, irrigation, and herbicide treatments, the biomass production was 4.6 t ha^{-1} in the fertilized and herbicide-treated plots; the Ni phytoextraction yield was 55 kg ha^{-1} (Figs. 2 and 3). In contrast, the yield was only 0.4 t and 4.3 kg Ni ha^{-1}, respectively, in the unfertilized plots with a significant difference ($p < 0.05$). In 2009, in the fertilized plots the biomass production was 9.0 t ha^{-1} and Ni phytoextraction yield was 105 kg ha^{-1}, which subsequently proved to be optimal for the prevailing edaphic conditions (Bani et al. 2015a, b).

3.1.3 Competition with Other Species and Density Effects

When enhancing natural vegetation stands by fertilization (Bani et al. 2015a), other species were reported on the plots, although their contribution to total biomass production was negligible (Fig. 2). Among weeds, *Chrysopogon gryllus* was identified as the most competitive species and *Trifolium nigrescens* tended to disappear with fertilization. A good weed control was obtained on the fields by addition of 120 kg ha^{-1} N fertilization and a single spraying with anti-monocot herbicide at the end of April 2005 (Bani et al. 2015a). During 2012–2014, the influence of plant density on Ni phytoextraction yield was evaluated for the case of sown/planted crops of *O. chalcidica* on two representatives ultramafic Vertisols at Pojskë (POJ) in the municipality of Pogradec (Fig. 3), and Domosdova (DOM) in the municipality of Prrenjas (Bani et al. 2015b). Three different plant densities were evaluated: (i) at the Domosdova site, *O. chalcidica* was either transplanted without density constraints (*ca.* 1.5 plants m^{-2}) (DOM-1) or at a density of 6 plants m^{-2} on an adjacent plot (DOM-6). This species was grown at a density of 4 plants m^{-2} in Pojskë (POJ-4). In 2014, the Domosdova field was mistakenly ploughed and was therefore unavailable for more cropping, hence only data for 2013 are available for this site. The biomass of DOM-1 (10.6 t ha^{-1}) was much higher, nearly twice as great as for DOM-6 (4.8 t ha^{-1}). Nickel yield followed the same trend: DOM-1 (77 kg ha^{-1}) was higher than DOM-6 (41 kg ha^{-1}) (Table 2). With a density of 4 plants m^{-2} (POJ-4), biomass was the highest (10.2 t ha^{-1} in 2013 and 8.8 t ha^{-1} in 2014); in 2014, *O. chalcidica* was in competition with its

Table 2 Physio-chemical characteristics of the soils of the test sites

Site	Pojskë	Bernstein	Koutsoufliani	Eidián
Country	Albania	Austria	Greece	Spain
Altitude (m)	700	620	930	430
Climate	Sub-mediterranean continental	Warm-summer humid continental	Sub-mediterranean continental	Humid temperate
Soil pH H_2O	7.5	6.1	7.2	5.8
Ca-total (g kg^{-1})	3.9	6.7	7	7.6
Mg-total (g kg^{-1})	60	103	138	45.1
K-total (g kg^{-1})	4.5	0.91	1.86	0.44
Ni-total (g kg^{-1})	3.14	1.45	2.35	0.97
Ni-DTPA (mg kg^{-1})	124	38.4	71.1	36.8

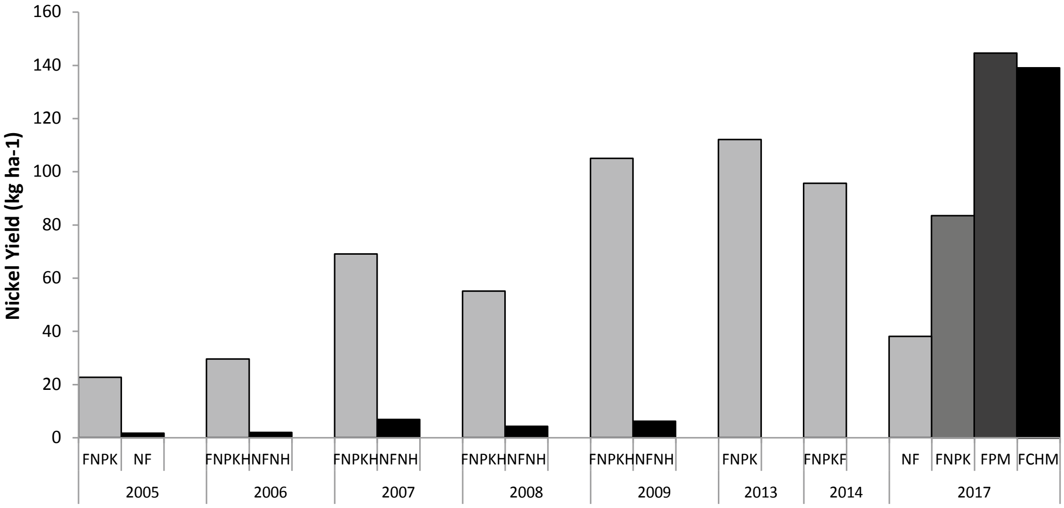

Fig. 3 Evolution of phytoextraction yield kg Ni ha^{-1} of *Odontarrhena chalcidica* during 8 years of field study at Pojskë, Pogradec, Albania

own spontaneous recruits. The Ni yields in the biomass of *O. chalcidica* for 2013 (112 kg per ha^{-1}) were higher than in 2014 (95.9 kg ha^{-1}) (Figs. 2 and 3).

According to the process for producing ANSH from *O. chalcidica* ash (Barbaroux et al. 2012; Zhang et al. 2014), it is crucial to evaluate the ash-producing yield and the percent recovery of Ni at 550 °C (Zhang et al. 2014). The total mass of ash that could be recovered was 1.3 t ha^{-1} for DOM-1 and 0.4 t ha^{-1} for DOM-6. Consequently, the total Ni mass in the ash of *O. chalcidica* in the DOM-1 treatment was 76 kg ha^{-1} with almost no loss of Ni during the combustion process. The quantity of ash of *O. chalcidica* produced in the DOM-6 treatment was only 41 kg ha^{-1}, also without significant loss of Ni during ashing. Using the optimized process to produce ANSH from *O. chalcidica* ash (Barbaroux et al. 2012), at least 45 g of ANSH (close to 100% purity) was obtained from one plant of *O. chalcidica*, which represents 450 kg ANSH ha^{-1} in the DOM-1 plot. Nickel phytoavailability is thought to have a stronger influence on uptake of this metal in shoots than does plant density (Bani et al. 2015b). It is important to note that the biomass yield of each plant did not impair Ni uptake, and that no strong dilution effect was observed in cropped *O. chalcidica* (Bani et al. 2015a). Therefore, on a given site, it is crucial to maximize the yield of plant biomass. Biomass yield at harvest under these edaphic conditions (i.e. ultramafic Vertisols of southeastern Albania) seems to reach a maximum of *ca.* 10 t ha^{-1} when using seeds of native populations (Bani et al. 2015a, b). Only genetic selection can further improve biomass yields (i.e. 20 t ha^{-1}) through more production of performing cultivars (Li et al. 2003), which are keys to increased Ni phytoextraction yield (Bani et al. 2015b).

3.1.4 Inorganic Fertilization Versus Farm Manure

In order to assess the effect of organic fertilization, three treatments were compared in the field trials of Pojskë, Pogradec, during 2016–2017 (Bani and Echevarria 2019): (i) POJ-1, a treatment with spontaneous vegetation composed of >90% recruits of *O. chalcidica* from spring of 2016 (0.3 ha). In this first plot, no treatments were applied (Note: NF –not fertilized). Different regimes of fertilizations were applied in the other two plots. (ii) POJ-2 plot was similarly covered by young recruits of *O. chalcidica* plants from spring of 2016 (0.3 ha). This plot was treated with mineral fertilizer (Note: FNPK–fertilized with NPK.), i.e. 50 kg ha^{-1} of N, P and K, respectively, or 3.3 kg ha^{1} NPK mineral fertilizer were added in this plot on April 2, 2017. (iii) POJ-3 plot in which *O. chalcidica* was planted at a density of 4 plants m^{-2} (1 ha) in April of 2017, was divided into four subplots. For a ploughed 400 m^{2} the first was not fertilized (NF). Three types of fertilization were applied on the three other subplots (100 m^{2} each) in November of 2016 to stimulate vegetative growth of seedlings, respectively, 15 kg ha^{-1} of N, P and K or 1 kg ha^{-1} 3x NPK mineral fertilizer were, respectively, added to subplot 2 (FNPK); N260:P105:K260 kg ha^{-1} or 175 kg ha^{-1} pig manure was added to subplot 3 (FPM - Fertilized pig manure); N260:P390:K260 kg ha^{-1} or 130 kg ha^{-1} chicken manure was added to subplot 4 (FCHM–fertilized chicken manure). On April 2, 2017, weeding was carried out mechanically for all POJ-3 subplots and 50 kg ha^{-1} N, P, and K were applied (i.e. 3.3 kg ha^{-1} NPK mineral fertilizer) only over the plants of subplot 2 in POJ-3 and 15 kg ha^{-1} N, P, K, or 1 kg ha^{-1} = NPK mineral fertilizer in subplots 3 and 4. During the vegetation period (late April-early May, 2017), the experimental plot POJ-3 was regularly irrigated by a hose using water from a natural stream in the area.

In the case of natural vegetation of *O. chalcidica* (POJ-2) the biomass of fertilized plot 1 was much higher ($3.^{1}$ t ha^{-1}), which is nearly three times as much per hectare than non-fertilized plots (POJ-1), and so the Ni yield of POJ-2 plot (39.1 kg ha^{-1}) was higher than that of POJ-1 plot (13.5 kg ha^{-1}). Secondly, in the case of *O. chalcidica* cultivated on POJ-3 plot, the biomass of fertilized subplots (6.32, 9.96 and 9.4 t ha^{-1}) was two and three times higher than non-fertilized subplot (2.8 t ha^{-1}), respectively, in the case of added mineral (FNPK) and organic (FPM, FCHM) fertilizers. Consequently, the Ni yields of fertilized subplots of POJ-3 were in the following order: FPM (145 kg ha^{-1}) > FCHM (139 kg ha^{-1}) > FNPK (83.4 kg ha^{-1}), all of which were higher than the non-fertilized subplot (NF 38 kg ha^{-1}). The Ni phytoextraction yield in pots grown for six months in the greenhouse experiment was 84.8 mg Ni pot^{-1} in the unfertilized treatment, 399 mg Ni pot^{-1} in the 260 N:105P:260 K kg ha^{-1} pig manure treatment, 265 mg Ni pot^{-1} in the 260 N:390P:260 K kg ha^{-1} chicken manure treatment, and 152 mg Ni pot^{-1} in the 50:50:50 + 15 kg ha^{-1} N, P, K treatment (Fig. 3). Manure addition (either chicken manure or pig manure) was by far the best fertilization option of all treatments in

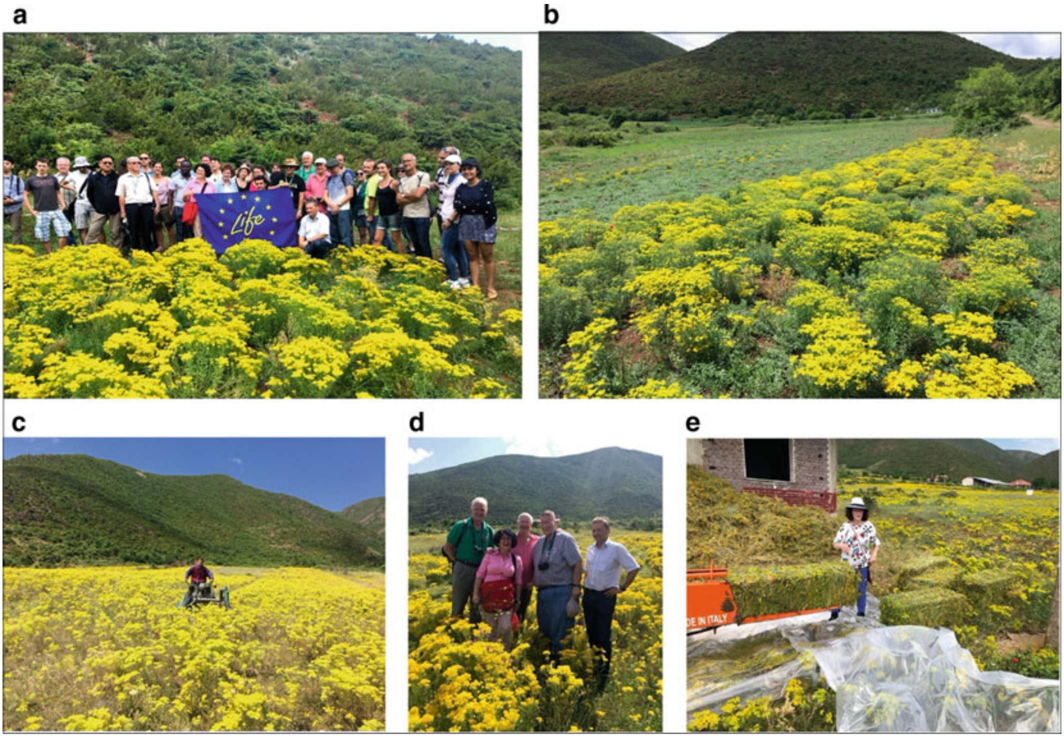

Fig. 4 Images of *O. chalcidica* crops at planting density of 4 plants m^{-2} during flowering and harvest in Albanian ultramafic site of Pojskë (2017)

terms of biomass production (plant height, weight, and number of stems were significantly increased), Ni uptake, and foliar Ni concentrations (Bani and Echevarria 2019) (Fig. 4).

3.2 Austria

A field experiment was set up in Bernstein in October of 2016 as described in Kidd et al. (2018). The main aim of the experimental work was to assess the phytomining efficiency of *O. chalcidica* and other hyperaccumulators on a serpentine site in the temperate climate zone. The initial experimental set-up consisted of 24 plots, 10 m^2 each. Average total soil Ni concentration was 1450 mg kg^{-1}, whereas the mean DTPA-extractable Ni concentration was 38.4 mg kg^{-1}. Further soil characteristics are given in Kidd et al. (2018). Overall, three experiments were carried out over three vegetation cycles. In contrast to the Mediterranean locations in Koutsoufliani and in Albania, in most treatments the seedlings were transplanted to the field in spring. The plants were then grown over the summer period and harvested in autumn.

Experiment 1 (2016/2017): All plots were fertilized with liquid pig manure in September of 2016. Seedlings of *Noccaea goesingensis* were transplanted in October of 2016, whereas seedlings of *O. chlacidica* were planted in April of 2017. Different plant densities and intercropping with *Lotus corniculatus* were tested for *N. goesingensis*, whereas intercropping with *L. corniculatus* and application of elemental sulfur (S^0) were tested for *O. chalcidica*. The largest Ni yield was found for *O. chalcidica* (Rosenkranz et al. 2019) (Table 3).

Experiment 2 (2018): Different mineral and organic fertilizer treatments were tested with *O. chalcidica*. In addition, a greater plant density (9.6 plants m^{-2}) was compared with the otherwise applied density of 4 plants m^{-2}. Pig and cow manure application led to the highest Ni

Table 3 Selected results on the experimental results achieved on the serpentine site in Bernstein

Species	Year	Treatment	Plot size (m^2)	Ni conc. (g kg^{-1})	Ni yield (kg ha^{-1})	Reference
O. chalcidica	2017	Fertilization with liquid pig manure + S^0	10	12.9	50.2	Rosenkranz et al. (2019)
N. goesingensis	2016/17	Fertilization with liquid pig manure, HD	10	9.8	30.6	Rosenkranz et al. (2019)
O. chalcidica	2018	Fertilization with cow manure	10	12.5	22.7	Hipfinger et al. submitted
O. chalcidica	2019	Fertilization with liquid pig manure	10	15.9	94.3	Unpublished data
O. chalcidica	2019	Fertilization with liquid pig manure, harvest in spring	10	11.7	79.9	Unpublished data
O. chalcidica	2019	Fertilization with liquid pig manure	250	15.0	114	Unpublished data

yield (unpublished data). Overall, shoot biomass and Ni yields were generally smaller than in the previous year, because seedling development was delayed due to significant competition by weeds (Table 3).

Experiment 3 (2019): Several hyperaccumulator species were compared for growth performance and Ni yield. The largest Ni yield was found for *O. chalcidica* (unpublished data). In addition, growth of *O. chalcidica* over winter and harvesting in spring was tested. Finally, a large-scale plot of 220 m^2 was set up for upscaling of previously observed results determined on 10 m^2 plots. On the large plot, a maximum of 130 kg Ni ha^{-1} was harvested (Table 3).

Overall assessment of the experimental results: In spite of the non-Mediterranean climate, reasonable Ni yields were achieved, especially in the last experimental year. However, due to several factors, the practical application of the agromining technology appeared limited. Firstly, all of the plants had to be germinated and pre-grown in a greenhouse for more than two months. Direct seeding in autumn was tested but was not successful. Secondly, the seedlings had to establish and develop in spring, thus severe weed competition likely limits growth of the hyperaccumulators if regular manual weed control is not done. In the last year (2019), a fleece preventing weed growth was successfully applied and plant yields were much larger than in the two prior years. Finally, in particular, the height of *O. chalcidica* plants was retarded compared to those grown on the southeast European sites and did not reach sufficient heights for mechanical harvesting. Overall, the results showed that sufficient Ni yields could be achieved on a non-Mediterranean site but require a great deal more effort compared to the indigenous areas. These additional efforts also generate additional costs, which would significantly limit profitability of agromining in this situation.

3.3 Greece

The Pindus Mountain Range is considered the ‘heart’ of serpentine areas in Greece and presents high floristic diversity. In the context of the Life-Agromine project, the first Ni agromining field trials were established in this area at Koutsoufliani, near the village Panagia at the borders of Thessaly, Epirus, and western Macedonia. The trials were carried out on an abandoned field with a mean total soil Ni concentration of 2347 mg kg^{-1} at an altitude of 930 m (Echevarria et al. 2017; Kidd et al. 2018).

The Ni agromining capacity of three high-biomass Ni hyperaccumulators (*O. chalcidica*, *Bornmuellera tymphaea*, and *B. emarginata*), native to the Pindus mountain range and closely related to serpentine substrates, is being evaluated in monoculture plots (three replicate 50 m^2 plots/species). *Bornmuellera emarginata* and *B. tymphaea* are Greek endemics, whereas *O. chalcidica* has a wider distribution (Euro + Med PlantBase). Field preparation (initial soil sampling, deep ploughing, transfer of a water tank, fencing) took place in March-April of 2017. The experimental plots were set up in May of 2017 through transplanting. Seedlings were produced in the greenhouse from seed collected from the surrounding and wider areas. No pesticides were used. NPK 15:15:15 fertilizer (1000 kg ha^{-1}) was applied after transplanting and once per year (early spring). Watering was only needed when transplanting the seedlings in the plots. The plots were subsequently under natural rainfall conditions. At harvest, plants were cut at 5–6 cm above ground and left to re-sprout (Kyrkas et al. 2019a, b).

After two harvests in 2018 and 2019, the results were particularly encouraging in terms of biomass production and Ni yield. Results of the first harvest carried out in June of 2018 are reported. The biomass production reached 8.1 t ha^{-1} for *B. emarginata*, 6.1 t ha^{-1} for *B. tymphaea*, and 13.5 t ha^{-1} for *O. chalcidica* with Ni yields of 151 kg ha^{-1}, 88.3 kg ha^{-1} and 106.3 kg ha^{-1}, respectively (Kyrkas et al. 2019a). The agronomic practices followed to date showed that *B. tymphaea* and *B. emarginata* seemed to be suitable as perennial crops through transplanting, whereas *O. chalcidica* may serve as an annual crop through transplanting or probably biennially through sowing (Kyrkas et al. 2019a, 2019b). This study is continuing.

The availability of seeds is a determining factor for upscaling Ni agromining in Greece and beyond. Creating a seed bank is a priority. For that purpose, the seed collection sites (already known and new ones) with all relevant information are being recorded through the Life-Agromine project, in order to create a database of the Greek biotopes of the species of interest that can be updated. Furthermore, a study of seed germination capacity of *O. chalcidica*, *B. tymphaea* and *B. emarginata* under different storage conditions in relation with time is in progress.

3.4 Spain

Agromining field trials have been carried out at an ultramafic outcrop under a humid-temperate climate in northwestern Spain. The experimental fields are located close to the village of Eidián (Galicia) (42°49′54.2″N, 8°00′13.4″W) at 430 m elevation. Mean annual precipitation is 1200 mm and mean average temperature 12 °C (Table 2). The soil is a Leptic Phaeozem (Magnesic), developed over serpentinized peridotite, has a sandy-loam texture, slightly acidic pH (*ca.* 6), with abundant organic matter (13%) and a pseudo-total Ni concentration of *ca.* 1000 mg kg^{-1} (available DTPA extractable Ni 36.8 ± 13.8 mg kg^{-1}). The first trials were initiated in 2015 for testing the performance for agromining purposes of the Ni hyperaccumulators *O. chalcidica* and *B. emarginata*. Plots of 50 m^2 (5 m × 10 m) were established in triplicate for each species, and the soil was fertilized with gypsum (1000 kg ha^{-1}) and inorganic NPK fertilizers (120:120:150 kg ha^{-1}). The plants were germinated and grown for 1 month in greenhouse before transplantation to the field site (Pardo et al. 2018) at a density of 4 plants m^{-2} following Bani et al. (2015b). During the first experimental year (June 2015–May 2016), the high precipitation and characteristics of the site led to waterlogging which, together with competition from weeds (mainly grasses), induced a serious plant mortality (up to 50–60% in some plots). Biomass obtained in the first harvest reached 1.0 ± 0.3 and 0.7 ± 0.2 t dry weight ha^{-1} for *O. chalcidica* and *B. emarginata*, respectively (Pardo et al. 2018). The Ni yield was also higher for *O. chalcidica* than for *B. emarginata* (4 kg and 3 kg ha^{-1}, respectively). During the second experimental year (2016–2017), a drainage system and weed control were implemented in the same plots, which led to improvements in the harvested biomass of

O. chalcidica (reaching 2 t ha^{-1}) and the Ni yield obtained from both species (on average 12 kg Ni ha^{-1}). An adjacent area in the same experimental site, where 20 m^2 plots were established, proved to be more favourable for the growth of the hyperaccumulators used. The biomass harvested in 2017 reached close to 10 t ha^{-1} for *O. chalcidica* and > 4 t ha^{-1} for *B. emarginata* with Ni yields of up to 45 kg ha^{-1} and 25 kg ha^{-1}, respectively. Nickel agromining was also evaluated with the local endemic *O. serpyllifolia s.l.* that grows spontaneously in fallow fields. The harvested biomass for this species was similar to that of *B. emarginata*, but Ni yield was lower (2–7 kg ha^{-1}) (Kidd et al. 2018).

Periodic analysis of the Ni concentration in shoots during plant growth indicated that Ni bioaccumulation was maximal at the mid-flowering stage and that this was the case for both *O. chalcidica* and *B. emarginata*, confirming this is the optimal stage for harvesting. Regarding the Ni distribution in plant tissues, Ni accumulation was strongly compartmentalized with the main contribution to Ni yields coming from leaves, especially those of flowering stems, but the contribution from flowers and fruits was also significant in the case of *B. emarginata* (Pardo et al. 2018).

The use of cow manure as fertilizer (10 t ha^{-1}) for agromining was also tested at the Eidián site. In the first trial, the biomass of *O. chalcidica* and *B. emarginata* obtained from manure-amended plots was similar to that from NPK-fertilized plots. However, Ni yield significantly decreased (*ca.* 50% lower). In the case of *O. serpyllifolia s. l.*, both the biomass and Ni yield obtained were lower using cow manure than with NPK fertilizer. In the second year of cultivation, the biomass and Ni yield of *O. chalcidica* in manure-fertilized plots was <50% of the values obtained in NPK-fertilized plots. Surprisingly, in the case of *B. emarginata* and *O. serpyllifolia s.l.*, the differences between the organic and inorganic fertilization treatments were not significant, although both biomass and Ni yield tended to be lower in manure-amended plots. The fertilization experiments continue in order to evaluate the potential usefulness of cow manure as a fertilizer for Ni agromining with *B. emarginata*.

Agromining experiments at Eidián also included trials that tested co-cropping of *O. chalcidica* with leguminous plants. Saad et al. (2018) observed that, in comparison with non-fertilized *O. chalcidica* monoculture plots, co-cropping the hyperaccumulator with *Vicia sativa* had positive effects on plant growth and Ni concentrations in shoots. These authors did not find significant differences in plant growth between co-cropping and fertilized (NH_4NO_3) mono-cropping treatments. Additional field experiments obtained a lower biomass of *O. chalcidica* when co-cropped with *Lotus corniculatus* than in monoculture in NPK-fertilized plots.

Biotechnological tools for improving the growth of hyperaccumulators, previously selected in experiments in the greenhouse, such as use of phytohormones (Cabello-Conejo et al. 2014b) or inoculation with plant growth promoting bacteria (PGPB) (Becerra Castro et al. 2013; Cabello-Conejo et al. 2014a), were also tested at field scale at the Eidián site. The experiments, carried out in two consecutive years, indicate that the plant growth regulator Promalin® (Kenogard, Barcelona, Spain) containing gibberellin and 6-benzyladenine, induces increases (20–70%) in the aerial biomass of *B. emarginata*. However, this phytohormone did not significantly affect the growth of *O. chalcidica* or *O. serpyllifolia s.l.* At field scale, growth of the three Ni hyperaccumulators studied was not significantly affected by the auxinic action Kelpak® (Daymsa, Zaragoza) applied every month during the growing season. Use of the PGP bacterial strains *Paenarthrobacter nitroguajacolicus* LA44 or *Pseudoarthrobacter oxydans* SBA82 as inoculants also improved the growth of *O. chalcidica* in field plots and significantly increased the Ni yield (Pardo pers. comm.)

4 Conclusions and Perspectives

Agromining field trials have been conducted almost continuously for 15 years in Albania and now for five years in Austria, Greece, and Spain.

These trials have allowed a complete and thorough understanding of the behaviour of Ni agromining crops under European conditions. Consequently, cropping systems have been designed that are adapted to each situation. The Ni hyperaccumulator *O. chalcidica* has real potential to become a cash crop in most of the situations. The species has a great plasticity (growing from almost sea level to *ca.* 2000 m elevation) and is adapted well to contrasted European settings. Results for this species were nearly similar in Austria, Greece and Albania. Those obtained to date demonstrate that it is necessary to sow or transplant *O. chalcidica* at a density of 4 plants m^{-2} on well-structured (ploughed) soils, under conditions of non-limiting Ni availability on ultramafic Vertisols (e.g. at Pojskë). Harvests can be carried out at the mid-flowering stage, which maximizes the recovery of Ni; it is now known that this stage corresponds to a massive phloem redistribution of Ni within shoots and to flowers. The fertilization patterns have been improved throughout the years for these soils, i.e. 120 kg N ha^{-1} with a split application and additional P, K, S and Ca fertilization (K_2SO_4 and $Ca(H_2PO_4)_2$) at 100 kg ha^{-1} for K and P, and 77 kg ha^{-1} for Ca. The effect of added farmyard manure (cow, pig, chicken) is rather contrasted among the different trial sites and their edaphic conditions. Whereas manure seems to be less efficient than full inorganic fertilization in acidic, low-available Ni soils (i.e. Eidián), it is much more efficient in high-available Ni soils (e.g. Albania). Specifically, at the first site, manure additions seem to enhance shoot biomass but strongly reduce Ni concentrations. In contrast, Albanian field trials show that both shoot biomass and Ni concentrations of shoots are strongly stimulated by manure additions as opposed to inorganic fertilization. Co-cropping *O. chalcidica* with legumes and stimulation of plant growth and Ni uptake through the inoculation of PGPR have also proved valuable alternatives or complements to a standard fertilization regime.

Crops are likely to export *ca.* 100 kg of Ni and K and more than 135 kg of Ca annually. Calcium and K should be returned by fertilization over the years to maintain fertility of the soil and suitability for agromining. Weeding is crucial and targets competing species as well as young recruits of *O. chalcidica* for maintaining an optimum density of the crop. In non-native sites, this is not a problem, however, the risk of dissemination of the species exists and it is recommended that weeds are managed with care. *Odontarrhena chalcidica* is harvested either by machine or by hand; the latter method proved to be the most effective and allowed biomass drying on the field as a hay crop. This biomass can then be baled, ready for combustion.

Two other plant species have confirmed a potential already described from pot trials, namely *Bornmuellera emarginata* and *B. tymphaea*. The first was tested several times under field conditions in Spain and the biomass and Ni yields were much lower than for *O. chalcidica*, whereas in its native area (i.e. Greece), it is the opposite. *Bornmuellera tymphaea* was only tested in Greece and after the second year, it proved to be a successful agromining perennial crop with yields that could reach those of the two other crops. Other native hyperaccumulator crops from Austria and Spain were not as successful.

In the frame of the Life-Agromine project, the technology was applied to all ultramafic terranes of the Balkans and showed that agromining has the potential to become an economically justifiable agricultural cropping system and hence enter traditional crop rotations. Continuation of the work is needed in order to evaluate the long-term positive effects of agricultural practices such as manure additions to the soil in agromining systems. It is also essential for a complete and improved ecosystem services assessment using Life Cycle Assessment (LCA) to quantify the provision of carbon storage and long-term effects on soil fertility. This assessment was completed for the first time for Ni agromining in Pojskë (Rodrigues et al. 2016).

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Element Case Studies: Nickel (Tropical Regions)

Philip Nti Nkrumah, Dulce M. Navarrete Gutiérrez,
Romane Tisserand, Antony van der Ent,
Guillaume Echevarria, A. Joseph Pollard,
Rufus L. Chaney, and Jean Louis Morel

Abstract

Substantial unrealized opportunities exist for economic Ni agromining in the tropics. However, until recently this technology has remained relatively unexploited in this part of the world. In this chapter, we discuss the progress of tropical Ni agromining in two regions, namely Southeast Asia and the neotropical region. Significant advances have been made in Ni agromining operations in Southeast Asia, particularly Sabah (Malaysia), in the past few years: (i) exploring for suitable locations, (ii) screening for hyperaccumulator plants in native flora, (iii) selecting candidate hyperaccumulator species with high biomass production and shoot Ni concentrations ('metal crops'), (iv) testing the agronomy of 'metal crops' to be used in viable agromining operations, and (v) demonstration of real-life agromining operations at field scale. The two most promising 'metal crops' in Sabah are *Phyllanthus rufuschaneyi* and *Rinorea* cf. *bengalensis*. However, Ni agromining developments in neotropical regions are still in their infant stages. Preliminary investigations have led to the discovery of a potential 'metal crop', *Blepharidium guatemalense*, having highly desirable attributes for possible Ni agromining. It is envisaged that tropical Ni agromining could be a productive alternative land use in these regions, and as such will significantly improve the livelihood of potential local 'metal farmers'.

1 Introduction

Nickel agromining involves growing selected Ni hyperaccumulator plant species ('metal crops') on Ni-rich soils (e.g. ultramafic or contaminated soils, mine spoils, or industrial sludge), followed by harvesting and recovery processes to extract valuable products, including Ni metal and salt (Chaney et al. 2007a, b; van der Ent et al. 2015a). Critical steps in developing Ni agromining

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P. N. Nkrumah (✉) · A. van der Ent · G. Echevarria
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane 4072, Australia
e-mail: p.nkrumah@uq.edu.au

D. M. Navarrete Gutiérrez · R. Tisserand · A. van der Ent · G. Echevarria · J. L. Morel
Laboratoire Sols et Environnement, Université de Lorraine, INRAE, UMR 1120, 54518 Vandoeuvre-lès-Nancy, France

A. Joseph Pollard
Department of Biology, Furman University, Greenville, SC 29613, USA

R. L. Chaney
Chaney Environmental, Beltsville, MD 20705, USA

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_17

include: (i) identification of potential locations, (ii) screening for hyperaccumulator plants, (iii) selection of potential 'metal crops', (iv) testing the agronomy of 'metal crops', and (v) evaluation of possible Ni recovery methods (Nkrumah et al. 2016a, 2018a, b, c, d; Chaney et al. 2018). The greatest potential for viable Ni agromining exists in the tropics as this region has large expanses of Ni-rich soils, coupled with a large number of Ni hyperaccumulator plants with desirable agromining traits (van der Ent et al. 2013a). Two main tropical regions are currently known to have a high potential for economic Ni agromining, Southeast Asia and the neotropical regions. Significant advances have been made in the development of Ni agromining in Southeast Asia in the past few years (van der Ent et al. 2015b, 2016, 2018, 2019a, b; Nkrumah et al. 2018a, b, 2019a, b, c, d), relative to neotropical regions (Navarrete Gutiérrez et al. 2018; McCartha et al. 2019). In Southeast Asia, particularly Sabah, Malaysia, extensive surveys have been undertaken to identify potential locations suitable for economic Ni agromining (van der Ent et al. 2016, 2018). In addition, recent intensive screening of the Sabah flora has led to the discovery of several new hyperaccumulator plants, the majority of which hyperaccumulate Ni (Nkrumah et al. 2018a, b; van der Ent et al. 2019b).

The agronomic management systems for selected hyperaccumulator plants have been tested in a large pot trial (Nkrumah et al. 2019b, c, d). A field trial also has been undertaken to provide 'real-life' evidence of the potential for tropical Ni agromining in Southeast Asia (Nkrumah et al. 2019a). In the neotropical regions, developments of Ni agromining are still in their infant stages, with many new hyperaccumulators having been identified very recently, and many more surely to be found. The recent discovery of the potential Ni 'metal crop', *Blepharidium guatemalense*, has opened up opportunities to further explore Ni agromining in these regions. Here, we discuss the advances of Ni agromining in Southeast Asia, and report on recent prospects in the neotropical regions.

2 Nickel Agromining in Southeast Asia

2.1 Substrates for Nickel Agromining in Southeast Asia

The cultivation of Ni 'metal crops' could be undertaken in Ni-rich areas, with Ni enrichment resulting either from geogenic (e.g. ultramafic soils) or anthropogenic (e.g. smelter contaminated soils; mine waste soils) sources (Nkrumah et al. 2016a, b, d). Ultramafic soils are characterised by relatively high concentrations of trace elements such as Ni and Mn together with the macro-element Mg, in contrast to inherently low concentrations of essential nutrients, particularly P, Ca and K (Echevarria 2018). To date, ultramafic soils have been the main target soils for exploring economic Ni agromining. In the tropical Far East (e.g. Malaysia, Indonesia, Philippines), extensive geochemical investigations have been undertaken to explore the suitability of these ultramafic substrates for agromining purposes (Nkrumah et al. 2019a; van der Ent et al. 2016, 2018). The ultramafic outcrops in Sabah are widespread, occupying an area of 3500 km^2 (Proctor 2003). Notably, there is high pedodiversity of ultramafic soils in Sabah, and these are broadly classified into four types, which include: (i) deep laterite soils (Geric Ferralsol), (ii) moderately deep montane soils (Dystric Cambisol), (iii) shallow skeletal soils at high altitude (Eutric Cambisol Hypermagnesic), and (iv) bare serpentinite soils (Hypereutric Leptosol Hypermagnesic) (see van der Ent et al. 2018). The high pedodiversity of these substrates suggests the need for careful physico-chemical investigations prior to selecting suitable sites for viable tropical Ni agromining (Nkrumah et al. 2019a).

High Ni phytoavailability is always a desired property for economic Ni agromining (Nkrumah et al. 2016a, 2018c). Extractability of Ni is mainly controlled by the soil pH and nature and proportion of Ni-bearing phases (Echevarria 2018). The weathering of ultramafic bedrock leads first to the formation of secondary phyllosilicates (Cambisol), then to amorphous and poorly crystalline Fe–Cr–Mn oxides, and finally to crystalline Fe-oxides (Becquer et al. 2006; Echevarria 2018). The Ni dynamics during such pedogenesis result in the formation of laterite soils consisting of a limonite (Fe-oxide) layer and a saprolite (Mg, Si-rich) layer (Gleeson et al. 2003). In laterite soils, Ni is mainly associated with crystallized Fe-oxides (e.g. goethite) and Mn-oxides (e.g. birnessite and lithiophorite), but in serpentinite soils this metal is predominantly associated with phyllosilicates and smectite clay minerals (Massoura et al. 2006). Whereas Ni extractability is high in secondary clay minerals (e.g. smectite), in goethite-dominant soils it is poor (Raous et al. 2013; Echevarria 2018). The three main ultramafic substrates present in Sabah are Cambisol, Ferralsol, and Leptosol (Nkrumah et al. 2019d). The dominant mineral phases of the Cambisol are phyllosilicates and smectite clay minerals, whereas in Ferralsol it is mainly goethite. Cambisols have relatively high Ni extractability (Massoura et al. 2006) and are by far the most suitable ultramafic substrate for tropical Ni agromining, at least in Southeast Asia (Nkrumah et al. 2019a). The physico-chemical properties of Ferralsol (e.g. poor water-holding capacity) and Leptosol (e.g. poor soil drainage) hinder their use for economic Ni agromining, but appropriate soil construction may enhance their suitability (see Nkrumah et al. 2019d).

Tropical Ni agromining is possible on large ultramafic areas (Cambisol) not used for food production or those having low productivity for food production (Nkrumah et al. 2019a). Soil pH, extractable Ni concentrations, drainage conditions, topography, site access, and rocky nature of substrates are some of the important parameters to consider when selecting ultramafic areas for Ni agromining purposes. Furthermore, extensive degraded sites from intense Ni laterite mining are potential areas for sustainable Ni agromining in the tropics (Fig. 1) (van der Ent et al. 2013a; Nkrumah et al. 2016b). Agromining could be an important part of a progressive rehabilitation strategy along the life cycle of Ni lateritic mining (van der Ent et al. 2013a; Erskine et al. 2018) together with appropriate soil construction that could increase economic returns.

2.2 'Metal Crops' for Nickel Agromining in Southeast Asia

Hyperaccumulator plants have the remarkable ability to accumulate high concentrations of trace elements into their living biomass (van der Ent et al. 2013b). For Ni, the hyperaccumulation threshold is set at 1000 µg g^{-1}; currently, there are more than 500 Ni hyperaccumulator plant species known globally (Reeves et al. 2018). In Southeast Asia, extensive field surveys have been undertaken to discover and study the biogeochemistry of Ni hyperaccumulator plants (van der Ent and Mulligan 2015; van der Ent et al. 2015b, 2019b). Recently, a new method of plant screening, referred to as 'Herbarium XRF Ionomics', has been employed to extract new elemental data from herbarium specimens using X-ray fluorescence spectroscopy (XRF), which has led to the discovery of 28 new Ni hyperaccumulator species in Sabah (Fig. 2a) (van der Ent et al. 2019b). These discoveries are followed by fieldwork and further investigations in order to unravel the biogeochemistry of these hyperaccumulators and also assess their suitability for use in Ni agromining (Fig. 2b) (Nkrumah et al. 2018a, b).

Criteria for selection of 'metal crops' include high biomass yield combined with high Ni concentrations in the above-ground biomass (>10 g kg^{-1}, so-called 'hypernickelophores') (Chaney et al. 2007a; Nkrumah et al. 2016a). The newly discovered hypernickelophores in the tropics possess some of the highest Ni concentrations ever recorded (Nkrumah et al. 2016a, 2018a, b; van der Ent and Mulligan 2015; van der Ent et al. 2019b). Presently, 16 plant species have been identified in the tropics as having exceptionally high potential for use as 'metal crops' for viable Ni agromining (Table 1). In contrast to temperate herbaceous 'metal crops' (e.g. *Odontarrhena chalcidica*), tropical 'metal crops' are perennial shrubs or trees that

Fig. 1 Degraded lateritic Ni mining areas in Southeast Asia are potential locations for economic tropical Ni agromining

Fig. 2 **a** Scanning herbarium specimens using X-ray fluorescence spectroscopy (XRF) at The Sabah Forest Research Centre (FRC) in Sandakan, Malaysia, and **b** employing XRF technique in the field to screen for hyperaccumulator plants

Table 1 Nickel hyperaccumulator species with over 1% Ni (‘hypernickelophores’) which have high potential for application as ‘metal crops’ in tropical Ni agromining operations

Species	Potential application area	Native distribution	Height (m)	Cropping system	Shoot Ni (%)
Actephila alanbakeri	Tropical Asia-Pacific Region	Southeast Asia	1–5	Ligneous shrub	0.1–1.5
Antidesma montis-silam	Tropical Asia-Pacific Region	Southeast Asia	5–20	Ligneous shrub	0.5–3.5
Blepharidium guatemalense	Tropical Central America	South-Eastern Mexico, Guatemala	5–20	Ligneous shrub	1–4.3
Buxus spp.	Tropical Central America	Cuba	0.3–12	Ligneous shrub	1–2.5
Leucocroton spp.	Tropical Central America	Cuba	1–3.3	Ligneous shrub	1–2.7
Orthion subsessile	Tropical Central America	Southeastern Mexico, Belize, Guatemala	1–15	Ligneous shrub	0.1–5.4
Phyllanthus spp.	Tropical Asia-Pacific Region	Southeast Asia and Central America	1–6	Ligneous shrub	2–6
Psychotria lorenciana	Tropical Central America	Southeastern Mexico, Guatemala	1–15	Ligneous shrub	0.1–2.1
Rinorea cf. *bengalensis*	Tropical Asia-Pacific Region	Southeast Asia	5–20	Ligneous shrub	1–2.7

Adapted from Navarrete Gutiérrez et al. (2018), Nkrumah et al. (2016a)

regenerate above-ground biomass rapidly after harvesting. For effective tropical Ni agromining, use of local ‘metal crops’ is recommended because of their adaptation to local climatic conditions and in order to avoid introducing exotic species into sensitive tropical ecosystems (Nkrumah et al. 2016b, 2019a).

In Southeast Asia, particularly Sabah, the most promising ‘metal crops’ are *Phyllanthus rufuschaneyi* (Phyllanthaceae) and *Rinorea* cf. *bengalensis* (Violaceae) (Fig. 3). *Phyllanthus rufuschaneyi* is known to be restricted to only two localities in Sabah (Bukit Hampuan and Lompoyou Hill) (Bouman et al. 2018). The local habitat is open secondary scrub that has been affected by recurring forest fires and excessive logging. These two species are adapted to young (eroded) hypermagnesian Cambisol developed on strongly serpentinized bedrock. These soils are characterized by unusually high Mg/Ca quotients, *circum*-neutral pH, and high extractable Ni concentrations (van der Ent et al. 2016). *Phyllanthus rufuschaneyi* accumulates up to 25 g Ni kg^{-1} in the leaf biomass and 12.3 g Ni kg^{-1} in twigs (van der Ent et al. 2015b). Nickel concentrations in the phloem sap and the seeds are also remarkable, reaching 10.5 and 17.5 g Ni kg^{-1}, respectively (van der Ent and Mulligan 2015). The high shoot Ni concentrations of *P. rufuschaneyi*, coupled with its high leaf fraction of biomass and multi-stemmed habit, rapid re-growth after coppicing, ease of propagation, and pest resistance, makes this species an ideal candidate for tropical Ni agromining (Bouman et al. 2018; Nkrumah et al. 2019a, b, c, d).

Rinorea cf. *bengalensis* is widespread in Southeast Asia (van der Ent and Mulligan 2015; Galey et al. 2017). As the taxonomic designation ‘cf.’ implies, there is uncertainty about the proper classification and nomenclature of these plants, and additional research on such fundamental topics is a high priority. This species occurs on both ultramafic and non-ultramafic soils, with only the populations on ultramafic soils hyperaccumulating Ni, thus making it a facultative hyperaccumulator (Brooks and Wither 1977; Pollard et al. 2014). In Sabah, *R.* cf. *bengalensis* is locally common on ultramafic soils, where it

Fig. 3 Selected ‘metal crops’ in Sabah, Malaysia: **a** *Phyllanthus rufuschaneyi*, and **b** *Rinorea* cf. *bengalensis*

can achieve a height of 25 m. This species can accumulate up to 17 g kg^{-1} Ni in leaves, whereas the Ni concentrations in green phloem in the trunk can be up to 80 g kg^{-1} (van der Ent and Mulligan 2015). *Rinorea* cf. *bengalensis* occurs on circum-neutral soils with relatively elevated phytoavailable Mg and Ni concentrations (van der Ent et al. 2016). Considering its previously observed growth rates in the wild and in pot and field trials, coupled with high shoot Ni accumulation, *R.* cf. *bengalensis* clearly has potential to be used as a ‘metal crop’ in tropical Ni agromining.

2.3 Agronomy of Nickel ‘Metal Crops’ in Southeast Asia

2.3.1 Inorganic and Organic Fertilizers

Large growth trials designed to develop agronomy of the tropical ‘metal crops’ (*P. rufuschaneyi* and *R.* cf. *bengalensis*) have been undertaken in Sabah (Fig. 4) (Nkrumah et al. 2019b, c, d). Inorganic (NPK) fertilization and organic matter amendments have positive growth effects on *R.* cf. *bengalensis*. In the case of *P. rufuschaneyi*, only N and P treatments yield strong positive growth responses, whereas K additions and notably organic matter amendments have adverse growth effects. Calcium and sulphate additions have no significant effects on the growth of *P. rufuschaneyi* and *R.* cf. *bengalensis*, but these amendments improve Ni uptake in these species (Nkrumah et al. 2019c). The increase in biomass in response to inorganic fertilization does not significantly reduce Ni concentrations in either species, except for N addition in *P. rufuschaneyi*. On the contrary, organic matter amendments significantly reduce soil Ni phytoavailability and consequently shoot Ni concentrations in both *P. rufuschaneyi* and *R.* cf. *bengalensis*. Notably, Ni uptake and growth responses to nutrient input are species-dependent (Nkrumah et al. 2019b, c). In summary, Ca, S and P additions seem to be essential in the agronomy of tropical ‘metal crops’ to be used in economic Ni agromining.

Fig. 4 Large pot trial employing *Phyllanthus rufuschaneyi* (small leaf blade) and *Rinorea* cf. *bengalensis* (large leaf blade) in Sabah, Malaysia: **a** the start of the experiment, and **b** at the end of the experiment

In contrast, traditional organic amendments appear not to be useful in tropical Ni agromining, at least when employing *P. rufuschaneyi*. However, some substrates may require organic matter amendments to support normal plant growth despite inevitable reductions in shoot Ni concentrations (Nkrumah et al. 2016a).

2.3.2 Soil pH Adjustments to Optimise Ni Uptake

The uptake of Ni by hyperaccumulator plants is influenced by soil pH (Kukier et al. 2004). Studies carried out in temperate ultramafic soils have shown that reducing soil pH does not increase Ni uptake in *Odontarrhena* Ni hyperaccumulator species (Li et al. 2003a, b; Kukier et al. 2004; Chaney et al. 2007a, b). All of these authors found that shoot Ni concentrations were highest at pH 6.5 in test soil with 22% iron. In contrast, the Cd-Zn hyperaccumulator *Noccaea caerulescens* has typical higher Cd and Zn uptake at lower pH until the pH is low enough to cause Mn or Al phytotoxicity (Wang et al. 2006). It is important to note that pH values of the native ultramafic soils where *Odontarrhena* species occur are commonly 6.5–8. In Sabah, Ni ‘metal crops’ are restricted to *circum*-neutral soil pH (van der Ent et al. 2016). Shoot Ni concentrations in *P. rufuschaneyi* decrease as soil pH drops below 6.0 (Nkrumah et al. 2019d). The most suitable ultramafic substrate in Sabah (Cambisol) for Ni agromining has *circum*-neutral soil pH (6.4). Therefore, reducing soil pH to increase soil Ni solubility may not be an important agronomic practice to consider in practical agromining in Sabah when employing *P. rufuschaneyi* on a Cambisol (Nkrumah et al. 2019a).

2.3.3 Harvest Method/Schedule

Many factors influence the management of propagation and harvest in tropical Ni agromining. This implies that planting and harvest management will be specific to each species and each location (Nkrumah et al. 2016a, 2018c). Currently, the local 'metal crops' used for tropical Ni agromining are perennial. Hence, annual coppicing can be an effective harvesting method, allowing 'metal crops' to re-sprout for the next growing season. It seems that defoliation in tropical 'metal crops' is low, thus harvesting could be done at multiple times for one year depending on plant growth and should be done before fruiting season. Manual harvesting directly in the secondary forest is also envisaged for non-endangered species, like *R.* cf. *bengalensis*.

2.3.4 Mass Propagation

Mass propagation of tropical 'metal crops' is essential for large-scale commercial Ni agromining operations in the tropics. Propagation by seeds is presently not feasible, at least for the 'metal crops' *P. rufuschaneyi* and *R.* cf. *bengalensis*. However, the seeds of *R.* cf. *bengalensis* show some promise, hence potential germination trials need to be considered in the near future. Likewise, research is needed to investigate the potential of mass propagation of *P. rufuschaneyi* via seeds. The current propagation method employed in the field trial in Sabah involves collection of wild saplings from the native habitats (Nkrumah et al. 2019a). This mode of propagation is laborious and may be challenging for commercial large-scale operations. The most effective mass propagation approach is via cuttings and this method needs to be trialled for field application. Preliminary investigations reveal that both *P. rufuschaneyi* and *R.* cf. *bengalensis* can be propagated effectively via cuttings using an appropriate rooting hormone.

2.4 Post-harvest Processing of Nickel

The recovery of Ni from harvested biomass of 'metal crops' is one of the key steps in Ni agromining operations. Traditionally, Ni metal or salt has been recovered from a high-grade Ni 'bio-ore' after incineration. Recent findings suggest the potential for Ni recovery also from freshly harvested biomass (Vaughan et al. 2017). This method of metal extraction seems more efficient and needs further research. In the traditional Ni recovery method, the bio-ore (obtained after incineration) of *R.* cf. *bengalensis* and *P. rufuschaneyi* contains 55 and 127 g kg^{-1} Ni, respectively (Vaughan et al. 2017). The biopurity of Ni obtained from 'metal crops' calls for further research to evaluate innovative recovery methods for maximising product recovery efficiency, and ultimately Ni profitability.

2.5 Large-Scale Nickel Agromining Southeast Asia

Large-scale demonstration of tropical agromining is required to provide real-life efficacy of agromining in the tropics. The first large-scale tropical 'metal farm' has been established in Sabah, employing *P. rufuschaneyi* on Cambisol (Fig. 5) (Nkrumah et al. 2019a), which is an important model for future commercial agromining operations in tropical regions. For large-scale tropical agromining employing *P. rufuschaneyi* considering a biomass of 200 g per plant, 25 g kg^{-1} shoot Ni concentrations, 5 plants per m^2, and one harvest per year, we can achieve a Ni yield of about 250 kg ha^{-1}, the greatest yet recorded globally; however, this result remains to be confirmed by field data (Nkrumah et al. 2019a). Preliminary field data suggest that the initial Ni yield estimation is conservative, because re-growth of coppiced *P. rufuschaneyi* generates more biomass (Fig. 5) and potentially more shoot Ni concentrations, leading to increased Ni yield. The method of 'metal farming' in Sabah mainly employs manual

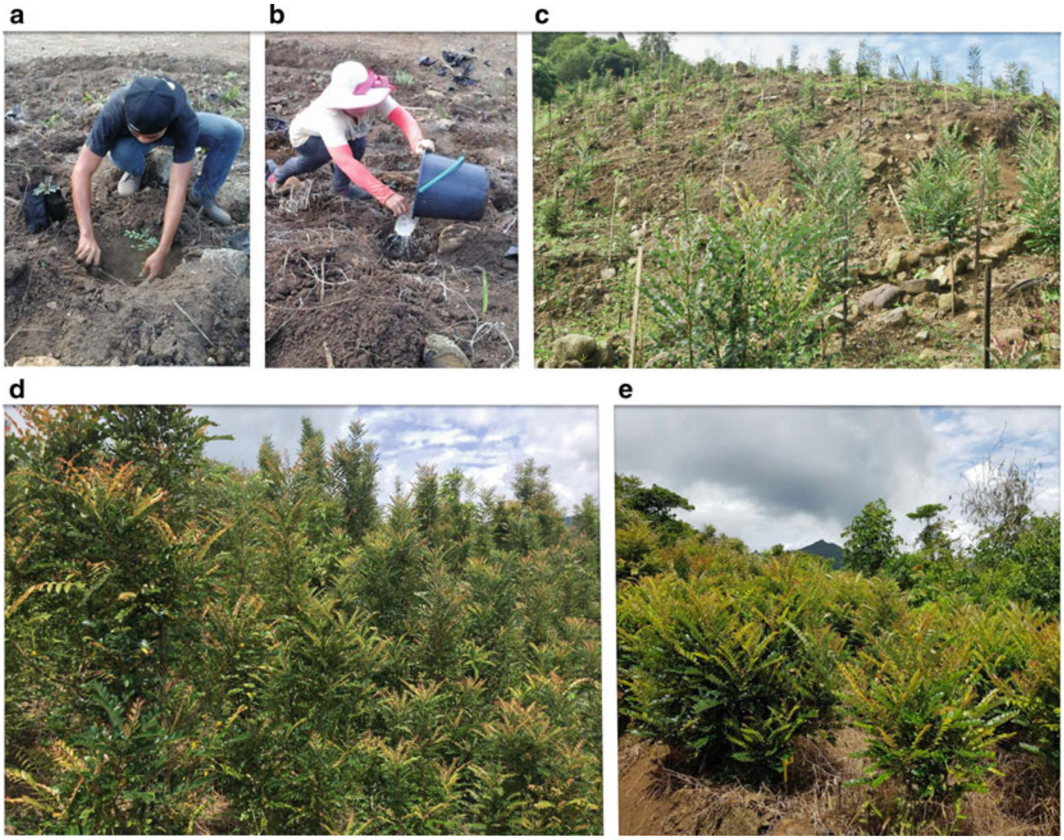

Fig. 5 The first large-scale tropical 'metal farm' in Sabah, Malaysia: **a**, **b** manual planting and watering of *Phyllanthus rufuschaneyi* wild saplings, **c** initial growth stage after establishment (9 months), **d** status of the field trial 2 years after planting, and **e** 1 year after coppicing (Adapted from Nkrumah et al. 2019a)

labour in its operation with relatively low production costs, similar to local agriculture, thus making Ni agromining in Sabah locally acceptable and more economically lucrative (Nkrumah et al. 2019a). Ultramafic soils are widespread in Sabah and efforts are underway to expand Ni agromining operations in this region. In the greater Asia-Pacific region (Indonesia, Malaysia, The Philippines, Papua New Guinea, and New Caledonia), tropical Ni agromining could replace existing marginal and abandoned agriculture on poor ultramafic soils (van der Ent et al. 2015a). In addition, agromining can be a complementary process to existing lateritic mining operations in areas having low-grade Ni soils or Ni mine waste materials, as a part of a progressive rehabilitation process after conventional resource extraction (Erskine et al. 2018).

3 Pioneering Studies on Nickel Agromining in the Neotropical Regions

3.1 Substrates for Nickel Agromining in the Neotropics

Hyperaccumulation of Ni on ultramafic soils in the neotropics has been reported from several regions, including Cuba, Brazil, Central America and Mexico (Reeves 2003; Reeves et al. 1996, 1999, 2007, 2018; McCartha et al. 2019). However, more extensive geobotanical field surveys are needed in order to determine the characteristics and extent of these areas, and their potential for Ni agromining. Three of the authors of this chapter (DNG, GE, AJP) have recently begun

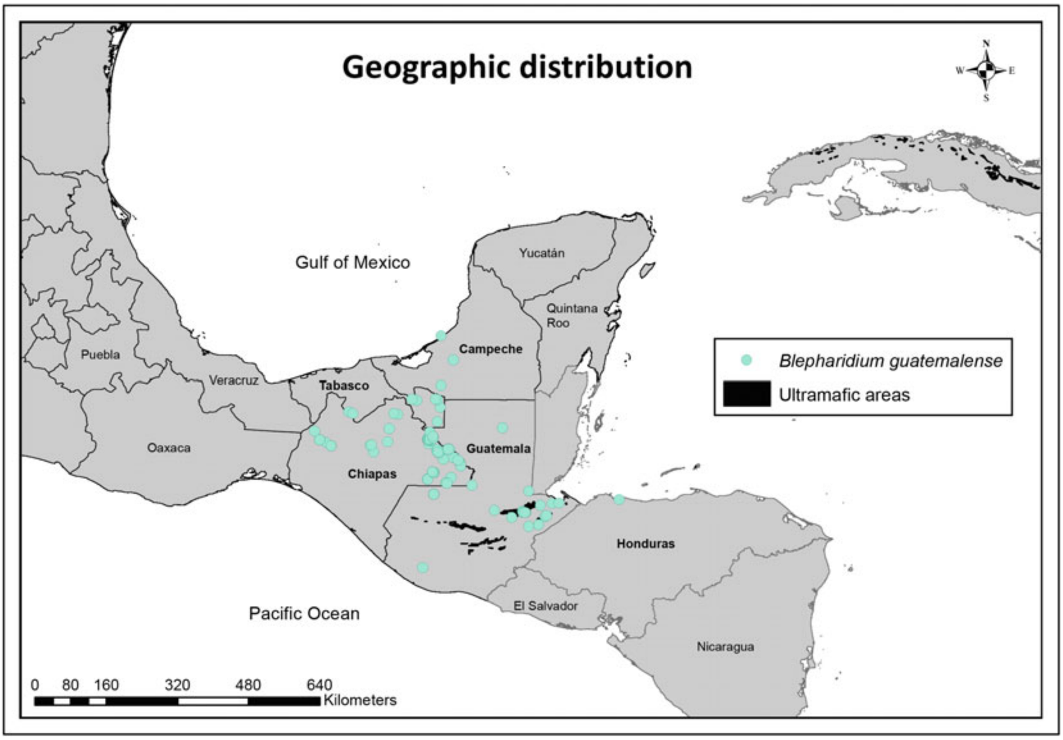

Fig. 6 Distribution of Ni hyperaccumulator plant, *B. guatemalense*, and ultramafic areas in the neotropical region

such studies in Mexico and Guatemala, with promising results. These findings are currently in preparation for publication; the most important initial results are summarized below.

In the specific case of Mexico, Ni hyperaccumulation was not found in the temperate ultramafic regions in the central and southern areas of the country (Navarrete Gutiérrez et al. 2018), but rather in the tropical evergreen forests from the eastern state of Veracruz and the southeastern states of Tabasco, Campeche and Chiapas, which are not shown as ultramafic on geologic maps (Fig. 6) (Navarrete Gutiérrez et al. 2018; McCartha et al. 2019). The origin of the Ni in the soils of those regions is different, having lower Ni concentrations compared to ultramafic soils. Unpublished data from recent field surveys reveal total soil Ni concentrations ranging from 80–500 mg kg^{-1} in Veracruz, 30–1600 mg kg^{-1} in Chiapas, and 200–500 mg kg^{-1} in Tabasco. Mineralogical analyses demonstrate that in Veracruz, Ni-rich soils are derived from weathering of volcanic rocks (e.g. basalt), whereas in Chiapas detrital sedimentary rocks composed of ferromagnesian clays are the main source of Ni in soils. Hernández-Quiroz et al. (2012) demonstrated that the main source of Ni (40–320 µg g^{-1}) in soils collected near a petrochemical facility in Tabasco was not anthropogenic but rather lithogenic and to a lesser extent pedogenic. In that study, large concentrations of Ni in those soils were attributed to volcanic ash additions (probably from the nearest volcano ‘El Chichon’) and to lateritic fragments identified in the upper part of soils (probably formed out of the ultramafic rocks situated in the margins of the Caribbean plate and transported by water to the alluvial plain of Tabasco). Although the volcanic activity may be contributing to Ni concentrations in soil through continual ash deposition, there is no study that confirms such a contribution in the specific regions where Ni hyperaccumulator plants are distributed.

Because of the variety of Ni-rich substrates in Mexico, their feasibility for Ni agromining should be studied individually. A general physicochemical characterization of the potential locations must be performed considering also geographic, topographic, cultural, and economic aspects. High available Ni concentrations in surface soils is a desirable feature in potential sites for Ni agromining (Nkrumah et al. 2016a). Our as yet unpublished data suggest that some sites in Veracruz have low DTPA-extractable Ni concentrations (<50.0 mg kg^{-1}), whereas in Chiapas it can reach 200 mg kg^{-1}. However, extensive studies are needed in order to identify all potential sites and to determine their Ni phytoavailability, as has been undertaken in Sabah (van der Ent et al. 2016, 2018, 2019a; Nkrumah et al. 2019a, d). In Veracruz, Ni hyperaccumulators occur mostly in protected forest areas, suggesting a low potential for agromining purposes. In Chiapas, large surfaces of degraded land, formerly forests and currently cattle ranches, seem to be suitable sites for agromining implementation. However, pot and field tests are first needed to determine characteristics of the most favourable substrate that will support economic Ni agromining in the region.

3.2 'Metal Crops' for Nickel Agromining in the Neotropics

In the neotropics, Cuba and Brazil are well-known hotspots of hyperaccumulator biodiversity having at least 128 and 30 hyperaccumulator plant species, respectively (Reeves et al. 1996, 1999, 2007, 2018). Some species of the genus *Buxus* (Buxaceae), *Leucocroton* (Euphorbiaceae), and *Phyllanthus* (Phyllanthaceae) occurring in Cuba and the Dominican Republic accumulate more than 10 g kg^{-1} Ni in shoot biomass and could have potential for Ni agromining. Nevertheless, scant information is available on these species, being indicative of the untapped Ni agromining potential in this region (Reeves 2003). A great effort and international collaboration are needed in order to elucidate the potential of these species for Ni agromining. Until recently, no hyperaccumulators were known from southern Mexico, Central America, and northern South America. Fortunately, recent publications have begun to correct this gap in our knowledge (Campbell et al. 2013; McCartha et al. 2019), and many additional hyperaccumulator species have been discovered through XRF screenings of herbarium specimens, combined with field research (currently unpublished). In total, at least 13 hyperaccumulator species from three families have been identified in this region: *Chionanthus panamensis* (Oleaceae), *Arachnothryx linguiformis*, *Blepharidium guatemalense*, *Psychotria costivenia*, *P. grandis*, *P. lorenciana* and *P. papantlensis* (Rubiaceae), *Mayanaea caudata*, *Orthion malpighifolium*, *O. montanum*, *O. oblanceolatum*, and *O. subsessile* and *O. veracruzense* (Violaceae). Table 1 highlights fast-growing and high biomass 'hypernickelophores' (>1 wt% Ni) in neotropical regions. *Blepharidium guatemalense* (Rubiaceae), an obligate ligneous Ni hyperaccumulator distributed mainly in the tropical perennial and sub-perennial rainforests of southern Mexico (Chiapas, Tabasco, and Campeche) and Guatemala, possesses inherent favourable attributes for Ni agromining (Fig. 7).

Preliminary studies demonstrate that *B. guatemalense* grows quickly (~1 m per year, large leaves ~40 cm long), produces high foliar biomass (~6 kg dry biomass, 8-year-old trees), and is easy to propagate via seeds (Fig. 8). Our field investigations in Chiapas reveal that *B. guatemalense* can accumulate up to 43 g kg^{-1} Ni in leaves, 20 g kg^{-1} Ni in roots and seeds, and 18 g kg^{-1} Ni in bark. The phloem sap is also highly enriched in Ni (Fig. 9). Currently, *B. guatemalense* is often used for rafters, beams, and supports of lowland houses due to strength of its wood (Fig. 10). The remaining biomass (e.g. leaves and small branches), so far unvalued, could then be used for economic Ni agromining purposes, but appropriate measures need to be put in place to ensure proper integration of emerging Ni agromining operations into the local context (Fig. 10). Pioneering field and laboratory studies are being carried out to develop suitable agronomic systems for *B.*

Fig. 7 *Blepharidium guatemalense* (Rubiaceae), an obligate ligneous Ni hyperaccumulator distributed mainly in the tropical perennial and sub-perennial rainforest of southern Mexico (Chiapas, Tabasco and Campeche) and Guatemala: **a** a branch with leaves and fruits, **b** leaf, **c**, **d** occurrence in the field, **e** a branch with leaves, **f** seed coat with seeds, and **g** a branch with leaves, fruits and flowers

guatemalense, which has high potential not only in tropical areas of south-eastern Mexico but also in some metalliferous regions of Guatemala and Central America. Other potential 'metal crops' such as *O. subsessile* (1–54 g kg^{-1} Ni) and *P. lorenciana* (1–21 g kg^{-1} Ni) require research in order to optimize Ni yield for economic Ni agromining.

3.3 Agronomy of Nickel 'Metal Crops' in Southeastern Mexico

3.3.1 NPK and Organic Fertilizers

Field data indicate that in its natural habitat, *B. guatemalense* can accumulate high Ni concentrations (average 13,500 mg kg^{-1}) in leaves even in soils having very low DTPA-available Ni (average 50 mg kg^{-1} DTPA-Ni) (Navarrete Gutiérrez et al. 2018). A recent six-month pot experiment demonstrated that inorganic fertilization significantly increases Ni shoot concentrations with no significant difference in biomass production (Fig. 8). Studies using manure compost did not show any positive effect on the biomass of *Odontarrhena chalcidica* (Broadhurst and Chaney 2016). Organic matter amendments had a negative effect on Ni uptake by *P. rufuschaneyi* (Nkrumah et al. 2019b). A six-month pot test using vermicompost (unpublished) showed a significant positive effect on the biomass of seedlings of *B. guatemalense* but decreased Ni shoot concentrations. Further research is required to evaluate the need for organic matter amendments in future Ni agromining, as some substrates may require organic amendments in order to support plant growth (Nkrumah et al. 2016a, b; 2018a).

Fig. 8 Progress of experimental work on *B. guatemalense* in southern Mexico: **a** germination trials, **b** manual watering, and **c** status of plants 6 months after planting

3.3.2 Harvest Method/Schedule and Ni Yield Estimation

It will be essential to estimate the annual Ni yield that could be obtained from the cropping of *B. guatemalense*. For this purpose, an open area used for cattle pasture in Chiapas (Ocosingo municipality), in which five- to eight-year-old trees of *B. guatemalense* are common, was chosen to test three possible harvesting frequency scenarios: (i) pruning five-year-old trees at a height of 1.5 m and harvesting only the leaf biomass once a year, (ii) pruning five-year-old trees at a height of 1.5 m and harvesting twice a year, and (iii) harvesting an eight-year-old tree at a height of 35 cm by harvesting the whole biomass (leaves, branches, fruits, flowers, and bark) (Fig. 11). Preliminary results suggest that scenario (ii) is the most suitable harvest method, which yields about 3 kg of dry biomass per tree. Field tests to obtain optimum tree density could require several years, but a spontaneous density of more than 2000 trees per hectare has been observed frequently in some open pastures in the region. The 'Ni farming' method would depend on the agronomic system used (e.g. scattered trees, live fences, dedicated planted areas, etc.), which must be adapted to the existing local systems and farmer needs. Assuming a monoculture with a density of 2500 trees per hectare, it could be possible to obtain ~8.5 tonnes of dry leaf biomass when harvesting five-year-old trees twice a year. Tree branches could also be harvested, adding 7 tonnes, resulting in a total yield of ~160 kg Ni per hectare. However, Ni yield could vary, since our preliminary data suggest that Ni uptake by *B. guatemalense* is highly dependent on Ni availability in the soils. Further studies may reveal potential Ni products that could be obtained from the dry biomass of *B. guatemalense* such as Ni metal, Ni salt, or Ni catalyst. Another potential farming scenario that is currently being tested in the field is biannual harvesting of trees present in 'acahuales'. These fields, initially mature tropical forests, are normally under 'slash and burn' agriculture and are progressively colonized by pioneer species, resulting in a secondary forest having a specific successional state. *Blepharidium guatemalense* is reported to be such a pioneer species, characterized by many small, wind-dispersed seeds and rapid growth. Preliminary results indicate a 20% lower growth rate in these secondary forests compared to that observed in open cattle ranches.

Fig. 9 **a**, **b**, **e** *Blepharidium guatemalense* tree with bright green phloem sap, and bark (**c**) indicative of Ni enrichment as demonstrated by a field spot test based on dimethylglyoxime (DMG) (**d**)

Seed propagation seems to be the best method of reproduction of this species since germination rate in natural conditions normally reaches 100% after 15 days (Fig. 10a). Laboratory tests confirmed its high germination rate: 80% after 45 days in an incubator at 30 °C. Seeds should ideally be collected during the fruit maturity period (April-May). Pot trials testing asexual propagation (cuttings) using phyto-growth hormones (Indole-3-butyric acid) did not result in a positive response, e.g. root development. However, further trials of this propagation method are needed, because there is high chance of success as demonstrated for *P. rufuschaneyi* and *R.* cf. *bengalensis* in Sabah (Nkrumah et al. 2019a).

4 Future Direction for Nickel Agromining in Tropical Regions

In the Asia-Pacific region, substantial unrealised opportunities exist for Ni agromining in Indonesia, Malaysia, The Philippines, Papua New Guinea, and New Caledonia (Fig. 12). In Southeast Asia, Sabah has been intensively explored in recent times for economic Ni agromining (van der Ent et al. 2016, 2018, 2019a, b; Nkrumah et al. 2019a, b, c, d). Ongoing field investigations are optimizing plant density, organic and inorganic fertilization, breeding techniques, co-cropping, coppicing, and mass

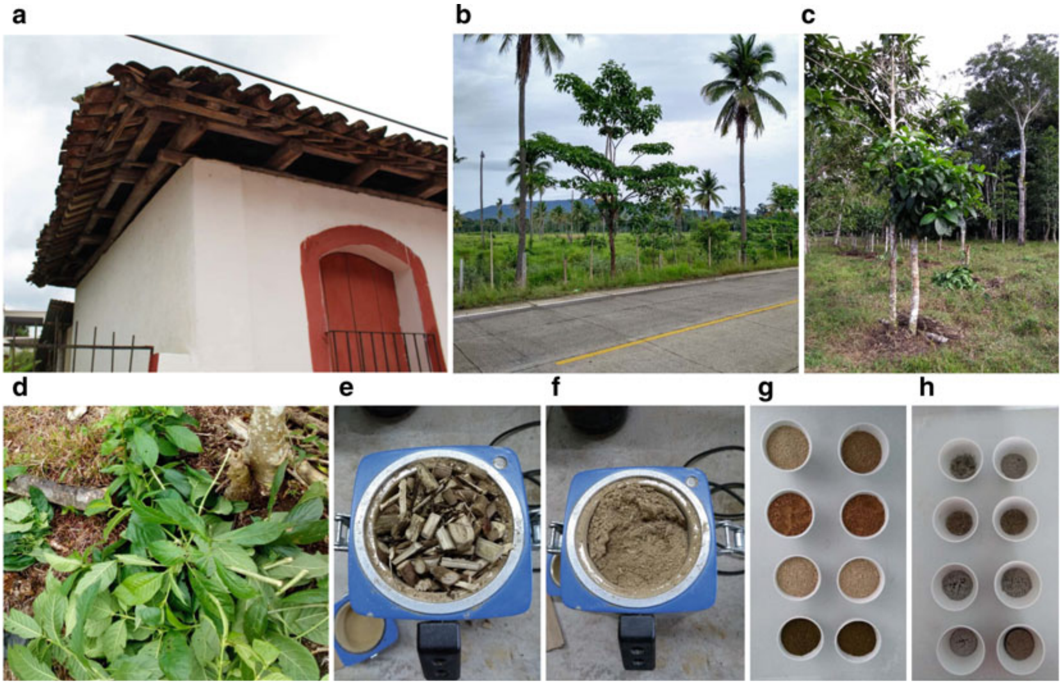

Fig. 10 Current uses of *B. guatemalense* as beams and supports of lowland houses (**a**) and live fence (**b**), and its occurrence in cattle pasture in southeast Mexico and Guatemala (**c**). Potential uses of *B. guatemalense* for Ni agromining: harvesting (**d**) and size reduction (**e, f, g**) prior to incineration (**h**)

propagation methods in order to optimise Ni yield for large-scale commercial operations (Fig. 13) (Tisserand et al. unpublished). Simultaneously, intensive biogeochemical studies are ongoing to unravel the dynamics of the Ni pools in a typical 'metal farm'. In addition, studies on the characterization of Ni (and other elements) and nutrient cycles in the native habitats of tropical hyperaccumulator plants are progressing. Investigations in other parts of the Asia-Pacific region need to be intensified, following the approach used in Sabah. Preliminary studies using XRF scanning of herbarium specimens of Papua New Guinean species have revealed promising prospects for hyperaccumulation discovery in the region (Do et al. 2020). Results of recent fieldwork in Indonesia has also highlighted a high potential for Ni agromining in the area (van der Ent et al. 2013a; Lopez et al. 2019).

In the neotropical region, preliminary explorations in Chiapas reveal high prospects for Ni agromining, but extensive investigations are required to fully realize any useful outcome. Intensive screening of local flora is needed to identify potential Ni hyperaccumulator plants. Furthermore, the agronomic systems of candidate 'metal crops' must be developed. Currently, the physico-chemical characteristics of potential locations for Ni agromining in Chiapas are unknown. The development of successful Ni agromining in the neotropical region can follow the approach employed in Sabah but must be adapted to local conditions in the region.

Fig. 11 Manual harvesting and bailing of twig and leaf biomass of *B. guatemalense* (**a**, **b**, **c**), and mechanical harvesting of *B. guatemalense* tree trunk in southern Mexico (**d**). Scientific cooperation with the Universidad Tecnológica de la Selva (Unidad Académica San Javier) in Chiapas

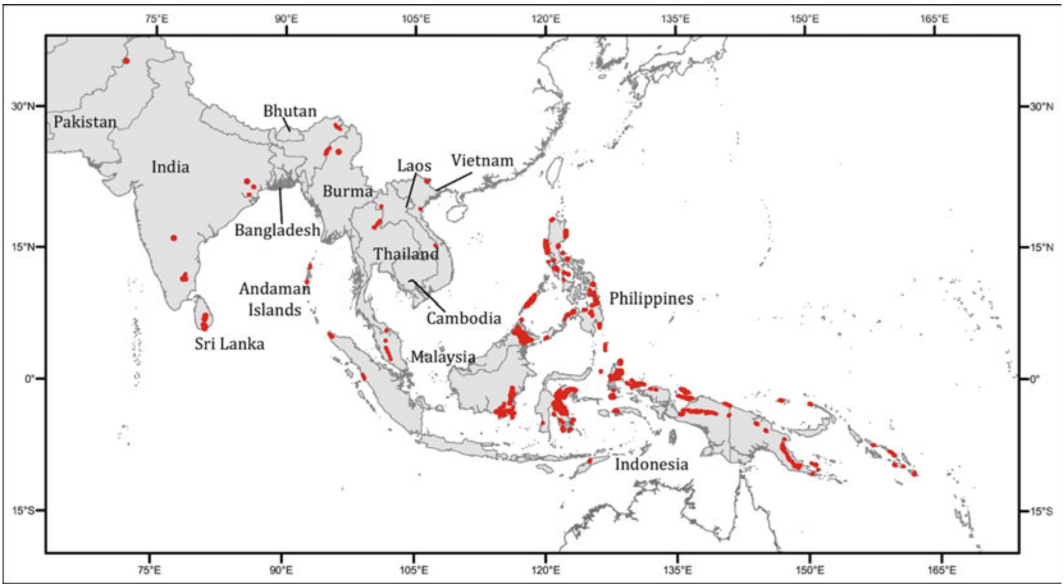

Fig. 12 Distribution of ultramafic substrates in Southeast Asia (Galey et al. 2017)

Fig. 13 Ongoing Ni agromining trials in Sabah, Malaysia (including plant density, fertilizer application, etc.) to optimize Ni yield of *Phyllanthus rufuschaneyi* (**a**, **b**). Some of the subplots are covered with organic matter composed of *P. rufuschaneyi* shoot biomass crushed with a shredder (1–3cm particle size) (**b**). Litter traps (wooden structure with green sieve) are installed to collect leaf litter (**a, b**)

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Element Case Studies: Cobalt

Michel-Pierre Faucon, Olivier Pourret, and
Bastien Lange

Abstract

Cobalt is economically considered as a critical metal. The globally most important Co ore deposits are found within the Katangan Copperbelt (Democratic Republic of Congo), where a great abundance of Cu- and Co-tolerant and accumulator plants have evolved. Cobalt mining activities in this region have disseminated large quantities of wastes into the environment during the last decades and become a major environmental issue. The reduction of environmental risks and Co dispersion can be explored by considering phytoremediation and/or agromining technics that select tolerant and putative hyperaccumulator plants from the regional pool of species. Accumulation of foliar Co to >300 $\mu g\ g^{-1}$ is rare globally and known principally from the Copperbelt. This chapter gives an overview of the current knowledge on Co accumulation by plants, examines the potential for Co bio-ore production using Co-accumulators from the region, and proposes a plant trait-based approach that maximizes ecosystem services provided by Co agromining.

M.-P. Faucon (✉) · O. Pourret · B. Lange
Institut Polytechnique UniLaSalle, AGHYLE,
Beauvais 60026, France
e-mail: michel-pierre.faucon@unilasalle.fr

1 Introduction

Cobalt is one of the elements defined as a critical metal for the next 5–15 years (Pourret and Faucon 2018). Demand for Co increased considerably after the Second World War, driven by the use of high-purity Co in jet engines and gas turbines. Cobalt demand and corresponding production have further accelerated in the past thirty years. This demand reflects the increased use of Co as an essential constituent of materials used in high-technology industries, including rechargeable Li batteries, superalloys, and catalysts (Crundwell et al. 2011). The economically most important ore deposits of cobalt, on a global scale, are found in the Katangan Copperbelt (Democratic Republic of Congo, DRC) where 41% of the known world reserves are identified (Slack et al. 2017). The Katangan Copperbelt acts as the major producer of cobalt (Fig. 1) accounting 70–71% of global production in 2018 and 2019 (USGS 2020). Smaller resources of Co originate from lateritic Ni mines worldwide (Slack et al. 2017). The average cobalt price has ranged from 30,000 to 40,000 US$/t in 2018–20 with a maximum trade value of >90,000 US$/t in early 2018 (London Metal Exchange values).

Over the past century, mining has disseminated large quantities of wastes into the environment and become a major environmental issue (Pourret et al. 2016). As a consequence, these activities have created secondary

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_18

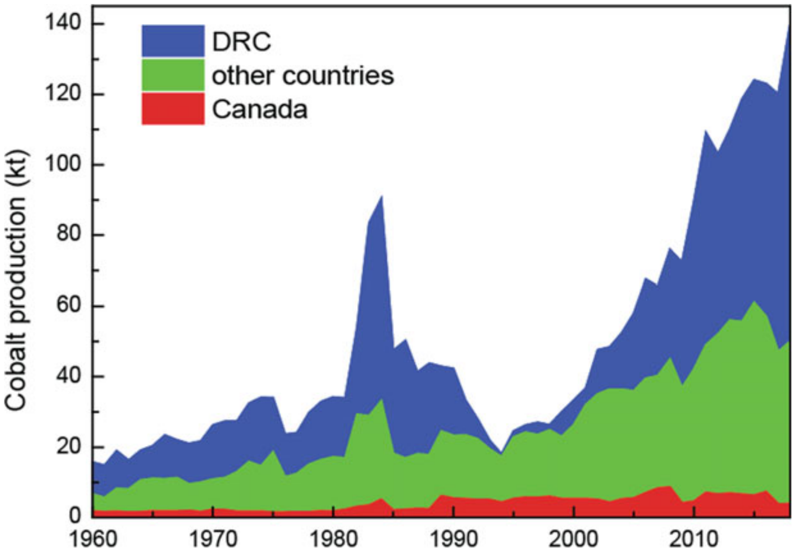

Fig. 1 Production of cobalt (data collected from mineral commodity summaries yearly published by USGS; https://www.usgs.gov/centers/nmic/mineral-commodity-summaries (USGS 2020)

metalliferous substrates with elevated concentrations of trace elements, including Co (more than 1000 times greater than the concentration in normal soil) (Faucon et al. 2011, 2012). Such exploitation results in contamination of huge areas of soil (over 30,000 km^2, around Kolwezi and Tenke-Fungurume mining centres) and implies soil degradation (Dupin et al. 2013). In southeastern Central Africa, contamination by trace elements is severe, as a consequence of the low pH of Ferralsol that favours trace element mobility in soils, soil erosion due to intense rainfall during the rainy season, and aerial dispersal of metal particles by wind during the dry season (Pourret et al. 2016). Moreover, depending on mineral residence and its aqueous speciation (Kaya Muyumba et al. 2019), Co is highly toxic and directly impacts human health, given that there is an elevated Co exposure in the general population of southern DRC (Banza et al. 2009; Cheyns et al. 2014; Squadrone et al. 2016).

Reduction of environmental risks and Co dispersion can be performed by phytoremediation and/or agromining technics that use tolerant and putative hyperaccumulator plants originating from natural Cu- and Co-enriched habitats (Ilunga et al. 2015; Boisson et al. 2015; Faucon et al. 2016). Agromining is a cost-effective and environmental sound method that develops an alternative type of agriculture on contaminated land based on phytomining principles (van der Ent et al. 2015). Agromining has been studied at field scale but to date is essentially limited to Ni (Chaney et al. 2007; van der Ent et al. 2015; Bani et al. 2015). Nevertheless, considering the large pool of Co accumulator plants recorded from southeastern DRC, Co agromining should be tested on Co-contaminated lands (Brooks et al. 1980; Reeves and Baker 2000; Faucon et al. 2007; Lange et al. 2017). Among these plants, which grow on naturally Cu- and Co-enriched soils, some accumulate Co in very high concentration (up to >1000 µg g^{-1}) and represent interesting candidate species (Kabeya et al. 2018; Lange et al. 2018) (Fig. 2). Cobalt accumulators from ultramafic soils naturally enriched in Co worldwide, such as *Berkheya coddii* (Asteraceae), could also be tested ex situ for attainable yields (Keeling et al. 2003).

Understanding the ecology and evolution of Co accumulators is essential primarily to develop Co phytomining at field scale. Recent research advances have shown that the poorly understood, large variations in Co accumulation documented at species and population levels can be explained by genetic variation, and also by variations in soil chemical factors involved in Co availability (Lange et al. 2017). Moreover, Co accumulators

Fig. 2 A few putative co accumulator plants. **a** *Anisopappus chinensis* Hook. f. & Arn. (Asteraceae), photo: Lange B., **b** *Haumaniastrum robertii* (Robyns) P. A. Duvign. & Plancke (Lamiaceae), photo: Faucon M.-P., **c** *Crepidorhopalon perennis* (P. A. Duvign.) Eb. Fisch. (Linderniaceae), photo: Faucon M.-P., **d** *Buchnera henriquesii* Engl. (Orobanchaceae), photo: Séleck M., **e** *Acalypha cupricola* Robyns (Euphorbiaceae), photo: Lebrun J., **f** *Triumfetta welwitschii* Mast. (Malvaceae), photo: Séleck M.

present high functional diversity (i.e. in life cycles, lateral spreading capacity, type and depth of underground system, growth phenology, N symbiotic fixation, etc.) (Ilunga et al. 2015), which has an influence on ecosystem processes such as biomass productivity, Co mobility and transfer, etc. (Faucon 2015). Cobalt agromining should consider both inter- and intraspecific variation of leaf Co accumulation and functional plant diversity, to design multi-services cropping systems.

In this chapter, we focus on putative Co hyperaccumulator plants and their possible use in Co agromining practices. We review recent advances in Co accumulation, examine the potential of a Co accumulator species in agromining, and proposes a plant trait-based approach to maximize ecosystem services provided by Co agromining.

2 Variation in Cobalt Accumulation

Plant populations tolerant to Co have been reported in the literature in several dozens of species collected in nature. However, the capacity to grow these plants under elevated Co concentrations without toxicity symptoms or growth inhibition has not been widely demonstrated experimentally. Similarly, elevated concentrations in plant leaves ($>300\ \mu g\ g^{-1}$) has been widely observed, but much less demonstrated under experimental conditions.

Field sampling over the past fifteen years has shown extensive inter- and intraspecific variation of Co accumulation by plants (Faucon et al. 2007; Lange et al. 2014). These variations still remain poorly understood but can be explained by variation in the total Co concentration in soil and the chemical soil factors influencing Co mobility and availability (Faucon et al. 2009). Natural Cu- and Co-enriched outcrops show a high soil total Co concentration (up to 3451 µg g^{-1}) from which a fraction is mobile (39–2146 µg g^{-1}) (Pourret et al. 2016). This finding could facilitate the high Co accumulation by *A. chinensis* grown on natural Cu–Co steppic savannas (up to 2822 µg g^{-1} foliar Co). Previous studies have also shown that Co accumulation by *Crepidorhopalon perennis* (Linderniaceae) and *A. chinensis* was strongly influenced by the mobile fraction of Co present in soil (ionic fraction) and by Co adsorbed onto the organic matter and Fe-oxide fractions (Lange et al. 2014). The rhizosphere processes, including interactions between roots and microbiota, are also expected to be fundamental to a better understanding of the ecology of Co accumulation variation (Lange et al. 2017).

Recent studies have tested the response of several populations of *H. robertii* and *A. chinensis* to ex situ Co exposure (Kabeya et al. 2018; Lange et al. 2018). True Co accumulation and translocation exists in *Anisopappus chinensis*, with up to 500 mg kg^{-1} Co reported in plant leaves. This species could be a good candidate to test its potential in Co agromining on secondary metalliferous habitats in DRC.

3 Potential of Cobalt Agromining

The best candidate species for Co agromining are plants with elevated extracting yields (i.e. biomass x Co shoot concentration). Cobalt agromining was first proposed by using Ni-hyperaccumulator species on ultramafic soils (Homer et al. 1991). However, Ni limits the uptake of Co in most Ni-hyperaccumulator plants (Malik et al. 2000) and hence also negatively influences the performance of Co agromining.

Cobalt agromining potential was evaluated using metallicolous populations of *A. chinensis* from Co-enriched soils having elevated leaf Co concentrations in nature (30–2822 µg g^{-1}) (Lange 2016). Mean shoot biomass of *A. chinensis* (n = 20) was 0.8 g (range: 0.14–3.79 g). Biomass productivity (Ybio) was estimated to be 16 g $m^{-2}y^{-1}$ that gives 160 kg $ha^{-1}y^{-1}$ (range: 20–320 kg $ha^{-1}y^{-1}$). Total phytoextracted Co (YCo=FCo.Ybio) could reach 176 g $ha^{-1}y^{-1}$ (max: 864 g ha^{-1}) (Lange 2016). Owing to large variations in the capacity to extract Co from the soil, agromining using *A. chinensis* should be augmented by plant selection and genotyping (Chaney et al. 2007). Additionally, achievable yields could be improved by including a multi-species system involving other Co-tolerant and accumulator species. Legume species such as *Crotalaria cornetii* (Fabaceae), which grows in natural and secondary metalliferous habitats (Leteinturier 2002), can improve the facilitation process, increase biomass productivity, and potentially the quantity of extracted Co. Other species such as *H. robertii* could also be introduced into the system (leaf Co concentration for this species can reach 4000 mg kg^{-1} in natural habitat) (Morrison et al. 1981). This first evaluation reveals limited achievable yields, but perspectives to test it at field scale remain interesting, especially for a multispecies system considering the functional diversity of Co-accumulator plants (species and populations) (Fig. 2) (Delhaye et al. 2016, 2018).

4 Functional Plant Diversity of Cobalt Agromining Systems

In the last decade, considerable progress in ecology has been achieved due to elaboration of the concept of functional traits. Functional traits are defined as morpho-physio-phenological traits which impact fitness indirectly via their effects on growth, reproduction and survival (Violle et al. 2007). Relationships among traits associated with the response of organisms to environmental factors such as resources and disturbances (response traits), and traits that determine effects of organisms on ecosystem functions (effect traits), have been studied recently in few processes and ecosystem services (Garnier et al. 2016; Faucon et al. 2017).

Phytoremediation and agromining generally attempt to study the effects of species or species mixtures on ecological processes in order to restore and design ecosystems independently of a functional trait approach (Faucon 2015). Agromining based on taxonomical description does not allow quantifying the effect of plant species on ecosystem processes and services. The challenge is to link data from the recent literature with below- and above-ground functional traits of plant and ecosystem processes, such as trace-element mobility, and water transfer and sediment transport, for improving ecological engineering and associated services, especially agromining yield, landscape restoration, reduction of trace element transfer and water contamination, carbon sequestration, etc. From 30 putative Co accumulator plants that present high variability of morphology, life cycle, and physiology (Ilunga et al. 2015; Malaisse et al. 2016), several traits could be selected to develop a multifunctional Co agromining system. Co accumulator plants that colonize metal-rich bare soils, present annual life cycle, growth phenology in wet season, depth 0–10 cm of underground system, bud bank by seeds, dispersule size <2 mm and dispersal mode by adhesion. Co accumulator plants growing in primary habitats are related to perennial species that present these following traits: high lateral spreading capacity, xylopodia, bulb or corm bud bank and dispersal on onset of wet season. Combination of contrasted traits of Co accumulator plants growing in secondary or primary habitats could offer a high functional diversity that would allow to increase the biomass productivity along all seasons and maximise ecosystem services for soil and water conservation, carbon sequestration and agromining yield (Ilunga et al. 2015).

5 Plant Functional Diversity to Improve Co Agromining

In Co accumulators, functional divergence of root traits should be considered for improving biomass productivity and yield from Co agromining. Effectively, the intercropping with functional diversity of crop species corresponds to temporal and spatial niche differentiation in resource acquisition for these species, associated with an increase in productivity compared to a monoculture (Li et al. 2014; Faucon et al. 2015). Spatial niche differentiation associated with root morphology and the capability to exploit nutrients in soils can also explain the way in which species colonize the soil profile and the total soil volume occupied by the crop (Lynch 2011). Physiological root traits should also be considered for improving Co agromining yield, especially root-induced processes such as releasing of protons and/or carboxylates that would directly affect Co availability in the rhizosphere (Lange et al. 2017). At opposite, functional diversity (FD) of leaf traits and plant height can present a negative effect on biomass productivity explaining by an increased light competition. Future investigations should be performed to understand the relationships between FD and ecosystem processes/services, in order to optimally design agromining systems.

6 Plant Functional Traits Involved in Landscape Restoration, and Co Transfer

Traits of perennial cycle, high lateral spreading capacity, and growth phenology in the dry season, will be prioritized for developing a dense cover to restore landscape, reduce runoff and soil erosion, and thus Co transfer (Egoh et al. 2011). Functional diversity should be considered for improving these ecosystem services, such as FD of deep underground systems, in order to improve root density and hence soil cohesion and stability (Gyssels et al. 2005) and growth phenology (both dry and rainy seasons) (Ilunga et al. 2015).

7 Conclusions

The use of Co accumulator plants in agromining is limited because of the strong dose-dependent accumulation response. However, Co agromining should be tested on Co-contaminated soils and tailings with high available Co, in order to maximise the extraction potential of such a critical and high-value metal. Perspectives include testing the technique at field scale on secondary metalliferous habitat, using one or several candidate species, and understanding the relationships between plant functional traits and ecosystem processes/services, for designing successful multifunctional and multi-services agromining systems.

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Element Case Studies: Selenium

Maria Clemencia Zambrano, Linxi Yuan,
Xuebin Yin, and Gary S. Bañuelos

Abstract

Plants in general are responsive to harsh soil environments, in particular when they are exposed to toxic and deleterious metals or metalloids such as arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), as well as excessive concentrations of selenium (Se). Nevertheless, not all plants exhibit similar responses when exposed to a metallicolous environment; they may display extreme metabolic strategies to prevent or avoid toxicity under these particular harsh edaphic conditions (e.g., As, Cd, Pb, Hg). Three major plant-based strategies to cope with metal toxicity are: exclusion, tolerance and/or hyperaccumulation. The metal-accumulating or metallophyte plants have become a major research focus in recent years. These plant species exert an enhanced capacity to extract metals from soil (phytoextraction). Their innate tendency to tolerate and accumulate various elements in their shoots (hyperaccumulation), has opened unlimited possibilities to explore, and to apply new plant-based technologies, e.g., phytoremediation, for potentially cleaning metal-laden soils.

1 Introduction

The concept of phytomining is a nascent green technology that will apply hyperaccumulators to degraded soils for the purpose of extracting metals, which can be harvested, dried and incinerated to collect bio-ore (Brooks et al. 1977, 1998; Chaney et al. 2007, 2018; Kidd et al. 2018; van der Ent et al. 2015). For this use, metallophytes are the preferred plant species for use in phytoextraction (Baker 1981; Baker and Whiting 2002; Farnese et al. 2016; Goolsby and Mason 2015; Ginneken et al. 2007; Prasad 2015; Selvi et al. 2019; Shrivastava et al. 2019; Suman et al. 2018; Whiting et al. 2004). Some of the primary and secondary accumulators of metal/metalloid used in phytomining and/or agromining technologies are: *Arabidopsis halleri*—zinc (Zn)/Cd (Weber et al. 2004), *Thlaspi*

M. C. Zambrano
Institute of Advanced Technology, University of Science and Technology of China, Hefei 230088, Anhui, China

L. Yuan
Department of Health and Environmental Sciences, Xian Jiaotong-Liverpool University, Suzhou, Jiangsu, China

X. Yin
Jiangsu Bio-Engineering Research Center of Selenium, Suzhou, Jiangsu, China

G. S. Bañuelos (✉)
USDA, Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, 9611 South Riverbend Avenue, Parlier, CA 93648, USA
e-mail: gary.banuelos@ars.usda.gov

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_19

goesingense—nickel (Ni) (Lombi et al. 2000), *Noccaea caerulescens*—Zn/Cd (Jacobs et al. 2017; Milner and Kochian 2008; Papoyan and Kochian 2004), *Odontarrhena chalcidica*—Ni/cobalt (Co) (Broadhurst and Chaney 2016; Whiting et al. 2003), *Brassica juncea*—Zn/Cd/Hg (Kidd et al. 2018; Prasad and Oliveira 2003; Raj et al. 2020; Schiavon et al. 2015; Steven and Culver 2019). Some hyperaccumulators also can accumulate metalloids like Se, which can be indirectly incorporated into the food web chain through agriculture activities after harvest (Bañuelos et al. 2015, 2016, 2017; Broadley et al. 2006; Dhillon and Dhillon 1991; Kidd et al. 2018; Pilon-Smits 2019; Schiavon and Pilon-Smits 2017a, b; Ros et al. 2016; White 2016, 2018; Wu et al. 2015; Zhao and McGrath 2009) and generate products of economic value (Schiavon and Pilon-Smits 2017a; van der Ent et al. 2015). With regards to Se, hyperaccumulators can also be used for biofortification strategies (described later) for increasing Se intake into animals and humans (Bañuelos et al. 2017; Sun et al. 2017).

Selenium hyperaccumulators such as *Stanleya pinnata* (Pursh.) Britton (Freeman et al. 2006, 2009, 2010; Freeman and Bañuelos 2011; Bañuelos et al. 2015, 2016; Lindblom et al. 2013; Parker et al. 2003; Schiavon et al. 2015; Wang et al. 2018), *Astragalus bisulcatus* (El Mehdawi et al. 2011, 2012; Feist and Parker 2001; Freeman et al. 2006; Parker et al. 2003; Pickering et al. 2003; Sors et al. 2009; Statwick et al. 2016; Valdez-Barillas et al. 2012) and the newly discovered Se hyperaccumulator *Cardamine hupingshanensis* from China (Brassicaceae) (Bai et al. 2008; Both et al. 2020; Shao et al. 2014; Tong et al. 2014; Xiang et al. 2019; Zhou et al. 2018), can play an important role in the Se cycle from soil to plant to human (Bai et al. 2008; Shao et al. 2014; Yuan et al. 2013). Selenium hyperaccumulators can be used for phytoextraction, agromining or for phytoremediation of Se (Broadley et al. 2006; Guerinot and Salt 2001; Pilon-Smits and LeDuc 2009; Schiavon et al. 2020; Schiavon and Pilon-Smits 2017a; Wu et al. 2015), as well as for their application in Se biofortification strategies (Bañuelos et al. 2015, 2016, 2017; Puccinelli et al. 2017; Ros et al. 2016; Schiavon and Pilon-Smits 2017a, b; Schiavon et al. 2017; Zhao and McGrath 2009; Zhu et al. 2009).

In this regard, biofortification leads to the nutrient enrichment of edible crops by growing in specific soils, applying agricultural practices with amendments (Bañuelos et al. 2015, 2016, 2017), or manipulating genetic activities to increase accumulation of essential elements like, iron (Fe), Zn and/or Se within crops, e.g., golden rice, high-Fe cassava (Global Panel 2015; Malagoli et al. 2015; Puccinelli et al. 2017; Trijatmiko et al. 2016; White and Broadley 2009), as well as nutraceutical compounds (e.g., antioxidants, carotenes, phenolics) in edible tissues (Brummell et al. 2011; Mabeyo et al. 2015; Malagoli et al. 2015; Wiesner-Reinhold et al. 2017; White and Broadley 2009). The success of biofortification relies not only on promoting the accumulation of essential nutrients, but also on enhancing bioavailability and assimilation of the nutrient by humans or animals (Malagoli et al. 2015; Sun et al. 2017). The agricultural-based strategy can focus on adding a source, e.g., inorganic Se, to produce Se-enriched food crops that will contribute to a greater Se intake in Se-deficient communities (Broadley et al. 2006; Dhillon and Bañuelos 2017; Global Panel 2015; Kápolna et al. 2009; Malagoli et al. 2015; Puccinelli et al. 2017; Schiavon et al. 2016; Schiavon and Pilon-Smits 2017a, b; Schiavon et al. 2017; White and Broadley 2009; Wiesner-Reinhold et al. 2017; Winkel et al. 2015). Alternative sources to using inorganic Se for a biofortification strategy is the application of organic Se, i.e., Se-rich plant tissues from a Se hyperaccumulator, as a green Se biofertilizer (Bañuelos et al. 2015, 2016, 2017; Chauhana et al. 2019; Schiavon et al. 2017; Rayman 2012; Wu et al. 2015), or the establishment of crops in Se-rich soils (Bañuelos et al. 2020). Consumption of Se-biofortified crops can increase Se intake for the general population residing in low-Se regions.

This chapter will focus mainly on three Se-hyperaccumulator plant species and their potential use in agromining and their use as a green Se biofertilizer in Se biofortification strategies.

2 Selenium: Its Chemistry in Soils and Its Impact on Plant Speciation

Selenium was discovered first by Jöns Jacob Berzelius (1817). This chemical element is found in group 16 on the periodic table with atomic weight 78.96 amu and 34 as atomic number (Terry et al. 2000). Selenium is classified as a 'metalloid', sharing chemical similarities to other members found in this group oxygen (O), sulfur (S), tellurium (Te) and polonium (Po). Selenium can be found in four different oxidation states: elemental selenium (Se^0), selenite (Se^{4+}), selenate (Se^{6+}) and selenide (Se^{2-}) (Garousi 2017). Inorganic oxyanions forms like selenite and selenate are found predominantly in soils that are alkaline and have well-oxidizing conditions (Bañuelos et al. 2017; Garousi 2017; Kabata-Pendias and Pendias 2001). Selenate has a higher water solubility and therefore, it is the predominantly inorganic form readily taken up by plant roots (Gupta and Gupta 2017; Terry et al. 2000; White 2016, 2018; Winkel et al. 2015). Selenium also can form complexes with carbon and other forms of organic matter forming seleno-compounds [e.g., selenocysteine (SeCys) or selenomethionine (SeMet)]. These seleno-compounds have been shown to serve humans in protection from oxidative damage, thyroid metabolism, and in anti-carcinogenic activities (Brummell et al. 2011; Dong et al. 2001; Domínguez-Álvarez et al. 2016; Kieliszek and Błazejak 2016; Rayman 2012). Besides the total Se uptake by the plant, speciation of Se, i.e., biotransformation of Se chemical forms, is one of the key processes related to biofortification and the nutritional bioavailability of organic Se species within edible plant tissues consumed by animals and humans (Bañuelos et al. 2015, 2016, 2017; Chauhana et al. 2019; Schiavon et al. 2017; Rayman 2012; Sun et al. 2017; Wiesner-Reinhold et al. 2017; Winkel et al. 2015; Wu et al. 2015).

The parental rock (e.g., igneous rocks, sedimentary rocks like sandstone, carbonaceous slit stones, and cretaceous shells) is the major component that influences the distribution of Se in soils after weathering (Garousi 2017; Kabata-Pendias and Pendias 2001; Sun et al. 2016; Terry et al. 2000). In comparison to O and S, Se is one of the rarest trace elements on the earth's crust (0.05 mg kg^{-1}) (Garousi 2017; Kabata-Pendias and Pendias 2001). Selenium can be found world-wide ranging from <0.01–2 mg kg^{-1} (Winkel et al. 2015) to highly Se contaminated soils in China with more than >3 mg kg^{-1} (Sun et al. 2016; Xing et al. 2015). These data demonstrated the non-homogeneous distribution of Se in natural soil conditions, which results in the low acquisition by plants in Se-depleted soils. Selenium is inversely found as clay content decreases. Therefore, sandy soils are found with the lowest Se content. Consequently, the rate of biotransformation from absorbed inorganic forms of Se into seleno-amino compounds in a plant depends on specific biochemical pathways, as well as different geochemical factors in the soil like, clay and organic matter contents, pH and redox potential (Eh), Se complexation and its availability in the water soluble forms at the rhizosphere fraction (Bañuelos et al. 2017; Kabata-Pendias and Pendias 2001; Terry et al. 2000; Winkel et al. 2015). Another important aspect in Se geochemical soil cycling is Se volatilization. This is a very important process that can take place in aqueous or terrestrial environments, releasing dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe) as the major volatile Se species (Sun et al. 2016). Bacteria found in Se rich soils play an essential role in this process and are also key components in Se remediation systems (Eswayah et al. 2016, 2017; Sun et al. 2016; Winkel et al. 2010).

3 Ideal Candidates for Selenium Agromining

Stanleya pinnata, Astragalus bisulcatus and *Cardamine hupingshanensis* are suggested plant species for use in agromining of high Se soils due to their ability to extract excessive amounts of Se from soil and accumulate Se to high concentrations that do not lead to toxicity. *Stanleya*, a perennial member of the Brassicaceae family, is a small genus, comprising seven species very well adapted to the western area of North America (Cappa et al. 2014). Prince's plume (*S. pinnata*), one of the most well characterized species, generates high biomass and grows as high as 1.5 and 2 m wide. These growth characteristics are ideal for its use as a Se agrominer (Feist and Parker 2001; Freeman et al. 2010, 2006; Freeman and Bañuelos 2011; Guerinot and Salt 2001). Several studies have shown that this primary Se-hyperaccumulator accumulates between 2000 and 5000 mg Se kg^{-1} DW on seleniferous soils (2–10 mg Se kg^{-1}) without suffering toxicity, while a non-Se accumulator plant will accumulate two orders of magnitude less Se (e.g., 25 mg Se kg^{-1} DW) (El Mehdawi et al. 2011, 2015, 2018; Feist and Parker 2001; Freeman et al. 2006, 2010; Freeman and Bañuelos 2011; Harris et al. 2014; Parker et al. 2003; Schiavon et al. 2015, 2017). Leaves, stems and roots of *S. pinnata* can contain high concentrations of Se up to 3500, 1500 and 2300 mg Se kg^{-1} DW, respectively in spring, while concentrations of Se decline sharply in old leaves (less than 200 mg Se kg^{-1} DW) in autumn. In the reproductive organs (i.e., flower and seed), concentrations can range from 1800 to 3300 mg Se kg^{-1} DW. These observations indicate a peak accumulation of Se in young tissues in spring, then Se is mobilized into reproductive tissues in summer, and falling back during autumn (Cappa et al. 2015; Galeas et al. 2007; Harris et al. 2014).

Astragalus bisulcatus, commonly known as two-grooved milkvetch or silver-leafed milkvetch, is a member of the Fabaceae family and is a primary Se hyperaccumulator (Alford et al. 2014; DeTar et al. 2015; Parker et al. 2003; Pickering et al. 2003; Sors et al. 2009; Valdez-Barillas et al. 2012). This species is a perennial plant native to North America and in contrast to *S. pinnata*, *A. bisulcatus* exhibits slow growth and produces less biomass (Alford et al. 2012; Feist and Parker 2001; Parker et al. 2003). However, this Se-hyperaccumulator can accumulate more than 10,000 mg Se kg^{-1} DW predominantly as methylselenocysteine (MetSeCys) in young leaves and reproductive organs (Cappa et al. 2014; Freeman et al. 2006, 2010; Statwick et al. 2016). Similar to that observed in *S. pinnata*, leaves of *A. bisulcatus* experience seasonal fluctuations in Se concentrations with the highest accumulation detected in young leaves in spring (up to 12,700 mg Se kg^{-1} DW) and the lowest concentrations in autumn (600 mg Se kg^{-1} DW) (Galeas et al. 2007). Speciation of the total Se showed that the predominant organic Se forms were 53% MetSeCys and 47% γ-glutamyl-Se-methylselenocysteine (γ-GMetSeCys) in the young leaf tissues. The absence of inorganic forms in hyperaccumulators could suggest their efficient assimilation in the Se pathway (Cappa et al. 2014, 2015; El Mehdawi et al. 2018; Freeman and Bañuelos 2011; Lima et al. 2018). Non-methylated organic forms [SeCys and SeCystine (SeCys$_2$)] were detected in *A. bisulcatus* in contrast to *S. pinnata* and *C. hupingshanensis* (Freeman et al. 2006), although SeCys$_2$ was the predominant form in *C. hupingshanensis* (Yuan et al. 2013). In mature leaves of *A. bisulcatus*, Se (VI) was found as the predominant inorganic form of Se (Pickering et al. 2003). During reproductive development, flowers and seeds of *A. bisulcatus* accumulated 9000 and 6500 mg Se kg^{-1} DW, respectively (Galeas et al. 2007). Overall, 10- to 14-fold higher total Se concentration was observed in young leaves of *A. bisulcatus* in comparison with mature leaves (Freeman et al. 2006). The Se fluctuations found within foliar tissues in Se hyperaccumulators is a distinctive characteristic of Se remobilization that may be helpful in determining harvest time of these plants.

Therefore, these Se-hyperaccumulators exhibited highly contrasting differences with non-hyperaccumulators, as well as intraspecific differences (species within genus *Stanleya*) (Cappa et al. 2014). Some hyperaccumulation criteria within plants are high Se:S ratios, high Se shoot to root ratios, and high source:sink Se ratios (Bañuelos et al. 2017; Bitterli et al. 2010; Cappa et al. 2014; Lima et al. 2018; White et al. 2007; White 2018). These listed higher ratios suggest upregulation of transporters for selenate in comparison with sulfate, as well as higher rates of translocation of Se within vascular tissues (Cappa et al. 2014; Chauhana et al. 2019; El Mehdawi et al. 2018; Harris et al. 2014).

The newly discovered plant species *C. hupingshanensis* (known also as bittercresses or toothworts) was identified growing in Enshi, the Se capital of the world in Western Hubei, China (Bai et al. 2008; Li et al. 2018; Yuan et al. 2013). This aquatic and subaquatic perennial plant species, belongs to the Brassicaceae family and it has been designated as a secondary Se accumulator due to its moderate ability to absorb and volatilize Se (Bai et al. 2008; Both et al. 2020; Xiang et al. 2019; Yuan et al. 2013; Zhou et al. 2018). In a morphological study, *C. hupingshanensis* exhibited erect stems, and taproots with multiple fine adventitious roots. This study suggested that the dense fine adventitious roots and the presence of lignified thickenings in the roots could be involved with the capture of the Se ions that might contribute to its Se hyperaccumulation. Also, tolerance under Se stress was suggested to be as result of the enrichment of polysaccharide present in the collenchyma cells walls (Xiang et al. 2019). However, this plant species is more like a Se hyperaccumulator than other secondary Se accumulators, e.g., *Brassica juncea* (Terry et al. 2000; Steven and Culver 2019) because *C. hupingshanensis* was able to accumulate 1965 mg Se kg^{-1} DW in shoots and 4414 mg Se kg^{-1} DW in roots, while growing in a Se-laden soil (10–70 mg Se kg^{-1}) in China (Yuan et al. 2013).

Recent findings suggest using *C. hupingshanensis* for Se phytoremediation in Se-contaminated regions of China, as well as utilizing the plant tissue after harvest as an organic Se biofertilizer in Se-deficient soils (Jiang et al. 2018; Shao et al. 2014; Yuan et al. 2013; Wu et al. 2015). Compared to *A. bisulcatus* and *S. pinnata*, more detailed information is needed on *C. hupingshanensis* as a Se-hyperaccumulating plant. Recent morphological (Xiang et al. 2019), transcriptomic analysis (Zhou et al. 2018) and localization and speciation studies (Both et al. 2020) have, however, provided new insights in the abilities and genes involved in *C. hupingshanensis* to hyperaccumulate, as well as the Se isoforms associated with speciation.

The use of these three Se hyperaccumulators make them clear candidates for agromining due to their ability to extract high concentrations of Se. However, only a few field studies have provided a detail description of total and extractable Se concentrations in soil and resulting Se concentrations in their plant tissues. For, example, Cappa et al. (2014) compared four varieties of *S. pinnata* (treated as distinct taxa) under field conditions. The authors found that only *S. pinnata* var. *pinnata* was the only true Se-hyperaccumulator found on the eastern side of the Continental Divide. In the leaves of *S. pinnata,* hyperaccumulation of Se exhibited 13-fold greater accumulation in comparison with the lowest Se accumulator, *S. tomentosa*. At the genomic level, variances in genome size within the *S. pinnata* species was observed, and *S. pinnata* was the only one non-diploid.

4 Enhanced Se:S Ratios, BCF and DC Factors

A higher Se:S ratio (Bañuelos et al. 2017; White 2016; White et al. 2007) is a distinctive characteristic of Se-hyperaccumulators, e.g., *A. bisulcatus* and *S. pinnata* (Cappa et al. 2014; El Mehdawi et al. 2018; Parker et al. 2003; Pickering et al. 2003; Schiavon et al. 2015), which allow them to be more suitable to colonize alkaline and well oxidized soils, predominantly enriched with high sulfate levels (Bañuelos 2001, 2006). In contrast to Se-hyperaccumulators, high sulfate concentrations in soils compete with selenate in non-Se hyperaccumulators due to Se uptake primarily occurring via sulfate transporters (Chao et al. 2014; El Mehdawi et al. 2018; Hesketh 2008; Schiavon et al. 2015; Shibagaki et al. 2002; White et al. 2004). In Se hyperaccumulators, there is a higher transcript expression of sulfur transporters (e.g., SULTR1 2) in roots and in shoots (Schiavon et al. 2015; Cabannes et al. 2011; Wang et al. 2018), as well as a higher expression of ATP dependent sulfurylases (APS) (Jiang et al. 2018; Pilon-Smits et al. 1999; Schiavon et al. 2015), which are suggested to be responsible for the high Se:S ratios in Se hyperaccumulators. The sulfur metabolic genes (e.g., *SULTR* and *APS*) are predominantly overexpressed if exposed to selenate/sulfate in competition assays and in comparison to secondary Se accumulators (Feist and Parker 2001; Freeman et al. 2010; Galeas et al. 2007; Jiang et al. 2018; Pickering et al. 2003; Pilon-Smits et al. 1999; Schiavon et al. 2015; Terry et al. 2000).

Constitutive upregulation of sulfate transporters have been observed at the molecular level using quantitative gene expression by real-time PCR reverse transcriptase (RT-qPCR) and transcriptomic analyses for Se hyperaccumulators and non-hyperaccumulators (El Mehdawi et al. 2018; Jiang et al. 2018; Lima et al. 2018; Schiavon et al. 2020; Wang et al. 2018). For example, higher transcript levels for genes involved in sulfur/selenate metabolism were observed in Se hyperaccumulator *S. pinnata* compared to the non-hyperaccumulator *Stanleya elata*. Genes upregulated in the Se hyperaccumulator *S. pinnata* (Wang et al. 2018) included *SULTR1;2* (high-affinity transporter facilitating uptake into roots), *SULTR2;1* (low-affinity transporter during xylem loading (translocation to shoot), *SULTR3;5* (root-to-shoot translocation), *SULTR4;2* (vacuolar efflux), *SULTR2;2* (phloem remobilization), *SULTR3;1* (chloroplast influx), *SULTR3;3* and *SULTR3;4* (unknown function). In contrast, reduced expression of *SULTR1;1* (plant root high-affinity H^+/sulfate cotransporter under low-sulfur conditions) was observed in *S. pinnata* (El Mehdawi et al. 2018). The accumulation of selenate in *S. pinnata* tissues was unaffected using competition experiments with Se and S, including fast response (1 h) or long term (9 days) with sulfur (S) in comparison with non-hyperaccumulators (*Stanleya elata* and *B. juncea*) (El Mehdawi et al. 2018). These results indicate that the higher expression of sulfate transporters ensure its translocation to above tissues (Lima et al. 2018; Schiavon et al. 2020).

In another experiment (in presence or absence of Se), an important enzyme involved in sulfur transport [ATP sulfurylase 2 (APS2)] showed over 120-fold higher elevated transcript levels in *S. pinnata* roots compared with *S. elata*. Furthermore, expression of this enzyme (APS2) was upregulated two–four fold higher in shoots of the Se hyperaccumulator (Jiang et al. 2018; Wang et al. 2018).

These findings suggest that despite the physical and chemical similarity of sulfate and selenate, high/low affinity sulfate transporters (H/LASTS) are highly expressed in Se hyperaccumulators in comparison with non-Se hyperaccumulators, which make them more suitable for agromining (El Mehdawi et al. 2018; Jiang et al. 2018; Lima et al. 2018; Pilon-Smits et al. 1999; Schiavon et al. 2015, 2020; Wang et al. 2018; White et al. 2007).

Nonetheless, Se accumulation varies even within Se hyperaccumulator populations. For example, Feist and Parker (2001) found variation in Se-accumulation ability in 15 different ecotypes of *S. pinnata*, indicating the importance of selecting the appropriate population for agromining field purposes. In their study, ecotype CO4 from Colorado showed the highest accumulation of Se in shoot tissue under the highest Se treatment (80 μM selenate) with 950 mg Se kg^{-1} DW compared to 200 mg Se kg^{-1} DW in ecotype UT5 from Utah. For this reason, genotype CO4 was suggested to be the most promising ecotype for Se phytoremediation strategies (Feist and Parker 2001; Parker et al. 2003).

In a field survey and common-garden experiment, Cappa et al. (2014) tested four *Stanleya* taxa and showed highly intraspecific variation in their abilities to hyperaccumulate Se, as well as their variation in the accumulation of Se:S in different plant tissues. Their findings were in agreement with those of Feist and Parker (2001) when they showed almost 100-fold variation in leaf Se accumulation and almost 300-fold variation in fruit Se accumulation in the field. *S. pinnata* var. *pinnata* was the only taxon hyperaccumulating Se levels exceeding 1000 mg kg^{-1} DW. The chromosomal number within *S. pinnata* showed a proliferation of tetraploids when grown on the western slope of the Rocky Mountains and diploids on the eastern slope in Colorado (Cappa et al. 2014). More studies are needed to examine how the accumulation of Se might need to address a possible genetic drift in differential pressures (e.g., soil levels of Se:S) (Cappa et al. 2014).

There are two parameters used to indicate how much of an element such as Se is taken up by the plant roots and transported to the shoots. The first parameter is called Biological Concentration Factor (BCF) (Bañuelos et al. 2017). For Se, the BCF is the ratio of Se concentration in dry shoots/total concentration of Se in soil (see Table 1). This ratio is very low in non-accumulators compared to the BCF ratios for *A. bisulcatus* and *S. pinnata* (>100), and 10.7 and 8.7 in roots and stems of *C. hupingshanensis,* respectively (Yuan et al. 2013). Once the BCF ratio reaches its plateau (indicating a homeostasis has been reached), there will not be an increase of Se uptake, irrespective of Se available in the rhizosphere. The BCF can be complemented with another parameter known as the Discrimination Coefficient (DC), as proposed by Bell (1992). This is the ratio of Se:S concentrations in plant tissue to SeO_4:SO_4 concentrations in soil, which is useful for detecting the preferences in uptake between these two analogues. Bell et al. (1992) indicated that *A. bisulcatus* exhibited a DC between 2.4–9.0 in comparison with *Medicago sativa* L. at 0.4–0.7. The DC indicates that *A. bisulcatus* selectively preferred selenate over sulfate (Bell et al. 1992), while DC values observed for *S. pinnata* ranged between 1.2–4.3 (as expected for a Se hyperaccumulator) among 15 ecotypes (Bell et al. 1992; Feist and Parker 2001). Both BCF and DC are useful parameters in selecting for plants growing in soils enriched with S and Se.

5 Continuous Accumulation of Se Without Phytotoxicity

Se-hyperaccumulator plants are equipped with a plethora of mechanisms that allow them to survive without experiencing Se-phytotoxicity under a continuous exposure to a high Se environments. In these plant species, Se is absorbed, metabolized and assimilated via reduction into selenoamino acids (e.g., selenocysteine, selenomethionine, selenocystathionine, Se-methylselenocysteine and dipeptide γ-glutamyl-Se-methylselenocysteine) (Chao et al. 2014; Schiavon et al. 2015; Shibagaki et al. 2002; Terry et al. 2000). Many of these amino acids are in methylated forms (Neuhierl et al. 1999; Terry et al. 2000) to prevent misincorporation during protein translation (Hesketh 2008; Terry et al. 2000). In *S. pinnata*, others have observed that selenoproteins are misfolded and then ubiquitinated and removed by the proteasome. These findings suggested that proteasome 26S could be a mechanism preventing Se toxicity (Sabbagh and Van Hoewyk 2012). Selenium hyperaccumulators have increased methylation of these selenoamino acids by the higher activity of the seleno-methyltransferase (SMT) enzyme, which incorporates a methyl group in the SeCys/SeMet (Ellis et al. 2004; LeDuc et al. 2004; Neuhierl et al. 1999). This chloroplastic enzyme was first characterized from *A. bisulcatus* and its overexpression in non-Se-hyperaccumulators (*Arabidopsis thaliana*, *B. juncea*) (Sors et al. 2009) showed increases in Se tolerance and Se accumulation (Ellis et al. 2004; LeDuc et al. 2004; Neuhierl et al. 1999). In *A. bisulcatus*, SMT activity is not induced by exposure to selenate/selenite but its expression is constitutive (Pickering et al. 2003). A high elevated activity of SMT in *S. pinnata* is suggested to be partially responsible for the successful Se hyperaccumulation and tolerance in this plant (Freeman et al. 2010). In *C. hupingshanensis,* 78% $SeCy_2$ and 12.91% SeMetCys were determined to be the most abundant forms of organic Se in leaves and roots. These findings indicate the existence of an alternative pathway for Se detoxification, however, the activity of SMT in *C. hupingshanensis* is still unknown (Yuan et al. 2013; Zhang et al. 2018). Several genes associated with Se response in shoot and root tissues were upregulated in the newly transcriptomic study on *C. hupingshanensis* exposed to two different Se concentrations (0.1 and 80 mg Se L^{-1} for 24 h) compared to control. Several genes encoding enzymes related to S metabolism were upregulated in the Se hyperaccumulator, which included; root-sulfite

Table 1 Selenium accumulation and bioconcentration Factor (BCF) in primary Se hyperaccumulators, secondary Se accumulators and non-accumulators grown in Se-laden soils

Plant species	Growing season (days)	Total Se in soil (mg kg^{-1} DW)[a]	Se in shoots (mg kg^{-1} DW)[a]	Bioconcentration factor (BCF)[b]	Reference
Se hyperaccumulators					
Astragalus bisulcatus	248	7.2	595 (80)	83	Retana et al. (1993)
Stanleya pinnata	180	9 (3.8)	1620 (600)	180	Freeman and Bañuelos (2011)
Secondary Se hyperaccumulators					
Brassica juncea	180	9 (3.8)	37 (9)	4.1	Freeman and Bañuelos (2011)
Cardamine hupinshanensis	100	46 (4.6)	539 (117)	12	Shao et al. (2014)
	100	25 (14.1)	432 (471)	17	Yuan et al. (2013)
Non-accumulators					
Grass Tallwheat	231	20.5	12 (0.9)	0.6	Retana et al. (1993)
Lettuce	Mature plants[c]	0.27	0.10	0.4	De Temmerman et al. (2014)
Spinach	Mature plants[c]	0.30	0.17	0.6	De Temmerman et al. (2014)
Leeks	Mature plants[c]	0.32	0.08	0.3	De Temmerman et al. (2014)
Celery	Mature plants[c]	0.31	0.11	0.3	De Temmerman et al. (2014)
Sugar Cane	Mature plants[c]	1.4 (0.7)	28 (18)	19	Dhillon and Dhillon (1991a)
Avena	60	2.5	47	19	Dhillon and Dhillon (2009)

[a] Values represent the mean followed by the standard deviation in parentheses
[b] BioConcentration factor (BCF) as the ratio of Se concentration in dry shoots/total DW concentration of Se in soil
[c] Growing season was estimated accordingly to growing seasons in Belgium. Data presented are from composited samples

oxidase (SOX), leaf-glutathione S-transferase 12 (GST) and aryl sulfotransferase (SULTs). These new findings indicate that *C. hupingshanensis* exhibited higher level of transcription for genes directly involved in S metabolism in comparison with other Se hyperaccumulators. This observation probably indicates that some of those genes also have better preference for Se over S. In this study, Zhou et al. (2018) suggested that due to the high upregulation of SOX, selenite might be first converted to selenate, following the incorporation into ATP by ATP sulfurylase (APS) (Jiang et al. 2018; Pilon-Smits et al. 1999; Schiavon et al. 2015). Finally, the reduced selenide by APS reductase (APR) is incorporated into SeCys in the root tissue of *C. hupingshanensis* seedlings (Zhou et al. 2018).

The methylated organic forms of Se found in hyperaccumulators not only prevent Se toxicity, but their presence makes their plant tissue potentially ideal for pharmaceutical uses, since these forms of Se in leaf tissue may possess anticancerous properties (Domínguez-Álvarez et al. 2016). Some of the organic forms like MetSeCys, and γ-glutamyl-Se-methylselenocysteine (GGSe MetSeCys) have shown natural anti-cancer properties and may play an important role in interfering with various types of cancer (Brummell et al. 2011; Dong et al. 2001; Domínguez-Álvarez et al. 2016). Speciation studies on these Se compounds showed that in young leaves of *S. pinnata,* 88% of the total Se was found as MetSeCys, and 12% as selenocystathionine (SeCysth) in leaf margins and tips (Freeman et al. 2006). In *A. bisulcatus*, the organic forms of Se were localized predominantly in trichomes: 53% as MetSeCys and 47% as γ-glutamyl-Se-methylselenocysteine (γ-GMetSeCys), although no SeCys or $SeCys_2$ species were detected (Freeman et al. 2006). In summary, the high enzymatic activity of SMT in Se hyperaccumulators and the potential beneficial forms of organic Se accumulated in shoots, make these Se-hyperaccumulators ideal candidates for agromining and also useful for Se biofortification strategies, as described by Bañuelos et al. (2015, 2016, 2017).

New insights about genes involved in antioxidant responses were detected in a transcriptomic experiment for the Se-hyperaccumulator *S. pinnata*. Antioxidants such as glutathione (GSH) were highly expressed (e.g., GSH1; GR2). In *S. pinnata* roots, the glutathione-S-transferase family was between 30- to 40-fold higher than the non-hyperaccumulator. Reacting oxygen species (ROS) enzymes, like GSH peroxidases (GPX2, GPX6 and GPX7), ascorbate peroxidase (APX1), thioredoxin peroxidase (TPX) and thioredoxin were also highly expressed in *S. pinnata* (Wang et al. 2018). Higher expression of other important genes encoding for hormones have been involved in abiotic and biotic stress tolerance such as jasmonic acid [3-oxo-2-2′-*cis*-pentenyl-cyclopentane-1-acetic acid (JA)], as well as genes from the ethylene family, were observed in *S. pinnata* compared to *S. elata* (Wang et al. 2018). The presence of such metalloids as Se, could trigger a hormonal response that might be effective for protecting the plant against abiotic stresses.

6 Higher Pest Self-defense Proteins Expression and Protection Against Herbivory

Accumulation of Se may provide protection against pathogens and/or herbivores to Se-hyperaccumulators (Freeman et al. 2009; Golubkina et al. 2014; Martens and Boyd 2002; Palomino et al. 2007). For example, Freeman et al. (2009) observed in a two-year field experiment that by exposing *S. pinnata* to either high (40 µM Se) or low (2 µM Se) levels of Se, they found a direct positive relationship between herbivory by black-tailed prairie dog (*Cynomys ludovicianus*) and plant Se accumulation. The results suggested that accumulation of Se in the plant tissues (~50–750 mg Se kg^{-1} DW) protected the plants against mammalian herbivory in their natural environment (Freeman et al. 2009). In this regard, Se accumulation by plants may be an effective way to reduce certain insect pest attacks, e.g., aphids (Hanson et al. 2004), caterpillars, snails or fungal infection (Hanson et al. 2003). Due to the observation that Se hyperaccumulators can reach concentrations greater than 1000 mg Se kg^{-1} DW, these plants can exert a type of pest control (Hanson et al. 2004). In another field experiment with Se and arthropods, both Se-enriched *A. bisulcatus* and *S. pinnata* showed fewer arthropods than non-hyperaccumulators (Galeas et al. 2008). The authors detected higher number of arthropods in plants containing Se concentrations below 600 mg kg^{-1} DW. As Se increased in plant tissues (up to 8000 mg kg^{-1} DW) less arthropods were feeding on them, suggesting that Se acted as an elemental defense. The authors also detected low bioaccumulation of Se within the arthropods feeding on the plants. Their levels of Se were at least one order less than in the plant tissue, indicating that if these Se-hyperaccumulator plants are used in agromining or used as an organic Se-biofertilizer to biofortify food crops (Bañuelos et al. 2015, 2016, 2017; Galeas et al. 2008), there is a minimal risk for bioaccumulation of Se into the trophic chain under these conditions.

Nematodes, a root herbivore pest was studied in response to their behaviour with Se-hyperaccumulators *S. pinnata* and *A. bisulcatus* in field and under greenhouse conditions (Prins et al. 2019). They observed a cycling of Se in the different tissues and detected that the numbers of nematodes diminished where Se concentrations increased in plant tissues. Importantly, their study showed that the higher concentration of Se by the hyperaccumulators were found predominately in the root epidermal cells, which is the first point of interaction with nematodes. This pattern of root Se allocation can prevent their access to the stele containing the vascular bundles in the Se hyperaccumulators (Prins et al. 2019). Similarly, high Se concentrations found in the margins of the leaves of Se hyperaccumulators, may have a possible role in protection to herbivory (Cappa et al. 2015; Freeman et al. 2006).

In a recent experiment by Steven and Culver (2019), four varieties of *B. juncea* were used to identify the beneficial and negative impacts of plant accumulation of Se on herbivore defense against *Pieris rapae* and on plant growth. Their study showed that there is a protective advantage for the plant when accumulating Se, but the Se concentration was negatively correlated with plant growth (e.g., number of flowers) and Se tissue concentrations greater than 500 mg Se kg^{-1} DW. In another experiment using *B. juncea*, this secondary Se accumulator did not possess a specialized mechanism to withstand high metal accumulation compared to Se hyperaccumulators (Lima et al. 2018). For secondary Se accumulators, moderate concentrations of Se are enough, however, to provide a protection mechanism against herbivory without disrupting plant growth. Because Se hyperaccumulators are able to accumulate much higher concentrations of Se than secondary Se accumulators without any effect on biomass, hyperaccumulators are more ideal to use for agromining.

Selenium hyperaccumulators not only have physiological mechanisms to survive in Se-enriched soils, they can reportedly promote Se uptake into neighbouring non-Se accumulators by plant interaction, as well as provide protection against herbivory attacks to adjacent plants (El Mehdawi et al. 2011). These events were observed in *Artemisia ludoviciana* and *Symphyotrichum ericoides* (non-accumulator plant species) cultivated in proximity with *A. bisultcatus* and *S. pinnata* (El Mehdawi et al. 2011). The non-accumulator plants, *A. ludoviciana* and *S. ericoides*, increased their growth, Se uptake (up to 20-fold) and increased herbivory protection and leaf S content by 40 and 25% respectively, when grown adjacent to Se-hyperaccumulators (El Mehdawi et al. 2011, 2012, 2015; Pilon-Smits 2019). These findings suggest the possibility to co-cultivate or intercrop Se hyperaccumulators to promote Se uptake into adjacent edible crops. The authors suggested that the presence of a Se hyperaccumulator may change the availability of certain nutrients in the rhizosphere, e.g., S, as well influence microbial communities due to increases in Se concentrations in the rhizosphere of the Se hyperaccumulators (Alford et al. 2014; Cochran et al. 2018; Di Gregorio et al. 2005; El Mehdawi et al. 2011; Sura-de Jong et al. 2015; Yuan and Zhang 2017). Rhizosphere communities (bacterial, archaeal and fungi) found on the same seleniferous soil were significantly different among Se hyperaccumulators and non-hyperaccumulators. Selenium hyperaccumulators may be able to select for specific plant rhizomicrobial communities that can affect plant abundance (Alford et al. 2014; Pilon-Smits 2019; Valdéz-Barillas et al. 2012). The microbial communities in the rhizosphere of the Se hyperaccumulators can also tolerate concentrations of Se up to 200 mM (Sura-de Jong et al. 2015).

For more information on the important associations between Se hyperaccumulators and microbial-fungal communities, relevant studies are discussed in detail by the following authors: Alford et al. (2012, 2014), Di Gregorio et al. (2005), Lindblom et al. (2013), Sura-de Jong et al. (2015), Tong et al. (2014), Valdez-Barillas et al. (2012), Wangeline et al. (2011).

Additionally, the coevolution of Se-resistant and tolerant insects and Se hyperaccumulators (*S. pinnata* and *A. bisulcatus*) have been studied, e.g., the wasp (*Acanthoscelides fraterculus* Horn, Coleoptera: Bruchidae) (Freeman et al. 2012). In this study, Freeman et al. (2012), found a high Se concentration in the seeds of *A. bisulcatus* (~5750 mg Se kg^{-1} DW) and *S. pinnata* (~1329 mg Se kg^{-1} DW), but in contrast, adult beetles and adult chalcid wasps found on the plants contained only 4–6 mg Se kg^{-1} DW and 9 and 47 mg Se kg^{-1} DW, respectively. The authors suggested that there is a possible efflux of Se by the beetles that can result in a decrease of Se in their tissues and consequently, allow them to continue feeding on Se-hyperaccumulator seeds without Se toxicity (Bañuelos et al. 2002; Freeman et al. 2012). Vickerman et al. (2004) showed using near-edge XAS analysis for the first time, the biotransformation in vivo of Se as it is taken up through three trophic levels, i.e., inorganic selenate to organic Se to volatile methylated Se. Their results showed Se biotransfer and different biochemical mechanisms that were engaged at each trophic level leading to Se detoxification. In their model, beet armyworm larva *Spodoptera exigua* (Hübner) (Lepidoptera; Noctuidae) fed on alfalfa leaves (*Medicago sativa* L., Condor CT) irrigated with inorganic 3.3 mg Na_2SeO_4 L^{-1} in water, converted selenate into organic Se. More than 57% of the Se taken up by alfalfa was transformed to organic Se forms, and 39% remained as inorganic selenate and 4% as selenite. In the third trophic level, after emergence of the endoparasitoid wasp *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) from *S. exigua*, the pupae contained only organic Se, e.g., selenomethionine and trimethyl selenonium cation, the same as its host without Se biotransformation. This result suggests that the parasitoid will experience greater toxic effects since it is exposed to unabsorbed selenate and organic Se forms from the gut of the herbivore. These studies investigated the trophic levels of crops and its microcosms, e.g., insects and parasitoids, and reflect other important components within phytoremediation systems and integrated pest management (IPM) techniques using a biological control (Vickerman and Trumble 2003; Vickerman et al. 2004).

7 Better Performance and Growth Under High Osmotic Stress

Freeman and Bañuelos (2011) and Retana (1993) suggested that the Se hyperaccumulators *S. pinnata* and *A. bisulcatus* are better equipped, and exhibited a dual ability to tolerate highly saline and boron-rich soil conditions, whilst hyperaccumulating Se (Freeman and Bañuelos 2011; Freeman et al. 2010; Retana et al. 1993). Under salinity conditions (electrical conductivity (EC) of 4.8–8 dS m^{-1}) *S. pinnata* was able to remove 30% of the total soil Se between 0 and 30 cm soil depth in two growing seasons (Freeman and Bañuelos 2011). Two ecotypes of *S. pinnata*, e.g., colorado 4 (CO4) and commercially available western native seed (WNS) exhibited better tolerance to high osmotic stress than other *S. pinnata* ecotypes when grown in high saline soils contaminated with boron (B) (Feist and Parker 2001; Parker et al. 2003). In addition, Retana (1993) evaluated Se uptake by *A. bisulcatus* and *A. racemosus* grown in a high saline/B soil (ranging from 5.2 to 14 dS m^{-1} and 7.8 to 17 mg B L^{-1}) and containing 0.1–1.6 mg S L^{-1}) under greenhouse conditions. The authors observed that *Astragalus* spp. removed 2–3.5 kg Se ha^{-1} from a total of 92 kg Se ha^{-1} soil between 0 and 90 cm in one growing season, while nonaccumulator grasses removed 100 g Se ha^{-1} under the same B and saline conditions (Retana et al. 1993). These observations suggest that Se hyperaccumulators such as *S. pinnata* and *A. bisulcatus* appear to be well suited for accumulating Se under high saline and B soil conditions.

8 Economics of Selenium Agromining

Economically speaking, the market price for certain elements will depend on their annual yield per unit area (amount of the element/biomass produced), as well as the distribution and concentration of the element, e.g., Se, in a particular geographical area. The economics related to Se hyperaccumulators to agromine for Se, showed that Se price per metric ton, (annual yield per unit area) is relatively high (US$52,000) (van der Ent et al. 2015). Planting the Se hyperaccumulators *S. pinnata, A. bisulcatus* and *C. hupingshanensis,* respectively, in Se-laden soils and utilizing the harvested biomass may provide some advantages and profit opportunities ideally situated for growers. For example, Se-enriched tissues can be carefully used as blend in cattle feed or used as biofertilizers in Se-biofortification strategies (Bañuelos 2001, 2006; Bañuelos et al. 2015, 2016, 2017; Freeman and Bañuelos 2011; Zhu et al. 2009). Another important feature that promotes the use of agromining Se, is that hyperaccumulators are grown in Se-laden soils, which in the western USA are generally of poor quality and would not support growing food crops. A drawback from using plants to agromine Se is that the percentage of Se removed seasonally will not be constant or predictable due to variable soil conditions, and consequently, Se concentrations in plant tissues will likely fluctuate (Galeas et al. 2007). Hence, economic value of plant products based upon tissue Se concentrations may also be unpredictable.

9 Applicability of Se Hyperaccumulators

Applying a plant-base agronomic technology (agromining) on soils with high levels of Se can produce a high Se enriched biomass after harvest. There are some ‘hot spots’ with naturally Se-rich soils in western China (Zhu and Zheng 2001; Wu et al. 2015) and in west side of central California (Bañuelos et al. 1993; Bañuelos 2006). Se-rich soils are necessary for Se agromining to produce Se-enriched plant material that can be potentially used as a Se biofertilizer for Se-deficient soils. Rates of biofertilizer application can only be determined after Se postharvest analyses has been performed on a continued basis. Depending on the Se concentration found in the harvested plant material, the Se-enriched plant tissue can be added to soil where edible crops are grown. In this regard, Bañuelos et al. (2015, 2016), established this first experimental approach by adding different rates of *S. pinnata* as an organic Se biofertilizer to the soil for growing broccoli and carrots under field conditions. The *S. pinnata* meal was Se-enriched with organic forms of Se such as 50% CysSeSeCys (oxidized selenocysteine), 44% C-Se-C, 3% SeOMet (oxidized form of SeMet) and 3% of inorganic SeO_3^{2-}. The application of this organic Se biofertilizer produced Se-enriched carrots with Se organic forms present as 17% SeMet, 33.4% CysSeSeCys, 7.6% MeSeCys and 9.5% SeO_4^{2-} and Se-enriched broccoli florets with 18% SeOMet, 23% CysSeSeCys, 55% of (SeMet and MeSeCys) and 4% SeO_4^{2-} (Bañuelos et al. 2015, 2016). The rate of application did not significantly affect speciation of Se within the plant tissue. It was not clear whether the application of *S. pinnata* influenced the organic forms of Se, e.g., MeSeCys, within the carrots and broccoli. Future studies should evaluate Se-biofortified foods for their MeSeCys content as influenced by source of Se applied to soil (inorganic *vs* organic), since MeSeCys has been suggested to play a role in human cancer prevention (Brummell et al. 2011; Dong et al. 2001; Domínguez-Álvarez et al. 2016; Kieliszek and Błazejak 2016). In addition, there are very few field studies that can provide data related to Se absorption by human consumption of Se-enriched crops produced from the application of organic Se biofertilizers. Similarly, pioneering work is currently in progress by Sun et al. (2017) in Belgium on evaluating bioaccessibility of Se by simulated intestinal studies from Se-biofortified food products. Therefore, it is imperative to encourage more research on bioaccessibility that can lead to better understanding of the effects of Se intake by humans and animals when using Se-biofortified food or feed products. Studies using plant-based Se biofertilizers are limited and there are no data available on their use in long term field studies, however, longer-term studies are currently in progress (Bañuelos et al. unpublished).

A biofortification strategy in Se-deficient soils will require application of Se biofertilizers or inorganic Se fertilizers that will supply additional

Se to crops (Kápolna et al. 2009; Wu et al. 2015). Meta-analyses on Se biofortification showed that inorganic Se applied as foliar application are the most common alternatives in the mitigation of Se deficient soils (Bañuelos et al. 2017; Broadley et al. 2006; Phiri et al. 2019; Ros et al. 2016). In contrast to inorganic Se fertilizers, growers using Se biofertilizers will need to find feasible, accessible, practical and economically-viable strategies to incorporate vast quantities of Se-enriched plant material into Se-deficient soil to biofortify staple crops.

10 Conclusions

All three species of Se hyperaccumulators *S. pinnata, A. bisulcatus* and *C. hupingshanensis* exhibited major advantages for agromining, which include: higher Se:S ratios, higher capacity for discriminating between these two analogs and better adaptability to saline and B soils conditions. Furthermore, due to the high Se concentrations in their tissues, Se hyperaccumulators are more pest-tolerant and deter herbivory when planted alone or with food crops. *Stanleya pinnata* plant meal has already been demonstrated to be effective as a Se biofertilizer in broccoli and carrot crops, but more research is needed for *A. bisulcatus* and *C. hupingshanensis* in their potential for use as Se biofertilizers. Assuming high Se soils are available, selecting, growing and utilizing a Se-hyperaccumulating species should be part of an agronomic strategy to provide growers with an additional source of Se, as Se biofertilizer for Se biofortification (Figs. 1, 2, 3 and 4).

Fig. 1 *Cardamine hupingshanensis* grown in China under greenhouse conditions

Fig. 2 *Cardamine hupingshanensis* grown and flowering in selenium soils in Enshi China

Fig. 3 *Stanleya pinnata* growing in selenium rich soils in California

Fig. 4 *Astragalus bisulcatus* growing in Wyoming (Courtesy of Matthew Lavin)

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Agromining of Thallium and Noble Metals

Brett Robinson and Chris Anderson

Abstract

Thallium is a relatively toxic and valuable element, for which there are fast-growing hyperaccumulators that have the highest bioaccumulation coefficients (plant/soil concentration quotients) of any non-essential element. As with other elements, many Tl-hyperaccumulating plants are in the Brassicaceae family. In contrast, hyperaccumulation of the precious metals Au, Pd and Pt is not recorded by any plant species. To achieve uptake of these noble metals, chemicals must be added to soil to induce metal solubility, for which cyanide has proven repeatedly to be the most effective agent to promote uptake. However, cyanide does not specifically target the noble metals. Increased solubility and uptake of more toxic Cu and Ag can limit the uptake efficiency of a phytomining or agromining crop (a co-metallic effect). Worldwide, there are numerous soils with a high Tl burden (>1.5 mg kg^{-1}) that are unsuitable for safe food production and have low value, and thus are ideal for agromining. Of the elements that could potentially be agromined, Tl has perhaps the greatest potential to be economically successful. Despite this promise, Tl has received relatively little attention. The geographical scope for noble metal uptake is in contrast, much more limited. Research is warranted on the discovery of new hyperaccumulators, the economics of recovering Tl and noble metals from biomass, and quantification of areas where agromining for these more valuable metals may be feasible.

1 Introduction

Thallium and the noble metals are distinct from other potentially agro-minable elements because of their extraordinary high monetary values (Anderson et al. 1999; Scheckel et al. 2004). Therefore, the threshold concentration of these metals in crops for successful agro-mining is significantly lower than for other elements. However, in all other respects Tl and the noble metals are distinct. Thallium is a chalcophile (sulfur-loving) element with a small but significant proportion of the total concentration occurring in soil solution. In contrast, the noble metals, of which the most important are Au, Pt, and Pd, are siderophiles (iron-loving) and characterized by inordinate lack of chemical reactivity—they are noble because they do not take part in

B. Robinson (✉)
School of Physical and Chemical Sciences,
University of Canterbury, Christchurch 8041,
New Zealand
e-mail: brett.robinson@kiwiscience.com

C. Anderson (✉)
School of Agriculture and Environment, Massey
University, Palmerston North 4414, New Zealand
e-mail: C.W.N.Anderson@massey.ac.nz

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_20

'common' chemical reactions. Therefore, the noble metals occur at exceedingly low concentrations in soil solution, and unlike Tl, have negligible interactions with plants unless lixiviants are employed to force them into solution. Owing to these differences, this chapter investigates the agromining potential of Tl and the noble metals separately.

2 Thallium

Interest in Tl agromining (phytomining) increased following Leblanc et al. (1999), who reported that *Iberis intermedia* from southern France (Fig. 1) accumulated >500 mg kg^{-1} (d. m.) in aerial tissues and that a crop grown on contaminated soil could return a net profit of more than US$1000 per hectare per annum. The metal price of Tl is increasing rapidly, from US $300 per kg in 1999 (Anderson et al. 1999) to US $7600 per kg in 2019 (Guberman 2020). This price increase has significantly changed the prospect of economically viable Tl agromining. In 1999, Anderson et al. (1999) concluded that a net return of US$1200 ha^{-1} yr^{-1} could be achieved with a 10 t ha yr^{-1} crop containing 800 mg kg^{-1} Tl. Today, assuming production costs from small-scale Tl agromining are US$1500 ha^{-1} yr^{-1}, twice that of wheat (Gloy 2015), that same crop would give a net profit of US $46,500 ha^{-1} yr^{-1}. Here, we aim to determine the current potential for Tl agromining and critical success factors, and identify fertile areas for future research.

Fig. 1 *Iberis intermedia* growing near St Laurent-le-Minier, southern France

2.1 Thallium in Soil

Globally, the background concentration of Tl in soil is <1 mg kg^{-1} (Anderson et al. 1999). Tremel et al. (1997a) reported a median value of just 0.29 mg kg^{-1} Tl in French soils, but with some soils containing geogenic Tl concentrations of up to 55 mg kg^{-1}. In some areas, such as Western Siberia, geogenic Tl can occur in soils at concentrations of 1.5–3.0 mg kg^{-1} (Il'in and Konarbaeva 2000). Being a chalcophile element, Tl is commonly associated with pyritic deposits. In China, natural soils associated with sulfide deposits contain 1.5–6.9 mg kg^{-1} compared to <0.5 in background soils (Xiao et al. 2004). Thallium is a co-contaminant in some mining ores and slags, as well as a product of coal combustion (Bunzl et al. 2001). Lis et al. (2003) reported that Zn-Pb ore mining and processing increased soil Tl concentrations by up to two orders of magnitude, with concentrations reaching 35 mg kg^{-1} in affected areas. Mine-affected soils near Keban, Turkey, contained 3.0–27.6 mg kg^{-1} Tl. In Poland, soil associated with historic mining of Ag, Pb and Zn contained an average of 20.8 mg kg^{-1} Tl (Woch et al. 2013).

Elevated Tl concentrations have been reported near W smelters (Cheng et al. 2013) as well as cement plants, with concentrations of 1.2–19.9 mg kg^{-1} Tl (Lee et al. 2015). Li et al. (2010) reported that continual irrigation of a cotton plantation with sewage increased soil Tl levels to about 0.42 mg kg^{-1}.

In soil, Tl is bound to quartz and aluminosilicate minerals, with lesser amounts associated with the organic fraction (Jacobson et al. 2005a; Gomez-González et al. 2015). Vanek et al. (2010) reported that Mn(III, IV) oxides, illite, and probably amorphous silicates were the dominant phases responsible for Tl retention in soils. Being typically monovalent and of similar ionic size, Tl may substitute for K in soil constituents (Tremel et al. 1997b).

2.2 Thallium in Plants

Of all non-essential trace elements, Tl has some of the highest bioaccumulation coefficients among plants, with even cabbage (*Brassica oleracea* var. *capitata*) having a bioaccumulation coefficient (BC) of 1–11 (Cheng et al. 2013; Jia et al. 2018). Brassicaceous species tend to accumulate more Tl than others (Tremel et al. 1997a), with kale having a BC of 80 in spiked soils, achieving a maximum concentration of 326 mg kg^{-1} Tl (Pavlíčková et al. 2011) and green cabbage surpassing 500 mg kg^{-1} (Xiao et al. 2004). In natural soils, the Tl hyperaccumulators *Biscutella laevigata* and *Iberis intemedia* have BCs of about 20.2 and 29.4, respectively (LaCoste et al. 1999); however, Wierzbicka et al. (2004) reported negligible amounts of Tl in the tissues of *B. laevigata.* In anthropogenically contaminated soil, *I. intermedia* can have a BC of >100 (LaCoste et al. 2001). In a mine spoil containing an average of 43 mg kg^{-1} Tl Wierzbicka et al. (2004) reported that *Plantago lanceolata* accumulated an average of 65 mg kg^{-1} Tl. In contrast, *Silene vulgaris* accumulated just 6.5 mg kg^{-1} Tl.

Plants access anthropogenic Tl in preference to geogenic Tl, which is poorly available for plant uptake (Al-Najar et al. 2005). *Iberis intermedia* significantly depletes the readily available fraction, which is then gradually replaced by residual fractions (Al-Najar et al. 2003). Importantly, high concentrations of Mo, Cu, Pb, Zn, Ag and As inhibit Tl uptake by plants (Sasmaz and Yaman 2006). Mn-oxides can reduce plant uptake of Tl, even in hyperaccumulating species (Grösslová et al. 2015), because they sorb significant amounts of Tl (Jacobson et al. 2005b). Thallium is taken up and stored by plants primarily as Tl(I) (Krasnodębska-Ostręga et al. 2012), although Sadowska et al. (2016) found traces of Tl(III) in *Sinapis alba.* Thallium appears to be transported primarily in the xylem with relatively little phloem mobility. The highest Tl concentrations are found in the water sinks, namely the leaves and transpiring sections of the stems (Jia et al. 2013).

2.3 Feasibility of Tl Agromining

At current prices, achieving a net profit of US \$1500 ha^{-1} yr^{-1} via Tl agromining would require the phytoextraction of 0.5 kg ha^{-1} yr^{-1}, assuming there is no return from combustion of the biomass. Given a crop of *I. intermedia* producing 10 t ha^{-1} yr^{-1}, a plant concentration of just 50 mg kg^{-1} Tl would be required. Using a bioaccumulation coefficient of 29.4 for geogenic Tl (LaCoste et al. 1999), *I. intermedia* could achieve this concentration on a soil containing just 1.7 mg kg^{-1}. Although there has been no comprehensive soil survey, the literature indicates that such areas should be widespread (e.g. Robinson et al. 2015).

Given the environmental and economic constraints of repeated plantings of crops for phytoextraction (Robinson et al. 2015), viable agromining of Tl is more likely to occur on anthropogenically contaminated areas that are unfit for other agricultural systems. The limited size of these areas may increase production costs, because of reduced economics-of-scale. Therefore, higher plant concentrations would be required, but these should be easily achievable with a hyperaccumulator species such as *I. intermedia*. While there are other common plants, especially members of the Brassicaceae that take up inordinate concentrations of Tl, these are unlikely to be useful for agromining since Tl toxicity appears at concentrations ≈221 mg kg^{-1} in the tissues of non-Tl-hyperaccumulating plants

(Babić et al. 2009). Therefore, there is an imperative need to search for more Tl-hyperaccumulating species, which would be expected to occur in sulfur-rich environments. Further research is also warranted on the processes by which Tl could be recovered from biomass and the possibility of producing valuable plant compounds containing Tl, such as organo-Tl complexes and Tl-nanoparticles.

3 Noble Metals

In contrast to Tl, there are no known hyperaccumulators of Au or the platinum-group metals (otherwise known as precious or noble metals). This is because noble metals are considered 'non-essential' in plant metabolism (Lambers et al. 2008) and have a low abundance and solubility in normal soils (Babula et al. 2010). There is little selective pressure for plants to evolve the hyperaccumulation of noble metals. The noble metal concentration in normal plants growing in mineralized soil is generally on the order of 10 ng g^{-1} (Anderson et al. 1998; Lintern et al. 2013). Considerably more data are available on the concentration of Au in plants relative to the platinum-group metals (PGMs). Few phytoextraction studies have focussed on the PGMs, and these have been predominantly restricted to Pd (Shi et al. 2020). There is, however, compelling evidence for an elevated concentration of noble metals in plants where geochemical conditions increase solubility. Elevated soil solution concentrations of chloride and thiosulfate ions derived through weathering processes, and cyanogenic compounds derived from biological pathways, are able to increase the solubility of Au (and PGMs) leading to a concentration of these metals in soil that is in excess of 'normal' levels. For example, Lintern et al. (2013) reported an Au concentration in *Eucalyptus* sp. leaves as high as 60 ng g^{-1} (dry weight).

Better understanding of the conditions under which the noble metals can be made soluble has led to mechanisms for promoting the solubility of these metals in soil solution. This has created the potential for noble metal phytomining or agromining, based on the principles of induced hyperaccumulation (Anderson 2005). Gold phytoextraction is extensively reported in the literature, with reviews by Wilson-Corral et al. (2012), Sheoran et al. (2013), and Novo et al. (2017). However, published data on Pd phytomining are rare. Several masters theses conducted at Massey University in New Zealand have focused on the subject (Walton 2002; Aquan 2015), the latter of these conducted as part of a G8 Materials Efficiency-funded collaboration between the University of York, University of British Columbia, and Yale University (the Phytocat project; www.phytocat.org). This work sought to induce the accumulation of Pd in plants such that the biomass could be used as a catalyst in chemical reactions (Parker et al. 2014). Palladium uptake was affected using both chemical (induced hyperaccumulation) and genetic mechanisms. Taylor et al. (2014) proposed that Au uptake was mediated by cation transporters in *Arabidopsis* sp. with similar observations apparent for Pd. This study indicated that there is potential to target uptake of PGMs through manipulation of the genetic pathways for metal uptake.

3.1 Lessons Learned from Applied Agromining Studies 1: The Impact of Co-metals on Noble Metal Uptake

The concentration of noble metal that can be induced into plants is proportional to the metal concentration in the growth medium. The Phytocat project, to the best of our knowledge, is the only example of a comprehensive study of PGM uptake by plants. The target Pd concentration in plants for this work was 1000 mg kg^{-1} (dry weight), which necessitated a concentration of metal in soil that was significantly higher than that found in mine wastes (generally <0.1 mg kg^{-1}). After extensive global exploration for a suitable growth medium, pot trials were conducted using gossan rock collected from an area of the Broken Hill district in New South Wales, Australia (Table 1). It is important

Table 1 Select metal concentrations in the Broken Hill gossan

Element	Concentration (mg kg^{-1})	Analytical method
Gold	2.5	INAA
Iridium	7.1	INAA
Osmium	3.8	INAA
Palladium	48	INAA
Platinum	15	INAA
Rhodium	4	INAA
Ruthenium	3	INAA
Copper	13,900	AAS
Nickel	6,350	AAS

INAA—Instrumental Neutron Activation Analysis; AAS—Atomic Absorption Spectroscopy

Fig. 2 Landscape in the Broken Hill region (left) showing a pit mined 100 years ago for Cu minerals (right). The dark coloured pieces of rock are the heavily mineralized gossan

to note that this gossan is not the one that developed above the giant Pb-Zn-Ag lodes in the main part of the district.

Magmatic PGM deposits are generally enriched in sulfide (Ludden 2012), and this has a significant impact on the pH of any ore or waste material that might be used for phytomining (generally a low pH). In the Broken Hill gossan extensive weathering of the original sulfide ultramafic ore body (weathering is required to form a gossan) has converted acid-generating sulfides in the rock into oxides. Therefore, the gossan is a high PGM material that could be suitable for phytomining. The gossan was hand-mined 100 years ago by miners looking for Cu. This has left pieces of gossan lying on the surface over a small area (Fig. 2). Total volume of the gossan is low; the available mass would be less than 1000 tonnes, thus making it uneconomic to a conventional mining operation.

Phytomining pot trials were undertaken at Massey University in 2015 (Aquan 2015). Initial attempts to grow *Brassica juncea* on the gossan failed (due to the high Cu concentration; Table 1); however, it was found that hemp (*Cannabis sativa*) was able to grow. Uptake of metals was induced by applying a dilute solution of sodium cyanide to the surface of the pots 33 days after germination. The final metal concentration in the dry biomass was as follows: Cu 6730 mg kg^{-1}, Ni 184 mg kg^{-1}, Fe 1290 mg kg^{-1}, Au 7.6 mg kg^{-1} and Pd 62.4 mg kg^{-1}.

This Pd concentration was significantly lower than anticipated based on the total metal concentration in the gossan and considerably lower than any economic threshold for phytomining (Wilson-Corral et al. 2012). The most likely reason is the relatively high Cu concentration in the plant. Cyanide induced a considerable increase in the concentration of Cu in the plants and this effectively killed the plants before they could accumulate appreciable Pd (and Au). This process has been described by Anderson et al. (2013) as a 'co-metallic effect', whereby more toxic metals also present in noble metal-rich soils (e.g. Cu and Ag) induce necrosis in plants used for phytomining. In media to be used for gold and PGM phytomining, it is likely that Cu will also be present, and the toxicity of Cu made soluble by the same treatments used to promote noble metal solubility seems to be a key limitation to the successful phytomining of precious metals.

3.2 Lessons Learned from Applied Agromining Studies 2: The Need for a Viable Biomass Processing Technology

Anderson et al. (2013) and Robinson et al. (2015) described how a lack of proven engineering flow sheets to recover precious metals from biomass is a major impediment to large-scale roll out of noble metal agromining or phytomining. Proposed processing techniques are based on the ashing of dry biomass and then wet chemistry (acid dissolution and solvent extraction) for recovery of target metals from the ash. Such techniques are, however, costly and environmentally challenging. For example, a waste stream of acid will require further processing before disposal. Trials in Indonesia have assessed how gold phytomining might be relevant to the sustainable management of artisanal and small scale Au mine waste in developing countries (Krisnayanti et al. 2012, 2016).

Krisnayanti et al. (2016) described the use of a simple smelting technique called the 'borax method' to recover precious metals from plant ash using Ag as a collector metal. The principle of smelting is to melt the metal constituents of a material being smelted. However, the energy required to melt Au is considerable. A flux is therefore used to lower the melting point of metals, and in the borax method the flux is borax ($Na_2B_4O_7{\bullet}10H_2O$). This technique is today being used extensively by artisanal and small-scale gold miners in the Philippines to recover Au from concentrates without the use of mercury. The borax method does rely on a high concentration of Au in the ore to be efficient (generally a concentrate with about 25% Au is smelted). In order to make the borax method effective for plants, Ag can be added to the smelt to act as a collector. The Ag melts and then 'collects' valuable metals in the smelt (Cu, Au, and Ag) to form a bullion product that can be sold for subsequent refining.

During a trial conducted on the island of Lombok (Indonesia) in 2015 the borax technique with Ag as a collector was demonstrated to an artisanal mining community (Krisnayanti et al. 2016). Tobacco was grown on mine waste with 1 mg kg^{-1} Au, treated with NaCN to induce uptake, harvested, sun dried, ashed, and then the ash was smelted (Fig. 3). The trial failed to induce significant Au uptake in the tobacco (final concentration was 1.2 mg kg^{-1}) and this was attributed to the high concentration of Ag induced into the plants (54 mg kg^{-1}). However, the mass of Ag added (31 g) increased to 39 g at the end of the smelt. This increase in mass was assessed to be predominantly Fe and Ag from the plant ash, indicating efficacy of the technique for recovering metals from the biomass.

The results from this work were by no means conclusive but indicated that simple techniques that are acceptable to artisanal mining communities and which use existing equipment that miners are familiar with do exist. This creates new opportunities for first-stage processing options for agromining or phytomining that can operate in remote field areas. The product from such options would not be a final metal, but value could still be attributed, leading to a financial reward for operating farmers/miners that might create sufficient incentive for them to

Fig. 3 Tobacco plants growing on cyanidation artisanal Au mine tailings in Lombok Island, Indonesia (left); borax smelting of the plant ash using an air-acetylene torch (right)

apply the technology. Refining of the bullion of a borax smelt could be readily done in existing facilities around the world.

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Element Case Studies: Manganese

Chuan Wu, Wenhui An, and Shengguo Xue

Abstract

Manganese, one of the important trace elements with different concentrations in living tissues, is also widely used in the metals industry. As an essential micronutrient for plants, taken up under the +2-oxidation state, it is crucial in reactions of some enzymes (malic dehydrogenase, oxalosuccinate decarboxylase, superoxide dismutase), and is an activator of those enzymes involved in the tricarboxylic acid cycle. In soils, Mn is commonly in the form of +4 and +3 valence-state oxides, which may be reduced to the +2 form in some ways, such as by acidifying the soil solution, under waterlogged soil conditions, by heating and drying, and by the activity of anaerobic and aerobic micro-organisms. Manganese can be taken up in high concentrations by plants growing in base-rich soils. Manganese hyperaccumulator plants have been defined by a threshold foliar concentration of over 10 000 μg g^{-1} dry weight (DW). Manganese toxicity in plants can cause stunting, chlorosis, curled leaves, or brown lesions, as well as inhibiting photosynthesis and respiration. Several elements such as P and Ca are reported to have important impacts on the uptake and accumulation of Mn by plants. Manganese may be stored in vacuoles, cell walls, the Golgi apparatus, and chloroplast lamellae, and can form black agglomerations in plant cells. In this review, Mn hyperaccumulator biomass disposal and ecological restoration of Mn tailings wastelands are also discussed.

1 Introduction

Metal mining is a major source of Mn contamination in soils (Singh et al. 2005). Pollution associated with mining operations and related tailings production may cause both direct and indirect economic losses each year (Liu and Shu 2003). Extractable Mn concentrations reported for non-polluted agricultural soils worldwide can reach 30.6 μg g^{-1} (Wang et al. 2008), 37.6–38.5 μg g^{-1} (Peris et al. 2007), or 58.5 μg g^{-1} (González and González-Chávez 2006); all of these values are considered to fall within the normal range for soils (<100 μg g^{-1}; Baker 1995). In contrast, studies of contaminated sites have reported known concentrations of Mn to reach 226 μg g^{-1} at a soil-ore crushing area and 550 μg g^{-1} in soil tailings at an abandoned mine site (Archer and Caldwell 2004), 179 524 μg g^{-1} in tailings of a Mn mine in Guangxi, China, and 289–1356 μg g^{-1} in heavily contaminated tailings (Li et al. 2007; Rivera-Becerril et al. 2013).

C. Wu · W. An · S. Xue (✉)
School of Metallurgy and Environment,
Central South University, Changsha, Hunan,
People's Republic of China
e-mail: sgxue@csu.edu.cn

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_21

Moreover, Boudissa et al. (2006) showed that in the vicinity of a Mn alloy production plant, mean concentrations of Mn in the soils were up to 283 000 μg g^{-1} because of direct deposition of the solid Mn-rich residue and atmospheric erosion of particles. Normal concentrations of Mn in plant tissues range from 15 to 150 μg g^{-1} (Reimann and Caritat 1998; Nagajyoti et al. 2010). Accumulation of high concentrations of Mn leads to a decrease in photosynthetic rates in leaves, brown spotting (also petioles and stems) over time that can increase in number and size, and necrotic lesions and even death of the plant with roots commonly becoming brown (Nagajyoti et al. 2010).

There is ample literature on the rehabilitation of Mn-contaminated mine lands (Xue 2002; Zhang et al. 2004; Yang et al. 2006). Paschke et al. (2005) provided a Mn toxicity threshold for grasses commonly applied in restoration, including redtop, slender wheatgrass, tufted hairgrass, big bluegrass, basin wildrye, and common wheat. It is necessary and important for the establishment of vegetation to stabilize a barren area and reduce environmental pollution (Krämer et al. 2000; Wong 2003). Phytostabilization for stabilizing toxic mine spoils and phytoextraction used for the removal of toxic metals from the spoils are widely used in remediation work often with some soil amendments added to aid the stabilization of mine spoils or to enhance metal uptake (Wong 2003). Despite poor physical structure and nutrient deficiencies in the local soil, the Mn mine waste site at Molango in Hidalgo State, Mexico, has been colonized by at least five plant species, which have tissues that accumulate Mn at normal levels (Fernando and Lynch 2015). Moreover, in Guangxi Province, South China, Li et al. (2007) used the local pioneer grass species *Digitaria sanguinalis*, *Imperata cylindrica* var. *major*, and *Erigeron canadensis* to colonize the Mn mine wasteland, combining the fast-growing indigenous species *Castanea henryi*, *Pinus massoniana*, and *Phytolacca acinosa*, in order to remedy and restore some functional uses of the wasteland (Li et al. 2007).

2 Manganese Hyperaccumulator Plants

Baker and Brooks (1989) defined Mn-hyperaccumulator plants by a threshold foliar concentration exceeding 10 000 μg g^{-1} DW. Currently, there are some 30 Mn-hyperaccumulator plants reported in the literature, including nine species listed by Baker et al. (2000). The first Mn-hyperaccumulator identified from PR China, *Phytolacca americana* (Phytolaccaceae), was reported by Xue (Figs. 1 and 2; Xue et al. 2003, 2004, 2008, 2010). Additional native Mn-hyperaccumulating plants have been discovered from other Asia-Pacific regions, such as *Polygonum pubescens* (Polygonaceae) (Hua et al. 2009), *Polygonum hydropiper* (Polygonaceae) (Ren et al. 2007), and *Chengiopanax sciadophylloides*. (Araliaceae). These include seven endemic species from New Caledonia reported nearly three decades ago (Jaffré 1977, 1980) but there have been limited follow-up studies. Furthermore, *Gossia bidwillii* (Myrtaceae) and *Phytolacca acinosa* (Phytolaccaceae) have been reported to be Mn-hyperaccumulators from Australia and China, respectively (Bidwell et al. 2002; Xue et al. 2004). Most recently, Xue et al. (2016) found that Mn in the leaves of *Chenopodium ambrosioides* in solution culture can reach the highest concentration of 26 070 μg g^{-1} when the Mn treatment concentration was increased to 8 000 μmol L^{-1}. In addition, Mn concentrations in the leaves of *Polygonum perfoliatum* (Xue et al. 2018) reached 13 138 mg kg^{-1} grown in 500 μmol L^{-1} Mn, whilst the Mn concentration reached its maximum of 41 400 μg g^{-1} in leaves in the 10 000 μmol L^{-1} Mn treatment. The concentrations in leaves of *C. ambrosioides* and *P. perfoliatum* were both higher than in roots. The heterogeneity trait of Mn hyperaccumulation was studied in an herbaceous species, *P. americana*, under greenhouse conditions (Xue et al. 2005), and in the tree *G. bidwillii* under natural conditions (Fernando and Batianoff 2007), both showing that the trait is constitutive in the former, whereas in the latter it was highly

Fig. 1 *Phytolacca americana* growing at Xiangtan manganese tailings (China)

Fig. 2 *Phytolacca americana* growing in South China

heterogeneous when growing on a variety of substrates. Studies have suggested that in Mn mining wastelands, the use of a Mn hyperaccumulator could be an effective measure for restoring the degraded ecosystem (Wang et al. 2018a, b). This field experiment showed that compared with a tailings site, the plant species diversity of a rehabilitation site was greater, and that the plant abundance of an external soil site was similar to that of the rehabilitation site. A rehabilitation scheme with hyperaccumulators could be recommended as the restoration model in Mn tailings wastelands (Wang et al. 2018a, b).

The Mn hyperaccumulator *C. sciadophylloides* inhabits temperate forested regions of Japan and can accumulate Mn concentrations up to 23 000 μg g^{-1} DW in shoots. These studies also showed that half of the Mn in leaves of *C. sciadophylloides* could easily be extracted with water, and almost all of the Mn could be extracted with hydrochloric acid (HCl). When the pH was adjusted to between 8 and 10, a highly purified Mn compound precipitated from solutions of the leaf ash. This study showed that the collection of Mn from *C. sciadophylloides* is relatively straightforward, making the plant a potential candidate for Mn phytomining.

The perennial herb *P. acinosa* that occurs in southern China was found to be a new Mn hyperaccumulator by means of field surveys on Mn-rich soils and in glasshouse experiments (Xue et al. 2004, 2008). Maximum Mn concentration in the leaf dry matter of plants living at the Xiangtan manganese tailings was 19 300 μg g^{-1}; the reported average concentration was 14 480 μg g^{-1} (Xue et al. 2004, 2008). Under nutrient solution culture conditions, *P. americana* can grow normally with Mn supplied at a concentration of 800 μmol L^{-1}, although with less biomass than in a control; the Mn concentration in leaves can reach its highest value (36 380 μg g^{-1}) at a Mn supply concentration of 12 000 μmol L^{-1}. This result gives a plant resource for exploring the Mn hyperaccumulation mechanism, and has the potential for application in phytoextraction of Mn-contaminated soils, and hence phytomining of Mn (Xue et al. 2004, 2008). The absorption and accumulation of Mn in the two comparative populations have been studied, including a population (MP) from Xiangtan Mn tailings and another (NMP) from a *Magnolia grandiflora* plantation having lower Mn status. All results to date indicate that the high tolerance and hyperaccumulation of Mn in *P. americana* are constitutive properties of the species (Xue et al. 2005, 2008).

To date, most Mn-hyperaccumulator plants have been found in New Caledonia and Queensland, Australia, and little is known about their ecophysiology. Fernando et al. (2008) reported on natural populations of one such species, the endemic shrub *Maytenus fournieri*. The average foliar Mn concentrations of two populations grown on ultramafic substrates having different soil pH values were very different from the average foliar Mn concentrations. Energy dispersive spectroscopy and scanning electron microscopy (SEM-EDS) analysis showed that Mn mainly occurred within epidermal tissues. This discovery, that most of the foliar Mn is highly concentrated in non-photosynthetic tissues, is similar to the research results obtained for other woody plants (Fernando et al. 2008).

Phytolacca americana (pokeweed) was found to be a new Mn-hyperaccumulator plant based on field surveys of Mn-rich soils and by hydroponic experiments. The maximum Mn concentration in leaf dry matter is 8 000 μg g^{-1} on Xiangtan Mn mine tailings, showing a high transfer factor (>10.8). Under hydroponic conditions, Mn concentrations increase with increasing Mn supply, reaching a maximum concentration of Mn in leaves of 47.1 μg g^{-1} at 50 mol L^{-1} external Mn supply (Yuan et al. 2007). Moreover, EDTA treatment was found to significantly inhibit root and shoot biomass and chlorophyll production of *P. hydropiper* and in soybean, whereas chlorophyll production of *P. americana* was the opposite, being clearly promoted by EDTA treatment (Yang et al. 2013). With or without EDTA treatment, the Mn concentrations in shoots of the two plants were significantly lower than the nominal threshold, indicating that these two plants only had hyperaccumulating ability when growing in the soils studied.

It has been found that *Schima superba* (Theaceae), a subtropical tree species occurring in the wasteland of a Mn mine in China, contained abnormally high concentrations of Mn in leaf tissue (Yang et al. 2008). A pot experiment showed that Mn in its tissues increased significantly with increasing Mn supply, and peaked (62 410 μg g^{-1}) in stems using a 150 mol L^{-1} treatment. The Mn concentrations in the aboveground parts of the plants in all treatments were higher than those in the roots. Most of the Mn was removed from the substrate to the upper parts; for example, 86% in the 150 mol L^{-1} treatment, suggesting that *S. superba* is probably a Mn hyperaccumulator. Liu et al. (2018) reported that *Celosia argentea* Linn. can hyperaccumulate Mn, with a maximum Mn concentration in leaves of 29 000 µg g^{-1}. Moreover, the transfer and bioaccumulation factors of Mn were >1, which showed that *C. argentea* has potential for Mn phytoextraction.

Some studies have shown that amendments can enhance the phytoremediation potential of Mn hyperaccumulators. Tang et al. (2020) suggested that the bacterial strain of WSE01 has potential to be used as a biocontrol and/or biofertilizing agent for application in the aquatic macrophyte *Myriophyllum verticillatum* and can assist in more effective phytoremediation of Mn-contaminated waters. *Enterobacter* sp. FM-1 is a potent bioaugmentation agent that facilitates Mn phytoextraction in *Polygonum hydropiper* and *P. lapathifolium* (Li et al. 2020).

Li et al. (2019a) investigated an enhanced phytoremediation technique for Mn-contaminated mine tailings by *Solanum nigrum* using biochar/attapulgite as soil amendments. In their greenhouse experiment, they showed increased removal rates of metals after the application, for Mn by 19.1%. In the field, the optimized combination of plant, microorganism, and amendment could enhance the effectiveness of phytoremediation for heavy metals such as Mn (Li et al. 2019b).

Yang et al. (2018) investigated the phytoremediation potential of two hyperaccumulator plants, *Phytolacca americana* and *Polygonum hydropiper*, on Mn-contaminated paddy soils using citric acid amendments. The mechanisms of citric acid treatment on Mn uptake by these two plants were analyzed. It was shown that the biomass of the two plants was both promoted under low-level application of citric acid, whilst root activity was also enhanced at low levels of citric acid. Under the low and intermediate levels of citric acid application, SOD, POD, and CAT activities increased sharply, which made the level of H_2O_2 very similar to that of the control, thus ensuring health of the plants. It was suggested that *P. hydropiper* in particular has potential for use in the phytoremediation of soil contaminated with relatively low levels of Mn.

A study by Nayak et al. (2018) isolated, characterized, and assessed the potential of rhizospheric bacteria to enhance growth and metal accumulation by the grass *Vetiveria zizanoides*. *Bacillus cereus* (strain T1B3) exhibited plant growth-promoting traits, and a removal capacity (mg L^{-1}) of 67% for Mn (50) during the active growth cycle in heavy metals-amended soils. Results indicated that inoculating the native *V. zizanioides* with T1B3 improved its phytoremediation efficiency.

A consortium of fungal isolates increased the shoot and root biomass of *Jacaranda mimosifolia* and improved the translocation potential of Mn (TF 2.93) from roots to shoots. This method also increased the total accumulated mass of Mn in the shoots and roots. Biochar applications improved Mn and Zn translocation and increased the total accumulated mass of shoot Mn. A photochemical profile, based on chlorophyll a fluorescence, showed that *J. mimosifolia* was tolerant to the contaminated soil and is a potential phytoremediator of Cu, Mn and Zn (Farias et al. 2020).

3 Manganese Toxicity in Plants

Excessive Mn exposure generally has direct cytotoxic effects on plants, such as inhibition of absorption and activity of Ca, Fe, and Mg, induction of oxidative stress, reduction of chlorophyll contents and therefore destruction of chloroplast ultrastructure, reduction of photosynthetic rate, and even plant death (Xue et al. 2018). Manganese is involved in some key plant physiological functions such as photosynthesis, redox processes, and mitigation of damage from reactive oxygen (ROS) (Graham et al. 1988). However, excessive cellular concentrations of Mn can cause oxidative destruction, hence Mn uptake, transport, and distribution within the plant must be strongly controlled (Ducic and Polle 2005). Elevated Mn^{2+} concentrations in tissues also drive toxic effects on photosynthesis and antioxidant enzyme activity.

Manganese toxicity is often the result of competitive interference with P, Ca, and Fe metabolism (González et al. 1998; St Clair and Lynch 2004; St Clair et al. 2005). Excessive Mn can cause phytotoxicity in plants (Demirevska-Kepova and Feller 2004); high concentrations will decrease plant growth and interrupt metabolic processes (Shi et al. 2006). Weng et al. (2013) also showed that Mn toxicity led to stunted growth, chlorosis, crinkled leaves, and brown lesions (speckles), as well as inhibited photosynthesis and respiration. The main effect of Mn toxicity in plants is the induction of oxidative stress. This effect is due to direct electron transfer in single electron reactions and reactive oxygen species (ROS) formation leading to increased metabolic disorders, and to deactivation and down-regulation of the enzymatic antioxidant defence system (Demirevska-Kepova and Feller 2004; Shi et al. 2006).

Fernando and Lynch (2015) suggested that there are two main conflicting theories for the mechanisms of Mn phytotoxicity and tolerance. One theory is that Mn toxicity occurs primarily in the leaf apoplast, whereas the other proposes that Mn toxicity develops through the disruption of photosynthetic electron flow in chloroplasts. The first theory is supported by the fact that the black spots on leaves are the most important indications of Mn phytotoxicity (Horst and Marschner 1978; Wissemeier and Horst 1992; Fecht-Christoffers and Horst 2006). However, the experiments carried out by these workers were mostly conducted in a low-light greenhouse and not in the natural environment, and there was no analysis of symplastic components. Another series of investigations have been performed under 'real' climatic conditions (González et al. 1998; González and Lynch 1999; St Clair and Lynch 2004, 2005; St Clair et al. 2005). In these studies, it was shown that Mn phytotoxicity is mediated via the effects of antioxidative enzymes such as ascorbate peroxidase and glutathione reductase (González et al. 1998; St Clair and Lynch 2004; St Clair et al. 2005). Chlorosis was recognized by Horiguchi (1988) as a consistent and notable symptom of Mn toxicity (González et al. 1998). These differing results may reflect the different light regimes used in the experiments, with the later studies being carried out under near-natural light conditions that represent the main driver of oxidative stress (Fernando and Lynch 2015). As well as exacerbating Mn toxicity by photo-oxidative stress, sunlight can also enhance foliar Mn accumulation (Fernando et al. 2009), as does acid rain that can change soil chemistry and increase Mn bioavailability in the soil (St Clair and Lynch 2005). The absorption and utilization of Mn by two distinct populations of *P. acinosa* have been studied by Xue et al. (2005). Their results showed that the high tolerance and extreme Mn accumulation ability by *P. acinosa* are constitutive properties. The effects of light, temperature, precipitation, and other climatic variables on the availability and toxicity of Mn have been discussed by Fernando and Lynch (2015).

Plants have developed a variety of mechanisms in response to Mn toxicity such as compartmentation, chelation, avoidance and exclusion, antioxidant production, and ionic by interactions. For example, a plant can remove metal ions from the cell or bind it to the inner cell compartment (Kim et al. 2004). In the work by Fernando et al. (2013), *G. bidwillii* chelated Mn and then accumulated it in leaves and stems, thereby reducing Mn toxicity, as also shown in the chelation in *P. perfoliatum* as a Mn-tolerant plant (Xue et al. 2018). In addition, some plants can also secrete organic acids, which assist in the detoxification of Mn (internally and externally) (Xue et al. 2018). For example, during a relatively low Mn addition, *Phytolacca acinosa* could exude and transport organic acids that enhanced tissue tolerance to Mn (Xue et al. 2004). Fourier transform infrared (FTIR) spectroscopy analysis showed that the absorption bands of *P. americana* differ greatly in carbohydrate and protein, mainly due to the exudation and transport of organic matter (Ren et al. 2008). This finding is consistent with results of more recent research by Xue et al. (2018), showing that low concentrations of Mn stimulated plants to produce organic acids and other exudates to overcome Mn toxicity. Release of Mn^{2+} from soil is important for the formation of Mn-oxides and for determining the bioavailability of Mn^{2+} in acidic soils. The ability of organic acids to promote Mn^{2+} release follows the following sequence: citric > tartaric > malic > lactic acid (Yang et al. 2011). In addition, the conversion of Mn to a metabolically inactive clathrate by the Mn-oxalate complex is a key detoxification mechanism (Dou et al. 2009). Manganese can also be separated into intracellular compartments, which is one of the main mechanisms of Mn tolerance (Xu et al. 2015). For example, Xue et al. (2018) showed that an over-supply of Mn increased soil cation exchange capacity by the demethylation of pectin in cell walls, and so increased Mn tolerance.

Another Mn tolerance mechanism results from its interactions with other elements, especially P, Ca and Fe (Esteban et al. 2013). Dučić et al. (2012) showed that the accumulation of Mn in epidermal cells indicates that the root endodermis hinders the transport of Mn and protects normal physiological processes in cells of *P. amelioration*. Moreover, Xue et al. (2018) demonstrated that the P content was high in acicular substances, and that P concentration and its precipitation with Mn may reduce the biological activity of Mn, which is the main mechanism recognized in phytoremediation studies of heavy metals (Hauck et al. 2003; Kochian et al. 2004).

4 Chemical Forms of Manganese in Plants and Its Interaction with Other Metals

An elevated Mn supply can also impose stress from competitive interference of P, Ca, Mg, K and Fe (St Clair and Lynch 2005; St Clair et al. 2005). Researchers have demonstrated that elements including Ca, Si, Fe and P exert a significant effect on the accumulation of Mn in plants. Among these elements, Ca is now considered the ‘hottest topic’ (Mukhopadhyay and Sharma 1991; Alam et al. 2006; Juice et al. 2006). However, Dou et al. (2009) have suggested that Ca oxalate crystals in the Mn-hyperaccumulator plant *Phytolacca americana* lack direct effects on the detoxification of Mn. Moreover, the presence of P and O in the Mn-containing crystals indicates that excess Mn could be deposited as phosphates, which may contribute to Mn accumulation and detoxification in this species. Xu et al. (2009) demonstrated that sufficient oxalic acid exists in *P. acinosa* to complex Mn. X-ray absorption spectroscopy data verify the existence of bivalent Mn in plant leaves, a large portion (90%) of which is Mn oxalate. Furthermore, oxalate biosynthesis was unaffected by Mn concentration because the density of oxalate crystals in the leaves of *P. acinosa* was unaltered when solution Mn was increased in this hydroponic experiment. Phosphate also has a significant impact on the tolerance of plants to heavy metals (Kochian et al. 2004). Much research has been devoted to the role that phosphate plays on Mn and other heavy metal immobilization by precipitation reactions (Hauck et al. 2003; Shao and Sun 2007). Using Energy-Dispersive X-ray (EDX) analysis, the density of phosphate accumulations in acicular substances was determined to be from 7.92 to 11.55% (Xue et al. 2016), which may have been caused by precipitation of Mn by phosphate.

It has been shown that oxidation caused by Mn can make changes at the cellular level in the plants (Najeeb et al. 2009), including variation of chloroplast swelling, cell size and shape, disruption of thylakoid membranes, and plastoglobuli augmentation. However, *Juncus effusus* showed a high degree of resistance to Mn toxicity with the chelates, which enhanced removal of Mn from the hydroponic solutions. We also studied the role of citric acid and EDTA on improving metal accumulation, plant growth, and Mn toxicity stress alleviation. Furthermore, EDTA can effectively promote the assimilation and translocation of Mn, and plant growth can be stimulated by citric acid application.

Shi and Zhu (2008) studied the influence of salicylic acid (SA) on the toxicity of Mn in cucumber plants (*Cucumis sativus*). Excess Mn caused serious growth inhibition, effectively promoting Mn accumulation in shoots and roots, and in addition, hindered the assimilation of Ca, Mg and Zn. However, additional amounts of SA reduced Mn translocation from roots to shoots and enhanced the inhibition effects of Ca, Mg and Zn.

5 Localization of Manganese in Plants

Compartmentation, exclusion, and detoxification mechanisms may all be involved in plant tolerance to excess Mn (Fernando and Lynch 2015). Compartmentation of heavy metals can reduce the damage to plant components such as cells, tissues, and organelles, making localization of heavy metals and plant ultrastructural analysis important for understanding these mechanisms (Weng et al. 2013; Rizwan et al. 2015; Wu et al. 2016, 2017). When plants are under stress due to exposure to toxic metals, changes in plant ultrastructure are the causes of irregular plant physiological effects (Xue et al. 2004; Keller et al. 2015). The cytoplasm is acknowledged as the site for cell metabolism. Damage to cytoplasm makes it shrink and disappear, producing vacuoles, and then metabolism ceases leading to cellular death (Xue et al. 2004; Fernando et al. 2006a, b).

Manganese is an important micronutrient for chloroplasts, as well as being involved in structural construction of various photosynthetic enzymes. However, excess Mn can cause serious damage to chloroplasts (Fernando et al. 2006a; Xue et al. 2016, 2018). Fernando et al. (2006a, b) showed that foliar Mn in *G. bidwillii* was primary localized in photosynthetic tissues, whereas in other studies the highest foliar Mn occurred in non-photosynthetic tissues as in *P. acinosa* (Robinson and McGrath 2003; Bidwell et al. 2004; Xu et al. 2006). Excess Mn may reduce the amount of Fe and Mg, and thus reduce the chlorophyll concentration in plants, because Fe plays an important role in chlorophyll biosynthesis (Shi et al. 2006). Moreover, excess Mn can also damage chloroplast structure, leading to a reduction in chlorophyll biosynthesis with a resulting decrease in photosynthetic rates (Shi et al. 2006). For example, Mn concentrations >3 000 μmol L^{-1} induced damage to the chloroplasts of pea plants and a change in the shape of thylakoids (twisted) (Doncheva et al. 2005). In addition, Dou et al. (2009) found that the deposits observed around vacuoles, showing a high Mn concentration in *P. americana*, caused damage to chloroplasts. It was also shown that lower concentrations of Mn did not destroy the ultrastructure of *P. americana* chloroplasts when they were exposed to a high Mn stress (12 000 μmol L^{-1}); the chloroplasts shrank, their outer membrane was disrupted, and the thylakoids were swollen, so that the chloroplasts became noticeably hollowed (Liang et al. 2011).

Doncheva et al. (2005) claimed that when the amount of Mn was increased, pea (*Pisum sativum* cv. 'Citrine') chloroplasts would be damaged, thylakoids would twist, and starch grains would mostly increase in terms of number and size. Moreover, this situation also led to a reduction in osmiophilic globules (Xue et al. 2016). However, a different study showed in contrast that with an increase in Mn concentrations, *P. americana* starch grains reduced in number and became smaller, whereas the number of osmiophilic globules increased (Liang et al. 2011). The differences between plant species may cause this effect; *C. ambrosioides* behaved differently to *P. americana*, as it lacked the detoxification mechanism.

One Mn localization study in *Gossia bidwillii* (Myrtaceae) by Fernando et al. (2006b) found that different parts of the plant had different Mn concentrations, with the greatest Mn density being located in the upper-layer palisade mesophyll and the lowest in mesophyll cells. According to Xu et al. (2006), Mn preferentially accumulates in the marginal part of the leaf of the hyperaccumulator plant *P. acinosa*. Moreover, based on a cross-sectional study of the leaf, the epidermis has higher Mn concentrations than the mesophyll. This distribution pattern was adjusted by transpiration rate. Manganese does not move in the plant after it has been transported from the roots to shoots of *P. acinosa*. Moreover, discovery of new Mn hyperaccumulators has led to renewed interest in the New Caledonian flora, and to Mn localization studies on *Virotia neurophylla* (Proteaceae), an endemic Mnhyperaccumuating species (Fernando et al. 2012). As in *G. bidwillii*, studies have found unusual spatial distributions of foliar Mn (Fernando et al. 2006b), with primary sequestration of Mn occurring within the palisade mesophyll cells.

Plants can evolve mechanisms to prevent toxicity from a metal, including efflux of metal ions from cells and sequestration of these ions into internal cellular compartments (Kim et al. 2004). Manganese can be stored in vacuoles, chloroplast lamellae structures, Golgi apparatus, and cell walls (Ren et al. 2007; Sytar et al. 2013). It was found that Mn accumulation in the cell walls and vacuole of leaf cells in *Polygonum hydropiper* might be one Mn tolerance and hyperaccumulation mechanisms (Wang et al. 2008). In addition, Xue et al. (2016) showed some structural variation in the leaf cells of *C. ambrosioides*, e.g., the formation of black agglomerations. Other researchers have also noted these black agglomerations in leaf cells (Doncheva et al. 2005; Papadakis et al. 2007; Dou et al. 2009), and those found in *P. americana* were actually Mn-oxides (Dou et al. 2009). By comparison with untreated controls, black agglomerations were also noted in *Polygonum perfoliatum* cells in Mn concentration treatments of 1000 and 10 000 μmol L^{-1} (Xue et al. 2018). The additional Mn-oxides result in brown spots and chlorosis on leaves (Liu et al. 2010). Chlorosis was found in the leaves of *C. ambrosioides*, which may due to high concentrations of Mn; the black agglomerations identified in this research may be Mn-oxides (Xue et al. 2016). Moreover, at 10 000 μmol L^{-1}, Mn was found to remain as acicular structures in cells and intercellular spaces of the leaves in both *C. ambrosioides* and *P. perfoliatum*. An energy spectrum study revealed that in acicular crystals, the concentration of Mn is much higher than in other locations, showing the tolerance and accumulation of Mn (Xue et al. 2016, 2018).

Pan et al. (2019) studied Mn subcellular distribution and chemical forms, as well as microstructure responses, of the mine ecotype (ME) and non-mine ecotype (NME) of *Xanthium strumarium*. Their work showed that >83% of Mn was localized in the cell wall and in soluble fractions, and that the Mn concentration in all fractions in the ME had a direct linear relationship with Mn treatment. The proportions of pectates and protein-integrated-Mn, phosphate-Mn, and oxalate-Mn forms were dominant in the leaves and stems of the ME, whereas in the NME relative proportions of inorganic Mn and water-soluble Mn forms in the roots were higher than in the other forms. Together, the combination of preferential distribution of Mn in the cell wall and soluble fractions, and storage of Mn in low-toxicity forms (such as phosphate-Mn, pectates, protein-bound Mn, and oxalate-Mn) might be responsible for alleviating Mn toxicity in the ME.

6 Mn Hyperaccumulator Biomass Disposal and Applications

Some Mn hyperaccumulators can grow rapidly with high biomass production for the remediation of Mn in contaminated industrial and mining areas. Moreover, some can even accumulate Mn above 10 000 μg g^{-1} on a biomass basis (Reeves and Baker 2000). For example, *P. acinosa* accumulated 19 300 μg g^{-1} (Xue et al. 2004) and *P. americana* was found to accumulate 8 000 mg kg^{-1} (Yuan et al. 2007) in different Mn mining areas in China. Improper disposal of this biomass could cause adverse impacts on human health and the environment (Abhilash and Yunus 2011). Previous studies have demonstrated several approaches for the proper disposal of heavy metal hyperaccumulator biomass, including direct disposal as hazardous waste, chemical extraction, composting, gasification, and pyrolysis (Ghosh and Singh 2005).

Direct biomass disposal to landfill is a problematic method and can cause secondary pollution by releasing the immobilized contaminants (Chalot et al. 2012). Herndon (2012) showed that plants take up soluble Mn^{2+} from the soil and store it as aqueous and organic Mn^{2+} compounds in biomass, which are then transformed into insoluble $Mn^{3+/4+}$ oxides during the decomposition process in soil. Handling large quantities of Mn-containing biomass is a problem for the environment and hence volume reduction is necessary. There are no reports available on the appropriate treatment and disposal of Mn hyperaccumulator biomass or on any domestic use, and therefore additional experimentation and validation are required to address this problem. Gasification is the most important route for the reduction of waste biomass and for the generation of electrical and thermal energy (Devi et al. 2003). This potential method for the recovery of energy from biomass by burning could help in disposing of biomass that has accumulated heavy metals and thus make phytoremediation more cost effective (Ghosh and Singh 2005). This combustion process produces valuable gaseous materials with high concentrations of H_2, CO and ash containing other toxic metals. However, utilization of these gaseous materials can cause additional environmental problems (Salo and Mojtahedi 1998; Kovacs and Szemmelveisz 2017).

Pyrolysis is a novel method for the disposal of heavy-metal contaminated biomass (Stals et al. 2010; Kovacs and Szemmelveisz 2017). The final products of pyrolysis can be divided into fluid oil, solid fractions, and gaseous phases (Lievens et al. 2008; Stals et al. 2010). During the process, organic matter of the biomass is destroyed, and immobilized heavy metals and stable carbon-enriched biochar are produced (Lievens et al. 2008; Kikuchi and Tanaka, 2012). Koppolua et al. (2003) reported that 99% of heavy metals are removed from the biochar produced from hyperaccumulator biomass by pyrolysis in a pilot-scale reactor. More recently, Wang et al. (2017) demonstrated that 91–99% non-volatile metal condensed biochar could be obtained from biomass of the Mn hyperaccumulator *P. acinosa* by a slow pyrolysis process. This biochar has an extraordinary capacity to remove heavy metals (Ag, Pb, Cu and Cd) from aqueous solutions. Pyrolysis can therefore be a very suitable technique for the disposal of Mn-hyperaccumulator biomass and has additional environmental applications.

Hyperaccumulator plants may be used for phytomining at contaminated mining sites (Baker and Brooks 1989; Nicks and Chambers 1998), to recover metals from plant biomass such as Ni (Zhang et al. 2014), Co (Boominathan et al. 2004), Au (Itouga et al. 2010), and even Mn for further industrial use. This approach is a novel, low-cost, and eco-friendly technology (Brooks et al. 1998). Manganese phytomining technology may provide high-quality Mn such as water-soluble Mn compounds, which are essential for steel production. Manganese phytomining with *Macadamia neurophylla* (a Mn hyperaccumulator) has been reported by Brooks (1997). Similarly, *Chengiopanax sciadophylloides* (also a Mn hyperaccumulator) has been used by Mizuno et al. for Mn phytomining. Phytomining technology has been established at the field-scale level in some countries (Robinson et al. 1997; Li et al. 2003; Bani et al. 2015) and is discussed in other chapters in this book.

7 Ecological Restoration in Manganese Tailings Wastelands

In the past few decades, different means have been tested for restoring Mn tailings wastelands with different substrate types, including amelioration with soil amendments and appropriate selection of plant species. Wang et al. (2018b) evaluated the plant species diversity on several sites having received three different treatment approaches: exposed tailings alone (ET), external soil amelioration (ES), and external soil amelioration + seeding propagation with the grass *Cynodon dactylon* (EC) (Table 1). Their records indicated that 29 species from 14 families were observed at the three sites after three years duration. Compared to the untreated tailings site, plant species diversity on the rehabilitation site was more than on the tailings site, and the plant abundance of the external soil site was similar to that of the rehabilitation site. Their study further showed that for these degraded ecosystems on Mn tailing wastelands, a man-made ecosystem with good function could be produced in a short time period using a combination of restoration of the pioneer plants by seeding, soil covering, and soil improvement. Large quantities of Mn and associated heavy metals have been produced by Mn mining, smelting, and other processes, with subsequent weathering and leaching of waste slags that can promote Mn entering surface run-off by different means, under the action of precipitation. Wang et al. (2018a) investigated the characteristics of excessive nutrient pollutants and heavy metals by using the method of bounded run-off plots on the Mn tailings wastelands after carrying out treatments, using three different approaches. The research showed that the maximum run-off occurred in rainfall events during July 2014, and that the basic order of run-off was EC > ET > ES area for the same rainfall event (Table 2). The concentration of total suspended solids (TSS) and chemical oxygen demand (COD) for the three ecological restoration areas was in the following order: ET > EC > ES area. Concentrations of Cr, Ni, Pb, Zn, Mn and Cu in surface run-off from the three restoration types all exceeded the Class V level of the Government environmental quality standard for surface water except for Cu in the EC and ES areas. The concentrations of heavy metal pollutants in surface run-off from the three restoration areas could be ranked as follows: ET > EC > ES area. This result suggested that the combination of external soil amelioration with *C. dactylon* seeding is a promising model for ecological restoration of the Mn tailings wastelands. These studies hence provide some theoretical basis for the assessment and treatment of surface run-off pollution of the metal tailings wastelands.

Table 1 Investigated plant species for three sites of manganese tailing wasteland in 2014

Family	Genus	Species	PFGs	Area
Poaceae	*Cynodon*	*Cynodon dactylon* (Linn.) Pers.	PG	Tailings site/external-soil site/rehabilitation site
	Pennisetum	*Pennisetum alopecuroides* (Linn.) Spreng.	PG	External-soil site/rehabilitation site
	Eleusine	*Eleusine indica* (Linn.) Gaertn.	PG	Tailings site
	Setaria	*Setaria viridis* (Linn.) Beauv.	AB	External-soil site
	Arundo	*Arundo donax* Linn.	PG	Tailings site/rehabilitation site
	Imperata	*Imperata cylindrica* (Linn.) Beauv.	PG	Rehabilitation site
Asteraceae	*Erigeron*	*Erigeron annuus* (Linn.) Pers.	AB	Tailings site/external-soil site/rehabilitation site
	Artemisia	*Artemisia argyi* Levl. Et Vant.	PG	External-soil site/rehabilitation site
	Ixeris	*Ixeris polycephala* Cass	PG	Rehabilitation site
	Xanthium	*Xanthium sibiricum Patrin* ex Widder	AB	Rehabilitation site
	Chrysanthemum	*Chrysanthemum coronarium* Linn.	PG	External-soil site
	Conyza	*Conyza canadensis* (Linn.) Cronq.	AB	Tailings site

(continued)

Table 1 (continued)

Family	Genus	Species	PFGs	Area
Polygonaceae	*Polygonum*	*Polygonum flaccidum* (Meissn.)Steward	AB	Tailings site/external-soil site/rehabilitation site
		Polygonum perfoliatum L.	AB	External-soil site/rehabilitation site
	Chenopodium	*Chenopodium ambrosioides* Linn.	PG	Tailings site/external-soil site/rehabilitation site
		Chenopodium album Linn.	AB	Tailings site
Solanaceae	*Solanum*	*Solanum nigrum* Linn.	AB	External-soil site/rehabilitation site
Apiaceae	*Torilis Adans.*	*Torilis scabra* (Thunb.) DC.	PG	Rehabilitation site
Phytolaecaceae	*Phytolacca*	*Phytolacca Americana* L.	PG	Tailings site/external-soil site/rehabilitation site
Amarantaceae	*Achyranthes*	*Achyranthes aspera* L.	PG	External-soil site/rehabilitation site
Moraceae	*Broussonetia*	*Broussonetia kazinoki* S. et Z.	SS	Rehabilitation site
Fabaceae	*Amorpha*	*Amorpha fruticosa* Linn.	SS	External-soil site/rehabilitation site
Convolvulaceae	*Argyreia*	*Argyreia seguinii* (Levl.) Van. ex Levl.	PG	Rehabilitation site
Rosaceae	*Rosa*	*Rosa multiflora* Thunb.	SS	Rehabilitation site
	Rubus	*Rubus corchorifolius* Linn.f.	SS	External-soil site
	Potentilla	*Potentilla chinensis* Ser.	PG	Tailings site
Verbenaceae	*Clerodendrum*	*Clerodendrum cyrtophyllum* Turcz.	SS	External-soil site/rehabilitation site
Papaveraceae	*Macleaya*	*Macleaya cordata* (Willd.) R. Br.	PG	External-soil site
Commelinaceae	*Commelina*	*Commelina communis* Linn	AB	Tailings site/rehabilitation site

* AB: annual and biennials, PG: perennial grass, including both rhizome grass and bunchgrass, SS: shrubs and semi-shrubs

** Adopted from Environ Sci Pollut Res, 2018, 25:24101–24110

Table 2 Changes of plant species diversity in three sites of manganese tailing wasteland

Area	Time	Species number	Average height (cm)	Average total coverage (%)	Plant growth situation
Tailings site	2012	10	10 ± 1.2^{f}	$<5 \pm 0.7^{e}$	Poor
	2014	11	15 ± 2.3^{e}	$<5 \pm 1.1^{d}$	Poor
External-soil site	2012	14	20 ± 3.4^{d}	35 ± 3.8^{c}	Better
	2014	16	40 ± 5.7^{c}	45 ± 5.3^{b}	Better
Rehabilitation site	2012	21	35 ± 4.6^{b}	95 ± 11.6^{a}	Fine
	2014	29	65 ± 8.1^{a}	98 ± 13.8^{a}	Fine

* Tailings site: exposed tailings, the control treatment; External-soil site: soil covering of 10 cm thickness; Rehabilitation site: soil covering of 10 cm thickness, soil improving (adding fowl dung) and seeding propagation of *Cynodon dactylon*

** Adapted from Environ Sci Pollut Res, 2018, 25:24101–24110

*** Different letters showed significant difference in the same column

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Element Case Studies: Arsenic

Tongbin Chen, Mei Lei, Xiaoming Wan,
Xiaoyong Zhou, Jun Yang, Guanghui Guo,
and Wen Cai

Abstract

Arsenic-contaminated soil is a major issue in PR China. The discovery of an As-hyperaccumulator fern, *Pteris vittata*, opens a door for phytoextraction of As-contaminated soils. In situ phytoextraction projects using *P. vittata* have been established that achieved high removal rates of As. The first phytoextraction project in the world was established in Chenzhou, Hunan Province. Subsequently, more phytoextraction projects were established in Guangxi Zhuang Autonomous Region, Yunnan Province, Henan Province, Hebei Province and Beijing. During these field-based projects, a series of technologies, including the fast reproduction of *P. vittata* sporelings, selection and cultivation of *P. vittata* ecotypes with higher As accumulating ability, and safe disposal and re-utilization of *P. vittata* biomass, were well developed. Incineration is one of the most commonly used safe-disposal technologies for the As-enriched hyperaccumulator biomass. Safe landfilling has been applied for disposal of the burned ash of *P. vittata* when the amount of that ash is small. Agromining of Ni has been successfully achieved, but agromining of As is at present only an idea, owing to the low commercial value of As. Nevertheless, production of a biofuel resulting from the incineration process, together with the recycling of As, could be a potential opportunity for agromining of this metalloid. Extraction and purification of other valuable compounds such as flavonoid in the hyperaccumulator might be another way to increase the economic benefit of As phytoextraction.

1 Introduction

Contamination by heavy metals/metalloids is a major issue in the world (Zucchi et al. 2005; Zimmer et al. 2011). Arsenic is a toxic metalloid, existing ubiquitously in the environment as a result of natural and anthropogenic activities. Chronic exposure to As through drinking water and/or staple foods is a major health concern globally (Eisler 1985; Sturchio et al. 2013). Arsenic has been identified as the most toxic element by the US Agency for Toxic Substances and Disease Register (ATSDR), from a priority list of 275 substances that were classified based on

T. Chen (✉) · M. Lei · X. Wan · X. Zhou ·
J. Yang · G. Guo · W. Cai
Institute of Geographic Sciences and Natural
Resources Research, Chinese Academy of Sciences,
Beijing 100101, China
e-mail: chentb@igsnrr.ac.cn

University of Chinese Academy of Sciences,
Beijing 100089, China

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_22

toxicity and potential for human exposure (ATSDR 2017). Considering the wide distribution and high toxicity of As, many efforts are being devoted to investigating suitable remediation schemes. Phytoextraction has increasingly attracted attention, since it is environmentally friendly, simple in operation, and economically efficient (Chen et al. 2002). In situ phytoextraction projects using the As hyperaccumulator *Pteris vittata* have been established on farmlands and residential areas in PR China and the USA, with high As removal rates recorded (Huang et al. 2007; Ebbs et al. 2010; Wan et al. 2016). Disposal of As-rich biomass harvested by a hyperaccumulator plant has received increasing attention. The potential economic value of heavy metals recovered in the harvests is the basis for the concept of phytomining.

Phytomining employs hyperaccumulator plants to take up metals from the growth medium into harvestable plant biomass. Harvesting, drying, and incineration of the biomass can generate a high-grade bio-ore (van der Ent et al. 2015). Agromining could provide local subsistence-level farming communities with an alternative type of agriculture on degraded lands: farming not only for food crops, but also for metals such as Ni. This technology also enables the extraction of metals from secondary resources (e.g. metal-enriched and contaminated soils, mineral wastes) that are unsuitable for economic mining, and thus for manufacturing high-value products (Zhang et al. 2016). Several ferns have been identified as As hyperaccumulators, including *P. vittata*, *P. cretica*, and more recently *P. melanocaulon* (Claveria et al. 2019; Zemanova et al. 2020). *P vittata* is a fern that has been widely applied to As-contaminated soils. Owing to serious As contamination in parts of China, several phytoextraction projects with a total area of 200 ha using *P. vittata* have been established (Wan et al. 2016). However, the low commercial value of As is one of the main reasons that restricts As phytomining as a viable technology. Recently, the production of a nano-material from the ash of incinerated *P. vittata* biomass, and recovery of biofuel or valuable compounds from the biomass, have provided novel directions for further research.

2 *Pteris vittata* and Its As-Accumulating Characteristics

The As hyperaccumulator *P. vittata* was discovered at the beginning of this century by two research groups, one from China and the other from the USA (Chen et al. 2000; Ma et al. 2001; Chen et al. 2002). *P vittata* has been demonstrated to have extreme tolerance to As. This fern is able to grow on soils having As concentrations as high as 4000 μg g^{-1}, and even on mine tailings with 23 400 μg g^{-1} As (Chen et al. 2002). It also shows an extreme As-accumulating ability. In a pot trial, the above-ground As concentration of *P. vittata* accumulated up to 7526 μg g^{-1} (Ma et al. 2001). The fern can accumulate As from different As compounds, including NaMMA, CaMMA, K_2HAsO_4, Na_2HAsO_4, and $Ca_3(AsO_4)_2$ (Tu and Ma 2002). The distribution of As in *P. vittata* is in the order pinnae > stems > roots; As contents in the pinnae are significantly higher than those in the rest of the tissues (Chen et al. 2002). This property favours the collection of harvests and concentrated As disposal. Arsenic in *P. vittata* resides mainly in the form of inorganic As(V) or As(III), together with small amounts of $FeAsO_4$, $AlAsO_4$, and $Ca_3(AsO_4)_2$ (Tu and Ma 2002; Zhang 2002). The extremely high concentrations of As that may be reached in *P. vittata* and its remarkable biomass produced under cropping conditions suggests that this fern is an appropriate plant for effective phytoextraction and potential agromining of As.

3 Case Studies of As Phytoextraction

In situ phytoextraction projects using *P. vittata* have been established on more than 10 sites in China. The first phytoextraction project in the

Fig. 1 Case study of As phytoextraction in Chenzhou, Hunan Province

world was established in Chenzhou, Hunan Province, in 2001 (Fig. 1). During this project, the effect of adding fertilizer and harvesting frequency on extraction efficiency was studied. CaH_2PO_4 was found to be the most efficient fertilizer, aiming to increase As removal from the soil. By comparing different harvest frequencies, three harvests a year was found to be the optimum schedule. After *P. vittata* had been cultivated for seven months, total As concentration in the soil decreased by 5.0 $\mu g\ g^{-1}$, with a remediation efficiency reaching 7.84% in the most efficient treatment (Liao et al. 2004). Based on the first successful phytoextraction project, additional phytoextraction projects were established in Guangxi Zhuang Autonomous Region, and in Yunnan, Henan, and Hebei provinces, and in the city of Beijing (Table 1).

The phytoextraction project established in Huanjiang, Guangxi Province, was the largest phytoextraction project (82.7 ha) in the world (Fig. 2); phytoextraction, intercropping, and phyto-barrier technologies were all used. The phytoextraction technology using *P. vittata* resulted in an overall annual As removal rate of 10%. After four years, As concentration in the soil met the national standard for farmland soil of 30 mg kg^{-1}. The phyto-barrier technology significantly decreased As concentration in agricultural products, at <5% the above-standard rate. The intercropping technology also reached an above-standard rate of As at <5%. As a result of the Huanjiang project, a new model for the "Government guidance, scientific and technological support, enterprise participation, and the implementation of farmers" was established and promoted for adoption at other sites.

The phytoextraction project in Jiyuan (Henan Province) was the first established in the temperate monsoon climate region of medium latitudes. Previous projects of this type were all carried out in the subtropical monsoon climate zone. *P. vittata* is naturally distributed only in this subtropical monsoon region. To overcome this

Table 1 Phytoextraction projects on As-contaminated soil in PR China

Location	Contaminant	Technology	Remediation efficiency
Chenzhou, Hunan Province	As	Phytoextraction	Through 3–5 year remediation, soil As content decreased from 50 μg g^{-1} to <30 μg g^{-1}, reaching the second national soil quality standard (GB15618-1995)
Shimen, Hunan Province	As	Phytoextraction and intercropping	Phytoextraction technology removed 15.9% As from soil each year In the intercropped system, agricultural products from intercropped cash crops meet the national standard
Huanjiang, Guangxi Zhuang Autonomous Region	As, Cd and Pb	Phytoextraction, intercropping and phytobarrier	Phytoextraction technology removed 10.5% Cd and 28.6% As from soil after 2-year remediation In the intercropped system, the yield of maize, rice and sugarcane increased by 154%, 29.6% and 105%, respectively; and the As, Cd and Pb concentration in corn kernel decreased by 39.0%, 4.1% and 4.9%, respectively In the phytobarrier system, the over standard rate of heavy metals in agricultural products was less than 5%
Gejiu, Yunnan Province	As and Pb	Phytoextraction	Phytoextraction technology removed 23.4 kg As, 11.3 kg Pb, 2.64 kg Cu, and 9.24 kg Zn per ha soil within two years
Huize, Yunnan Province	As	Phytoextraction	Phytoextraction technology removed 12.0% As from soil each year
Jiyuan, Henan Province	As, Cd and Pb	Phytoextraction	Phytoextraction technology removed 13.9% As, 0.5% Pb and 16.1% Cd from soil each year After 2-year remediation, 338 g As, 36.2 g Cd and 104.5 g Pb were removed from each Mu soil
Fangshan, Beijing City	As	Phytoextraction	Phytoextraction technology removed 17.2% As from soil each year
Dabaoshan, Guangdong Province	As, Cd, Cu, Pb and Zn	Intercropping and phytobarrier	Products of cash crops met the national standards
Yangshuo, Guangxi Zhuang Autonomous Region	As, Cd	Phytoextraction, intercropping of *P. vittata* and Cd accumulating *Amaranthus cruentus* L	Annual removal rate of As and Cd reached 10.0%, respectively
Baoding, Hebei Province	As	Phytoextraction	Phytoextraction technology removed 13.0% As each year

limitation, a series of measures was established to assist *P. vittata* survival during cold winters; with a suitable insulating cover material, the winter survival rate of *P. vittata* can reach as much as 45% (Zhang et al. 2017). After a two-year remediation, the removal rate of As from soil was 13.9%, validating the possibility of utilizing As hyperaccumulation in northern China.

Scientists from the University of Florida found an even higher As extraction ratio. After

Fig. 2 Case study of As phytoextraction in Huanjiang, Guangxi Zhuang Autonomous Region

P. vittata had been cultivated for two years, the average concentration of As decreased from 190–150 μg g^{-1}, indicating a removal of 26.3% (Kertulis-Tartar et al. 2006). Such a difference may result from different pollution sources and pollution extents. The As contamination in soil reported by the Kertulis-Tartar et al. study resulted from the use of chromated copper arsenate (CCA), involving very high concentrations of As, whereas projects carried out by our group dealt with farmland soil contaminated by mining and smelting activities, with the average concentration of As in soil being much lower, in the range of 30–50 μg g^{-1}.

During the establishment and duration of these case studies, several funding and manpower enabling steps received focused public attention and publicity, as described below in details.

3.1 Fast Reproduction of *P. vittata* Sporelings

Obtaining an adequate supply of *P. vittata* sporelings is the first vital step in a successful phytoextraction project for clean-up of As-contaminated soil. Being a pteridophyte, *P. vittata* is from an ancient division of the plant kingdom, and as such requires spores to propagate. The minute spores are only tens of microns in size and therefore can store only a very limited amount of nutrients. After germination of the spores, a sexual fertilization process is necessary for the formation of diploid sporophores. These processes have special requirements for both light and moisture. Through a series of studies on germination and fertilization processes, optimized parameters were proposed (Wan et al. 2010). After optimization, the reproduction time was shortened by ~27%. Fast reproduction technologies for *P. vittata* sporelings were established and implemented in all of our large-scale projects.

3.2 Harvesting and Incineration of the Hyperaccumulator Biomass

Pteris vittata is a perennial fern. With the roots kept in soil, the above-ground parts can be harvested several times a year. The effects of harvesting on As accumulation and phytoextraction efficiency of *P. vittata* were studied. Our results showed that repeated harvesting increases both parameters. Through this field practice, 16 kg As, 8.5 kg Pb and 9.2 kg Zn can be removed from one hectare of soil each year by harvesting twice per year (Xie et al. 2010).

The disposal of hyperaccumulator plants enriched with toxic As is an essential step for an As phytoextraction project. Direct disposal to hazardous waste landfill, incineration/ashing, and liquid extraction are possible methods for the disposal of hyperaccumulator biomass. Among these, incineration is here proposed as the most feasible, being an economically acceptable and environmentally sound method (Sas-Nowosielska et al. 2004). Incineration can decrease the volume and weight of the hyperaccumulator biomass by as much as ~90%. However, during incineration, the risk caused by emission of As to the atmosphere deserves special attention. Data obtained on this process by Yan et al. (2008) have shown that 24% of the total As accumulated by *P. vittata*, which contained a high concentration of As (1170 μg g^{-1}), was emitted at 800 °C, and that 62.5% of emitted As (0–800 °C) is volatilized below 400 °C. Based on such empirical results, reagents such as CaO that can immobilize As during the incineration process could be added, in order to reduce emission of As to the atmosphere. Recent study further optimized the incineration parameters by using three *P. vittata* biomass with different As concentrations from three sites in China, based on an incineration equipment with a disposal capacity of 600 kg d^{-1}. Arsenic-fixing agent (main composition being calcium) led to a recovery rate of As by 76% (Lei et al. 2019).

3.3 Establishment of the First National Soil Remediation Industrial Park

The first national soil remediation industrial park was established in Shimen, Hunan Province, China. The Huangchang realgar mine is located in Shimen County, Hunan Province, with a mining history of >1500 years until closure in 2011. The As-enriched wastewater, dust, and waste residues produced during mining and smelting processes at this mine resulted in major As contamination of the local environment, with soil As concentrations reaching 0.5% (w/w). Phytoextraction, intercropping, and phytostabilization technologies have been established there since 2014. Different combinations of technologies were applied to soils with varied contamination extents. For heavily contaminated soils, *P. vittata* was planted, in order to reduce As release through runoff. For moderately contaminated soils, *P. vittata* and low-As-accumulating cash crops were intercropped, to gradually remove As and at the same time produce economic benefits. For slightly contaminated soils, low-As-accumulating maize or rice was planted. Furthermore, a series of supporting equipment was established, including an intelligent production line for sporelings breeding, an automatic disposal equipment for hyperaccumulator biomass, production lines for As mobilizer and immobilizer, and a control centre. This industrial park has an integrated function of science and technology transformation, and science and technology incubation and science popularization, which has become a model for the development of similar soil remediation projects elsewhere.

3.4 Reuse the Biomass of As Hyperaccumulator as Resources

In the early days of the development of phytoextraction, safe disposal of hyperaccumulator biomass was considered unsustainable. However, current practices require the re-use of harvested biomass as a resource, not a waste product. Agromining is proposed as a method to reuse the biomass of hyperaccumulator plants, and has been successfully applied to Ni hyperaccumulators (Echevarria et al. 2015; Kidd et al. 2015; Rosenkranz et al. 2015, and Chapter “Element Case Studies: Nickel” this volume). Nonetheless, the potential for As phytomining is not as promising as that for Ni, owing to the low commercial value of As and its compounds. Recently, some studies have explored a hydrothermal method to convert biomass into biomass fuels having high calorific value (Srokol et al. 2004). In this case, not only valuable Ni was recycled from plants, but also a biofuel was obtained. Carrier et al. (2011) applied supercritical conditions to safely dispose of the biomass of *P. vittata*, transforming the biomass into a liquid fuel.

The preparation of nano-metallic materials from hyperaccumulator plants is another approach to potential resource utilization. This

Table 2 Total flavonoid concentrations in *P. vittata*

Population of *P. vittata*	Tissue	Concentration (%)
GX	Root	6.21
GX	Shoot	6.08
YN	Root	6.93
YN	Shoot	12.05
HN	Root	4.04
HN	Shoot	4.65

approach provides new insights for the recycling of hyperaccumulators and a novel route for further development of green nanostructure syntheses (Qu et al. 2012). At present, scientists from the Chinese Academy of Sciences and Cranfield University, UK, are attempting to enhance the commercial value of harvested *P. vittata* biomass. In addition, evaluation of the potential medicinal properties of this fern is in progress. Total flavonoids in different tissues of *P. vittata* have been tested (Table 2). Huge differences have been identified among different populations and tissues. The highest concentration of total flavonoids reached 12%, which may be sufficient to bring in economic benefits. Additional technologies for the extraction and purification of such flavonoids are the focus of ongoing research.

Currently, the primary value of As phytoextraction is still in the removal of toxic As from mine waste or soil, thereby increasing the value of the land (Corzo Remigio et al. 2020). Hopefully, the recovered post-process energy and extraction of active ingredients from the biomass can significantly increase the financial viability of phytoextraction projects and reduce the environment impacts of contaminated biomass disposal (Jiang et al. 2015).

4 Future Prospects

China is a country having the highest geological As reservoir in the world. However, the grades of unmined As ore in this country are quite low as a result of long-term exploitation of high-grade ores. Concurrently, problems that have been recognized in As-contaminated soils have enabled the advancement of phytoextraction technology for this metalloid. Agromining can remove As from contaminated soil and at the same time provide high-grade raw materials for mining (incinerating ash of hyperaccumulator biomass). However, the low commercial value of As has hindered the development and viability of As agromining. Recently, a move in this positive direction has progressed because of the proposed use of As biomass for the production of biofuel, nano-metal materials, and valuable compounds. Due to the 2016 enactment in PR China of the Soil Pollution Prevention and Control Action Plan, it could be expected that many more soil remediation projects will be established in PR China. Further development of phytoextraction technology and a concomitant increase in the amount of *P. vittata* biomass, demands an appropriate recycling method for this species. The Institute of Geographic Sciences and Natural Resources Research, the Chinese Academy of Sciences is actively developing potential commercial utilization of the products from the *P. vittata* biomass. Hopefully, as a result of these research activities, a completed industrial-scale chain of phytoextraction and agromining for As-contaminated soil will be established in the next years.

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Element Case Studies: Cadmium and Zinc

Longhua Wu, Pengjie Hu, Zhu Li,
Wenzhong Xu, Tong Zhou, Daoxu Zhong,
and Yongming Luo

Abstract

Sedum plumbizincicola (Crassulaceae), a new *Sedum* species, was originally discovered in 2005 in Zhejiang Province, eastern PR China, and was identified as a Cd-Zn hyperaccumulator in 2007. During the past decade, great efforts have been made to understand its metal-accumulating capacities, physiological and molecular mechanisms for metal hypertolerance and hyperaccumulation, and enhanced measures of phytoextraction, as well as field applications of phytoremediation practice and disposal of harvested biomass. This chapter provides a brief review of the progress on phytoremediation of Cd- and Zn-contaminated soils using this species. Agronomic measures to enhance Cd and Zn phytoextraction efficiency using *S. plumbizincicola* were studied, including cultivation management, intercropping with other plant species, and nutrient management. Changes in soil and plant metal uptake were investigated during long-term and repeated phytoextraction of Cd- and Zn-contaminated soils using *S. plumbizincicola*. Field assessment results confirm that phytoextraction using *S. plumbizincicola* is a promising technique for the remediation of slightly Cd-polluted soils without the need to halt normal agricultural production.

1 Introduction

Heavy metals such as Zn and Cd are common pollutants that derive mainly from anthropogenic activities such as mining and the application of phosphatic fertilizers. Zinc is an essential element to plants but can be highly toxic at high concentrations. Cadmium is a non-essential element and also is highly toxic to both plants and animals, even at low concentrations. Among the various remediation techniques available, phytoextraction—the use of metal hyperaccumulator plants to remove pollutants from soils—is considered a cost-efficient and environmentally friendly method, although it still faces many challenges (McGrath et al. 2006).

The success of metal phytoextraction relies on several aspects. Firstly, an ideal hyperaccumulator plant produces a large biomass, grows rapidly, and is easily cultivated. Secondly, sound agronomic measures such as optimum moisture,

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L. Wu (✉) · P. Hu · Z. Li · T. Zhou · D. Zhong · Y. Luo
Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, People's Republic of China
e-mail: lhwu@issas.ac.cn

W. Xu
Key Laboratory of Plant Resources, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, People's Republic of China

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_23

nutrition, planting density, and harvest strategies are required. Intercropping with other crop species, for example, might be a feasible method of remediating contaminated soils without halting routine agricultural production. Thirdly, the harvested biomass must be safely treated. *Sedum plumbizincicola* (Crassulaceae), a newly described *Sedum* species, was originally discovered in 2005 in a Zn-Pb mine area of Zhejiang Province in eastern PR China (Wu et al. 2006). This species was identified as a Cd-Zn hyperaccumulator in 2007. Subsequently, much effort has been made to understand its metal-accumulating capacity, physiological mechanisms used for metal hypertolerance and hyperaccumulation, enhancing measures of phytoextraction, field application practices for phytoremediation, and disposal of harvested biomass. This chapter provides a brief review of progress made in the phytoremediation of Cd- and Zn-contaminated soils using this remediation plant species.

1.1 Characteristics of *Sedum plumbizincicola*

When our research team undertook extensive field reconnaissance to search for metal-hyperaccumulating plant species in Zhejiang Province, eastern PR China, some unusual and isolated populations that superficially resemble *S. alfredii* but produce four-merous flowers, were found in Chun'an and Lin'an counties (Wu et al. 2006), together with four other species, *S. alfredii*, *S. emarginatum*, *S. hangzhouense*, and *S. bailey* (Fig. 1). Based on geographic distribution, growth habit, phenology, macromorphological characters, stem and leaf anatomical features, seed micromorphology, and nrDNA internal transcribed spacers (ITS) sequence data, formal description of the new species *S. plumbizincicola* and clarification of the affinities between *S. plumbizincicola* and closely-related taxa were conducted (Wu et al. 2013a).

Sedum plumbizincicola is a perennial, light green or yellowish-green herb and is known only from the type locality, Zitong town (Zn-Pb mining areas), southwest of Hangzhou City in west Zhejiang Province, eastern PR China. Annual rainfall here varies from 980 to 2000 mm and occurs mainly in the summer, with a mean annual temperature of 15–18 °C. Soils in this area are typically sandy, acidic, highly leached, and commonly thin. This species has a strong ability to hyperaccumulate Zn and Cd, and is promising for restoration of metal-polluted soils by phytoremediation. The specific epithet *plumbizincicola* refers to the distribution of the species in the Pb and Zn mining areas of west Zhejiang Province.

1.2 Hyperaccumulation Ability for Cadmium and Zinc

The potential ability of *S. plumbizincicola* to extract Cd and Zn from contaminated soils has been demonstrated in glasshouse and field experiments. A field survey was conducted at a mining area in Chun'an county, Zhejiang Province, where the soils were strongly enriched with Cd (36–157 mg kg^{-1}), Zn (1930–7250 mg kg^{-1}), copper (Cu) (530–8340 mg kg^{-1}), and Pb (71–6940 mg kg^{-1}) due to pollution from mine tailings and processing of wastewater (Hu et al. 2015). *Sedum plumbizincicola* was the predominant species in this area, in which shoot Cd and Zn concentrations ranged from 574–1470 mg kg^{-1} and 9020–14,600 mg kg^{-1}, respectively. The metal concentrations in plant shoots were >100 mg kg^{-1} for Cd and nearly or over 10,000 mg kg^{-1} for Zn, these levels defining Cd- and Zn-hyperaccumulation ability according to the criteria of van der Ent et al. (2013). Moreover, Cd concentrations in leaves were approximately twice as high as in stems. Zinc concentrations in leaves were somewhat higher than, or equal to, those in stems. In a hydroponic experiment, Cao et al. (2014) recorded the Cd and Zn concentrations in shoots (7010 and 18,400 mg kg^{-1}, respectively), which were about seven-fold and five-fold higher than those in roots (840 and 3000 mg kg^{-1}, respectively) after exposure to 100 µM $CdSO_4$ and 600 µM $ZnSO_4$, respectively. In a pot experiment using 108 agricultural soils

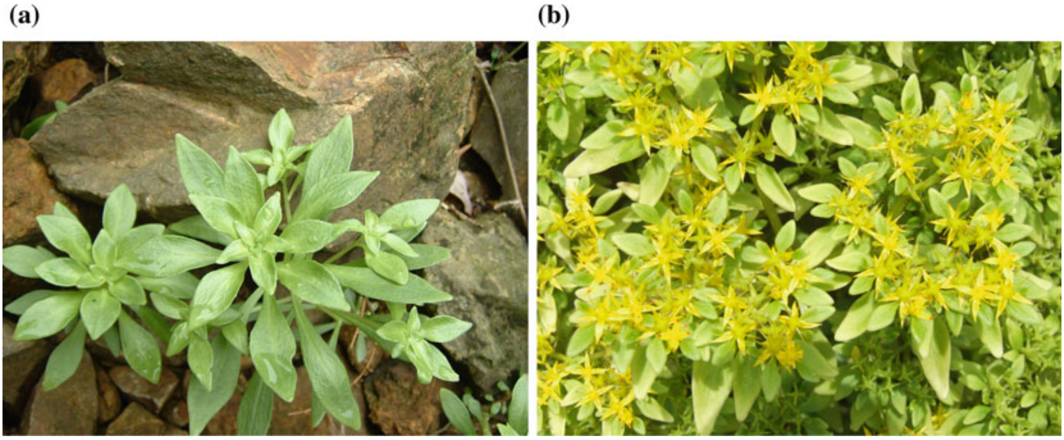

Fig. 1 *Sedum plumbizincicola* in its **a** vegetative and **b** flowering states in the original habitat

representing a wide range of soil types, Wu et al. (2018) and Zhou et al. (2019) found that the mean shoot biomass was 8.70 g pot^{-1}, ranging from 1.38 to 16.6 g pot^{-1}. Low soil pH limited plant growth, which might be caused by aluminium (Al) toxicity. The shoot Cd and Zn concentrations ranged from 0.66 to 1188 mg kg^{-1} and 87.6 to 14,779 mg kg^{-1}, respectively. The mean values of the bioconcentration factor (BCF) for Cd and Zn were 41.2 and 8.31, respectively. The mean Cd removal rate was 30.3% in the acid soils (pH <6.5) and 5.2% in the alkaline soils (pH >7.5). Optimum pH for phytoextraction with *S. plumbizincicola* was 5.5.

1.3 Spatial Distribution of Elements in *Sedum plumbizincicola*

Heavy metal sequestration in less-metabolically active parts of plants such as the epidermis and trichomes is one possible mechanism of heavy metal detoxification in hyperaccumulators. The spatial distribution of Cd, Zn, and other nutrient elements in frozen-hydrated sections of *S. plumbizincicola* from a mine area were quantitatively determined using cryo-micro-PIXE (Proton-induced X-ray Emission) (Hu et al. 2015). In roots, Zn and Cd were concentrated in the cortex but were low in the central stele. In stems, taking tissue area (or weight) into account, 22.1, 46.2, and 31.8% of total Zn mass and 17.1, 71.6, and 11.3% of total Cd mass were partitioned in the epidermis, cortex, and central cylinder (including the pith), respectively. In leaves, high concentrations of Zn were noted in the upper and lower epidermis. Cadmium was distributed more or less homogeneously in the entire leaf section, somewhat enriched in the vascular bundle, and slightly depleted in the spongy mesophyll. Considering tissue area, 23.8, 31.4, 23.8, 20.2, and 0.9% of total Zn mass was distributed in the upper epidermis, palisade mesophyll, spongy mesophyll, lower epidermis, and vascular bundles, respectively. However, 52.2 and 29.1% of total Cd mass was distributed in the palisade and spongy mesophyll, respectively. These data indicate that the preferential sequestration of Zn in stem and leaf (and to a lesser extent, Cd in the stem) into the epidermis is an important mechanism for hyperaccumulation and detoxification in *S. plumbizincicola*. Nevertheless, the parenchyma cells, e.g. mesophyll in the leaf, cortex, and pith in the stem, play more important roles in storage and detoxification of Cd than Zn within *S. plumbizincicola*. This result also indicates that different storage and detoxification strategies may exist for Cd and Zn in the shoots of *S. plumbizincicola*.

Additionally, in hydroponically cultured *S. plumbizincicola* supplied with Cd and Zn (Cao et al. 2014), micro-PIXE analysis and tissue fractionation showed that young and mature leaves of *S. plumbizincicola* exhibit different accumulation and distribution characteristics for metals. The concentrations of Cd and Zn in young leaves (4330 and 9820 mg kg^{-1}, respectively) were about six-fold and two-fold higher than those in mature leaves (636 and 2620 mg kg^{-1}, respectively). Zinc was predominantly localized in epidermal cells in both young and mature leaves, but large amounts of Zn were also found in the mesophyll cells of young leaves.

1.4 Molecular Mechanisms of Metal Hyperaccumulation in *Sedum plumbizincicola*

Sedum plumbizincicola, in contrast to non-accumulators, requires highly efficient mechanisms of Cd and Zn transportation and detoxification for its hyperaccumulation and strong resistance. On the basis of transcriptome analyses, metal hyperaccumulation has been associated with some metal transporter genes that are expressed at higher levels in hyperaccumulators than in non-hyperaccumulators. However, as a consequence of the lack of genetic transformation of Cd-hyperaccumulator species, improved understanding of the genes' importance in metal hypertolerance and hyperaccumulation remains unclear. In order to investigate the *in planta* functions of metal transporter in *S. plumbizincicola*, a reliable genetic transformation system was developed for *S. plumbizincicola* and the non-hyperaccumulating ecotype of *S. alfredii* (Liu et al. 2017a, b). Multiple-shoot clumps of *S. plumbizincicola* and *S. alfredii* induced from stem nodes were selected as target material for *Agrobacterium tumefaciens*-mediated genetic transformation. The *A. tumefaciens* infected explants were cultured on shoot induction medium with 20–30 mg l^{-1} hygromycin selection for several weeks. The hygromycin-resistant shoots were cut for rooting on a phytohormone-free MS medium containing the antibiotic. Transgenic plantlets were confirmed by histochemical β-glucuronidase (GUS) assay or polymerase chain reaction (PCR) verification for use in further experiments.

Heavy metal ATPases (HMAs), the P_{1B}-type ATPases subfamily, play an important role in transporting heavy metal ions. HMA3 proteins participate in Cd ion transport and detoxification through vacuolar sequestration. In non-accumulating plants, HMA3 is mainly expressed in the root tonoplast. The homolog of heavy metal ATPase 3 (*SpHMA3*) gene was isolated from *S. plumbizincicola* and identified to be highly expressed in shoots (Liu et al. 2017b). The *SpHMA3* protein was also tonoplast-localized and showed Cd-specific transport activity. Using the efficient genetic transformation approach for *S. plumbizincicola* and the non-hyperaccumulating ecotype of *S. alfredii*, RNA interference (RNAi)-mediated silencing and over-expression of *SpHMA3* were performed in the *Sedum* species. The *SpHMA3*-RNAi lines were hypersensitive to Cd, and growth of shoots and young leaves was severely inhibited by Cd but did not affect the accumulation of Cd in the shoots. These results suggest that *SpHMA3* plays an important role in Cd detoxification in young leaves and stems by sequestrating Cd into the vacuoles but does not directly regulate Cd hyperaccumulation. Another homologue gene of heavy metal ATPase 1 (*SpHMA1*) was also isolated and functionally identified from the *S. plumbizincicola* (Zhao et al. 2019). The *SpHMA1* protein was localized to the chloroplast envelope. *SpHMA1* RNA interference (RNAi) transgenic plants and CRISPR/Cas9-induced mutant lines showed significantly increased Cd accumulation in the chloroplasts compared with wild-type plants. Chlorophyll fluorescence imaging analysis revealed that the photosystem II of *SpHMA1* knockdown and knockout lines suffered from a much higher degree of Cd toxicity than the wild type. These results suggest that *SpHMA1* functions as a chloroplast Cd exporter and protects photosynthesis by preventing Cd accumulation in the chloroplast in *S. plumbizincicola*. These studies showed that *SpHMA3* and *SpHMA1* are highly expressed in shoots to form an efficient system for Cd detoxification in leaves of *S. plumbizincicola*. *SpHMA1*, as a Cd transporter localized at the chloroplast envelope, functions to export Cd from chloroplasts to cytoplasm and protect the photochemical reactions in leaf cells of the Cd hyperaccumulator *S. plumbizincicola*. In order to maintain the cytosolic Cd at low levels, another heavy metal ATPase, *SpHMA3*, functions to transport Cd from the cytoplasm into the vacuoles. Thus, both *SpHMA1* and *SpHMA3* are important for Cd hypertolerance in *S. plumbizincicola*.

1.5 Rhizosphere Acidification and Microbiome of *Sedum plumbizincicola*

Plant roots can significantly alter soil pH and the chemical concentration and distribution of different elements in the rhizosphere environment. A recent study showed that Cd bioavailability in the rhizosphere of *S. plumbizincicola* can be influenced by root-induced effects on soil pH (Sun et al. 2019). The Cd-hyperaccumulator *S. plumbizincicola* and the Cd non-hyperaccumulator ecotype *S. alfredii* were both grown in four different Cd-contaminated soils. It was found that both *Sedum* species showed root-induced increases in soil acidification (i.e. soil pH decreases of 0.1–0.62 units), which were clearly associated with greater root density of these plants. Remarkable excess cation uptakes by both *Sedum* species were detected and likely represent a driving factor for the root-induced acidification. However, soil solution Cd concentration in the rhizosphere of *S. plumbizincicola* was much higher than in bulk soil in Orthic Acrisol (+342%) and in Hydragric Antrosol soils (+296%). The hyperaccumulator *S. plumbizincicola* had larger root systems, higher acidification ability, and was associated with greater soil solution Cd concentration than *S. alfredii*. Spatial patterns of root distribution and soil pH were similar among *Sedum* plants; however, spatial patterns of Cd concentration differed across polluted soils. It was suggested that rhizosphere acidification induced by *S. plumbizincicola* plants can play an important role in soil Cd mobilization, but overall effects on soil Cd bioavailability will depend on intrinsic biogeochemical properties of the soil.

The link between soil microbiome and plant performance remains poorly understood during trace-metal phytoremediation by hyperaccumulator plants. Hou et al. (2019) found that an exogenous plant growth promoting the bacterium *Rhodococcus* sp. NSX2 could enhanced Cd accumulation in *S. plumbizincicola* by reshaping the rhizosphere microbiome. High-throughput sequencing and random matrix theory (RMT)-based network analysis imply that NSX2 made the bacterial network in the rhizosphere more complex through cooperating positively with the indigenous bacteria and that increased phytoremediation efficiency strongly correlated with the reshaped topology of the bacterial network. Another study by Hou et al. (2017) confirmed that the rhizosphere microbiome could affect Cd and Zn accumulation in *S. plumbizincicola*. The soil microbiome was modulated directly using gamma irradiation and vancomycin amendment. Results showed that plant Cd and Zn contents in the vancomycin-amended treatment were about 38–46% and 35–53% lower, respectively, than those in the other treatments. The bacterial functional profiles were very different following treatment with vancomycin compared with the other treatments at KEGG orthology hierarchy level 3, whereas the bacterial composition and diversity were not. Genes related to up-regulated biofilm formation and down-regulated siderophore synthesis may partly contribute together to low trace-metal accumulation in the plant following vancomycin treatment. Therefore, it was suggested that specific bacterial community function rather than bacterial community diversity and composition affect trace-metal uptake by *S. plumbizincicola*.

1.6 Agronomic Measures to Increase Cadmium and Zinc Phytoextraction

Sedum plumbizincicola is suitable for clonal reproduction. The shoot branches are usually selectively picked for reproduction. A tissue culture method has been established to provide large amounts of plant material (Wu et al. 2010).

For field application, appropriate plant density, soil moisture, light, and harvest strategy have been optimized for plant biomass and Cd and Zn uptake, and for minimal time required for the phytoremediation period. A field plot experiment was established to study the effects of planting density on growth and heavy metal uptake by *S. plumbizincicola* (Liu et al. 2009). The results showed that when the planting density was 15 × 15 cm (4.4×10^5 plants ha^{-1}), *S. plumbizincicola* had the highest yield as well as Cd and Zn removals. Neither lower nor higher densities were preferable. Humid-dry conditions (~70% of water-holding capacity) are preferable for plant growth and metal accumulation, and not extremely dry or flooded conditions (Cui et al. 2009). *Sedum plumbizincicola* is a shade species, and Zn and Cd accumulation did not decline when irradiance decreased to 50% of the control level (Li et al. 2010). *Sedum plumbizincicola* is also a perennial and can be harvested two or three times each year, for example before a hot summer and cold winter. During cutting, retaining 3–5 cm of stubble will promote multiplication vegetatively (Li et al. 2009).

Much effort has been devoted in the past few years to exploring the feasibility of simultaneous safe grain production and phytoremediation of heavy metal-contaminated soils using *S. plumbizincicola* in major food crop areas of China. *Sedum plumbizincicola* is also suitable for rotation with rice in southern China, e.g. growing *S. plumbizincicola* from October to June and rotating rice from July to October. A pot experiment was conducted to explore the effects of different rates of sulfur (S) addition associated with alternating drying and wetting on a *S. plumbizincicola*-rice rotation in Cd-contaminated neutral and calcareous soils (Wu et al. 2019). Results showed that the oxidation of added S under aerobic conditions significantly decreased soil-solution pH and increased soluble SO_4^{2-}, Cd, and Fe concentrations in both soils. During the rice growing season, the soil solution redox (Eh) decreased to <-200 mV and solution pH increased to neutral during the first few days of flooding. Soluble SO_4^{2-} and Cd in the S treatments decreased significantly with increased duration of flooding. Moreover, S addition promoted shoot Cd concentrations of *S. plumbizincicola* by 1.7–5.5 times on calcareous soil and 1.7–2.3 times on neutral soil, compared to the controls. Rice yields increased but Cd concentrations decreased at suitable S addition rates. It was suggested that appropriate S amendment combined with water management can be a feasible strategy to enhance Cd remediation efficiency of the hyperaccumulator and reduce accumulation of Cd in rice grains in this rotation (Wu et al. 2019).

Shade tolerance ensures that *S. plumbizincicola* can be employed to intercrop with tall straw plants such as maize, sorghum, sugarcane, and wheat (Fig. 2). In an 8-year field phytoextraction experiment of maize/*Sedum* intercropping, soil Cd and Zn decreased from 3.50 to 0.54 and from 1799 to 1465 mg kg^{-1} (Deng et al. 2016). Nevertheless, the roots of *S. plumbizincicola* may activate soil Cd and increase Cd availability, which may increase the heavy-metal risk in intercropping or plant rotation. For example, Zhao et al. (2011) found that compared with mono-cultured wheat, intercropping with *S. plumbizincicola* significantly increased soil $NaNO_3$-extractable Zn and Cd, which increased metal uptake by wheat. Shen et al. (2010) also reported that Zn and Cd concentrations in rice rotated after *S. plumbizincicola* were higher than the control, while the amendment of phosphate fertilizer (particularly Ca–Mg phosphate) in rice season decreased metal accumulation in rice and promoted rice production as well. The activation mechanism of soil metals by roots of *S. plumbizincicola* has been unclear up to now. Soil type and plant species may be involved (Ju et al. 2015). Therefore, such intercropping should be done carefully.

1.7 Nutrient Management

The effects of nutritional status on plant growth and metal accumulation by *S. plumbizincicola* were investigated (Hu et al. 2013; Arnamwong

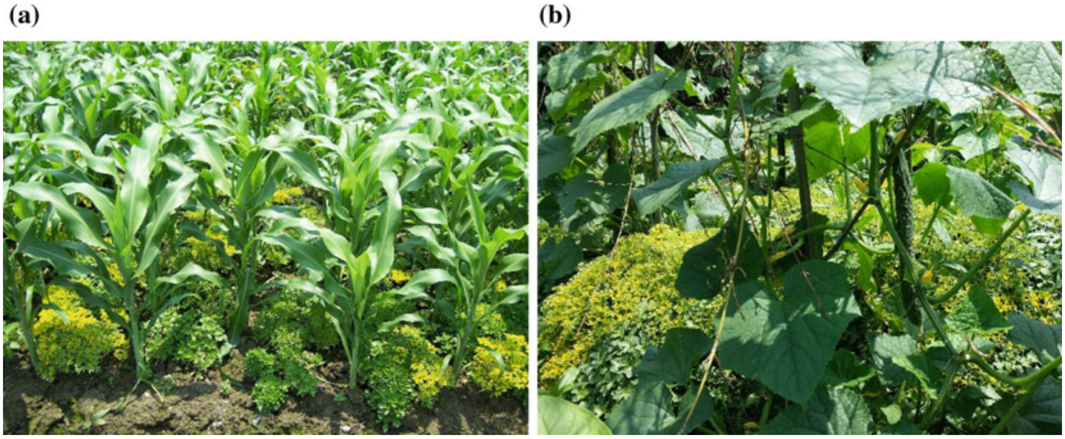

Fig. 2 Field practice of intercropping of *S. plumbizincicola* with **a** maize and **b** cucumber

et al. 2015). Hydroponic experiments were designed to evaluate whether and how the form of N (NO_3^- vs. NH_4^+) influences Cd uptake, and translocation and subsequent Cd phytoextraction by *S. plumbizincicola* (Hu et al. 2013). The results from a 21-day hydroponic culture showed that shoot biomass and Cd concentrations were 1.51 and 2.63 times higher in the NO_3^--fed than in the NH_4^+-fed plants. Furthermore, a short-term (36 h) ^{107}Cd uptake and translocation experiment using a positron-emitting tracer imaging system showed that the rates of Cd uptake by roots, and transport to the shoots, in the NO_3^- treatment were higher than in the NH_4^+ treatment. Moreover, NO_3^- treatment increased Cd, Ca, and K concentrations but inhibited Fe and P uptake in the xylem sap. Therefore, *S. plumbizincicola* clearly prefers NO_3^- to NH_4^+ nutrition for Cd phytoextraction under hydroponic conditions. The effects of different N fertilizers on shoot yield and Cd and Zn accumulation by *S. plumbizincicola* were also investigated using pot experiments (Arnamwong et al. 2015). In this study, the soil was contaminated with 0.99 mg kg^{-1} Cd and 241 mg kg^{-1} Zn. The results suggested that treatment by urea + DCD (dicyandiamide, a nitrification inhibitor) was a benefit for Cd phytoextraction by *S. plumbizincicola*. Apart from N, low P and high K levels were recommended for metal phytoextraction by *S. plumbizincicola* (Shen et al. 2011).

1.8 Other Measures

Organic materials having different functional groups can be used to enhance metal bioavailability. It was found that amendment of the soil with ground rice straw or ground clover increased by 1.92 and 1.71 times the Cd concentrations in shoots of *S. plumbizincicola* compared to the control soil (Wu et al. 2012). Therefore, these traditional organic materials can be effective and environmentally friendly in enhancing the phytoremediation efficiency of Cd-contaminated soil. Furthermore, Zhou et al. (2018a) found that rice straw amendments had no significant effects on soil pH, DOC, or Cd concentrations in bulk soils during repeated phytoextraction. However, rice straw amendments were associated with significant increases in available soil nutrient contents, increases in the percentage of oxidizable Cd fractions, and the mass of soil particulate organic matter (POM) fractions, and with decreases in POM Cd concentrations after repeated phytoextraction. These findings suggested that POM fractions may represent important accessible pools of Cd for uptake by *S. plumbizincicola* plants.

Sedum plumbizincicola usually adapts best to acidic soil. But for strongly or extremely acidic soil, the application of a proper dosage of lime was thought to benefit the growth of this species and metal uptake as well (Han et al. 2013). It has also been suggested that an appropriate application of sulfur fertilizer promotes the growth of *S. plumbizincicola*, and that the addition of calcium magnesium phosphate fertilizer reduces the concentration of active heavy metals in the soil solution and the contamination risk for subsequently grown vegetables (Ren et al. 2013). The amendments of organic fertilizer, perlite, and vermiculite in metal-contaminated calcareous soil significantly enhanced the shoot biomass production and Zn and Cd uptake by *S. plumbizincicola* (Zhou et al. 2018b). On the other hand, treatment with EDDS increased metal concentrations in shoots of *S. plumbizincicola*, although EDDS also resulted in high soil concentrations of soluble metals and consequently a high risk of groundwater contamination (Wu et al. 2012).

2 Repeated Phytoextraction of Cd-Zn-Contaminated Soils Using *Sedum plumbizincicola*

Phytoextraction using hyperaccumulator plants requires long periods of remediation time and successive crops to remove excessive metals from contaminated soils and achieve permissible levels (Koopmans et al. 2008). Short-term remediation cannot give an accurate prediction of plant metal uptake and soil metal changes during the phytoextraction process. Thus, plant growth, metal uptake, and soil metal changes were investigated during repeated phytoextraction of Cd- and Zn-contaminated soils using the hyperaccumulator *S. plumbizincicola*.

2.1 Plant Metal Uptake and Soil Metal Changes in Soils During Repeated Phytoextraction

Two levels of Zn- and Cd-polluted soil (0–15 cm) were collected from an agricultural field, namely lightly polluted soil (S1) collected 150 m from the pollution source (a Cu smelter) and a highly polluted soil (S4) collected only 30 m from the smelter. These two polluted soils were then mixed in proportions of 1:2 and 2:1 by weight, to gain another two contamination levels (S2 and S3). These four soils were then remediated by successive plantings with *S. plumbizincicola* in a pot experiment (Li et al. 2014b). The main properties of S1, S2, S3, and S4 are listed in Table 1. *Sedum plumbizincicola* grew well during repeated phytoextraction and the plants did not have visible metal-toxicity symptoms. Cadmium concentrations in the plant shoot decreased for early crops of the repeated phytoextraction process, but for later crops plant Cd levels were relatively constant, especially in soils S1 and S4. Plant Zn decreased in the first three crops in S1 but increased in the first four crops of S2 and S3, and then remained stable for crops S1, S2, and S3. During the repeated

Table 1 Chemical properties of four contaminated soils with different pollution levels (Li et al. 2014b)

Soils	pH (H_2O)	Total C	Total N	Total P	Total K	CEC (cmol (+) kg^{-1})	Total Zn	Total Cd
		(g kg^{-1})					(mg kg^{-1})	
S1[a]	6.47	42.2	3.75	0.24	21.6	16.1	321	1.11
S2	6.95	37.6	3.19	0.22	22.3	16.6	2367	5.82
S3	7.13	33.7	2.84	0.24	24.4	14.4	4343	10.6
S4	7.24	29.1	2.21	0.22	22.9	11.9	6499	15.3

[a] *S1* lightly polluted soil; *S2* and *S3* intermediate polluted soils; *S4* highly polluted soil

phytoextractions, S4 had similar plant Zn concentrations for all crops.

Changes in total Cd and Zn in the four contaminated soils with and without phytoextraction were investigated. Compared to the unplanted controls, after nine repeated phytoextractions over about four years, both total Cd and Zn decreased greatly: soil Cd and Zn decreased by 89.1, 96.8, 90.1, and 80.1% and 64.2, 70.1, 32, and 24.8% in S1, S2, S3, and S4, respectively. The removal efficiencies of metal were also high at relatively low levels of soil contamination, but in the more highly polluted soils the removal efficiencies were low, especially for Zn. This pattern indicates successful phytoremediation of the metal-contaminated soil was achieved by use of the hyperaccumulator plant.

Metal fractions extracted by the BCR (Community Bureau of Reference) method were used to discriminate among different degrees of metal availability during repeated phytoextraction. For the unplanted soils, over 90% soil Cd and Zn was partitioned mainly into the acid-soluble and reducible fractions, but Zn in S1 was partitioned into the acid-soluble fraction, and then into the residual, oxidizable, and reducible fractions. Phytoextraction induced the largest decrease in the acid-soluble fraction, followed by the reducible fraction, and had no significant effects on the oxidizable and residual fractions. This trend indicates that the acid-soluble fraction represents the most plant-available fraction, followed by the reducible fraction; *S. plumbizincicola* can remove these two fractions of Cd and Zn, but the oxidizable and residual fractions were unavailable to this species. Measures could be taken to release the acid-soluble Cd and Zn or to redistribute the reducible fractions in order to enhance remediation efficiency.

2.2 Plant Metal Uptake and Metal Changes in Soils During Repeated Phytoextraction

Samples of four metal-contaminated soil types were collected from agricultural fields in different parts of China. Two acidic soils were collected from Huludao in Liaoning Province, north PR China, and Dabaoshan in Guangdong Province, southern PR China, and are termed here HLD and DBS, respectively. The calcareous soils were collected from Shuanglingdong and Zhujiawu in Zhejiang Province, eastern PR China, and are referred to as SLD and ZJW, respectively. Selected chemical properties of the contaminated soils are shown in Table 2. These four soils were remediated by successive plantings of *S. plumbizincicola* in a pot experiment (Li et al. 2014a).

Shoot Zn and Cd concentrations in each crop were determined in order to investigate the changes in plant metal uptake during repeated phytoextraction. Shoot Zn concentrations in the calcareous soils (ZJW and SLD) were much lower than those in the acidic soils (DBS and HLD). As the crops of phytoextraction increased, shoot Zn concentrations in DBS and HLD and shoot Cd concentrations in HLD decreased markedly. However, there was no obvious decreasing trend in shoot Zn or Cd during

Table 2 Chemical properties of four contaminated soils without and/or with long-term phytoextraction (Li et al. 2014a)

Soil	pH		TOC (g kg^{-1})		CEC (cmol (+) kg^{-1})	Cd (mg kg^{-1})	Zn (mg kg^{-1})
	NP[a]	P	NP	P	NP	NP	NP
DBS	3.71	3.87	24.3	23.7	8.31	0.42	291
HLD	4.77	4.71	15.1	13.2	15.4	8.68	476
SLD	7.74	7.71	28.4	25.7	18.9	16.9	1308
ZJW	7.14	7.72	20.6	19.5	11.2	2.28	1201

[a] *NP* without phytoextraction; *P* the soil was repeatedly phytoextracted by the hyperaccumulator *Sedum plumbizincicola*

repeated phytoextraction in ZJW and SLD, or in shoot Cd in DBS. Furthermore, a slight Cd increase in the later crops was found compared to the first several crops. This result indicates that metal uptake in plants under low available metal conditions depends on both soil and plant metal processes such as rhizosphere activity.

Both soil total Zn and soil Cd decreased markedly after repeated phytoextraction seven times. The highest decrease was found for the acidic soil HLD having high concentrations of Cd; after repeated phytoextraction, soil total Zn and Cd decreased from 476 to 229 and 8.68 to 0.93 mg kg^{-1}, respectively. Compared to the unplanted control soil, the soil total Cd and Zn in P7 soils (seven phytoextractions) from HLD, DBS, ZJW, and SLD declined by 89, 64, 38, and 37%, and by 52, 37, 19, and 12%, respectively. In addition, after phytoextraction with seven successive croppings, available Zn and Cd declined markedly in all four soils. Compared to the soils that were not exposed to phytoextraction, after seven remediation croppings, NH_4OAc-extractable Zn decreased 98, 97, 58, and 30% in HLD, DBS, ZJW, and SLD, respectively; NH_4OAcextractable Cd decreased by 98, 95, 41, and 57%, respectively. The equivalent Zn values extracted by $CaCl_2$ were 94, 92, 27, and 24% for HLD, DBS, ZJW, and SLD, respectively, and for Cd were 96, 87, 60, and 68%.

The total or available metals extracted by chemical agents are based on theoretical equilibrium conditions and thus cannot reflect metal re-supply processes usually occurring in the rhizosphere. The metal resupply capacity from the soil solid phase can be evaluated by the response time (the characteristic time taken by the system to approach equilibrium, Tc), and by the metal desorption rate constant (k^{-1}) measured by the diffusive gradients in thin-films technique (DGT). Effects of phytoextraction on these two parameters were studied further by Li et al. (2016).

In the unplanted soils, Tc values were much lower in the calcareous soils (SLD and ZJW) than in the acidic soils (DBS and HLD), suggesting a fast metal supply from the solid phase to solution in the calcareous soils. It is reasonable for a longer response time to occur for acidic soils than calcareous soils. Acidic soils have high metal contents in soil solution but small metal pool sizes in soil solid phases, which leads to a large equilibrium shift induced by DGT metals removal. After phytoextraction, the Tc values of Zn and Cd markedly decreased in the acidic soils but increased in the calcareous soils. During repeated phytoextraction, the k^{-1} value of Cd remained constant in DBS, suggesting that the same mechanism regulates metal desorption from the solid phase to soil solution. However, k^{-1} values of Cd and Zn in all of other soils declined greatly after phytoextraction. This result suggests that different metal pools regulate metal releases to soil solution during repeated phytoextraction, and that the residual metal in soil after phytoextraction might have stronger combining forces to the soil solid phase.

2.3 Field Application of Phytoextraction with *S. plumbizincicola*

Sedum plumbizincicola was employed for field phytoextraction of heavy metal-contaminated soils beginning in 2006. To date, research and demonstration bases have been established in several provinces in PR China, namely Zhejiang, Hu'nan, He'nan, Jiangxi, Jiangsu, Guangdong, Guizhou, etc. (Fig. 3). This species uniformly exhibits extremely strong adaptation under diverse climatic, edaphic, and topographic conditions. *Sedum plumbizincicola* has shown much greater efficiency in the removal of Cd from slightly to moderately polluted acidic soils than from neutral soils.

A field experiment on a total area of one hectare was conducted to evaluate Cd removal efficiency from a contaminated acid paddy soil (pH 5, Cd 0.6 mg kg^{-1}) by repeated phytoextraction using the hyperaccumulator *S. plumbizincicola* from October to June each year (Hu et al. 2019). Results showed that *S. plumbizincicola* grew well with a shoot dry biomass of 1.8–5.9 t ha^{-1} and a Cd concentration of 51.4–94.9 mg kg $^{-1}$ and Cd accumulation of 169–

Fig. 3 Demonstration base of Cd phytoextraction with *S. plumbizincicola* in polluted acid soil in Xiangtan, Hu'nan Province

353 g ha^{-1}. The Cd removal rate from rooting depth (the top 15 cm) of the arable soil ranged from 13.8 to 34.7% each season with an average of 21.8%. In plot 1, the soil total Cd concentration decreased from 0.64 to 0.29 mg kg $^{-1}$ after two seasons of phytoextraction. The soil Cd concentration decreased mainly within the depth range 2–10 cm that corresponded with the root distribution pattern of *S. plumbizincicola*. Subsequent Cd risk assessment in rice showed that grain Cd concentrations in phytoextracted soil decreased significantly compared to non-phytoextracted soil. Brown rice Cd in lower accumulating rice cultivars MY12085 and MY12086 was <0.2 mg kg^{-1} (Chinese standard), while additional immobilization was needed in the case of higher accumulating cultivars. This field study strongly indicates that phytoextraction of slightly contaminated acid soils using *S. plumbizincicola* is feasible for depressing total Cd concentration to meet the statutory limit within a relatively short time, e.g. 2–5 years. Appropriate utilization of rice cultivars and soil amendments in the phytoextracted soils may further contribute to safe agricultural production.

3 Treatment of *Sedum plumbizincicola* Biomass

The safe and economic disposal of harvested hyperaccumulator plants for avoiding secondary environmental pollution is a challenging problem. Compared with the other general disposal methods of composting, disposal to hazardous waste landfills, or compaction and ashing, the incineration and pyrolysis of harvested biomass are generally recommended for contaminated biomass owing to issues of high-volume reduction rates and energy-reuse efficiency (Sas-Nowosielska et al. 2004; Demirbas 2010).

3.1 Volume Reduction and Drying of Fresh *Sedum plumbizincicola* Biomass

The water content of fresh *S. plumbizincicola* biomass after harvest is usually >90% and a large area and a long time interval are needed to dry this biomass. Rapid volume reduction and drying are therefore necessary for further disposal of the biomass. A feasible plan is to cut and crush the fresh biomass to produce a residue and liquor. The residue can then be dried quickly and the liquor containing heavy metals and its chemical oxygen demand (COD) can be treated by physical, chemical, or biological methods. Wang et al. (2017) found that when the pulp yield was set to 60%, the volume of residue decreased by 71.1% and the drying time was shortened to one-third. The Cd concentration in the liquor increased with the increase of pulp yield. Based on a Cd ion-selective electrode method, 2% of the total Cd in the liquor was thought to be in the Cd^{2+} form (Zhang et al. 2018). Under optimal conditions of flocculation precipitation, the removal rates of Cd, Pb, Zn, and COD in the liquor were 81.9, 77, 76.4, and 55%, respectively. A heavy-metal capture reagent such as trimercaptotriazine can be used for the further removal of metals from the liquor (Wang et al. 2017).

3.2 Incineration Products of *Sedum plumbizincicola*

Sedum plumbizincicola dry biomass sampled from different polluted fields was treated by incineration processes. The experimental facility comprised a flow control valve and meter, a flue gas absorption device, a horizontal quartz tube with a surrounding electrically heated furnace, and two high-pressure gas sources (O_2, N_2). Experiments were performed at temperatures from 350 to 950 °C and air-flow rates of 0.5, 0.75, 1 and 1.2 L min^{-1}. The Thermal Gravity (TG) analysis curves show that the mass percentages of bottom ash relative to original biomass after incineration generally decreased with increasing temperature in the horizontal tube furnace, and that the weight losses of *S. plumbizincicola* were 6.17 to 10.8, 49 to 50.4, and 82.7 to 89.4% at three temperature steps of <185 °C, 185 to 400 °C, and 400 to 697 °C, respectively (Zhong et al. 2015).

The distribution of heavy metals in flue gas is an important basis for using the gas cleaning system for disposal of *S. plumbizincicola*. With total Cd and Zn concentrations of 152 and 7480 mg kg^{-1}, respectively, occurring in *S. plumbizincicola*, the data show concentrations of these metals in control flue gas were 0.101 and 46.4 mg m^{-3}, respectively, during the incineration process (Wu et al. 2013b). The additives aluminium oxide (Al_2O_3), CaO, kaolin, and activated carbon decreased the Cd concentration by 33, 45.2, 91.2, and 97.6% in the flue gas, thus meeting the Chinese emission standard. About 21.6, 50.8, 88.1, and 99.1% of the Zn in the gas was removed after use of these adsorbents. By comparison, activated carbon was more effective than the other three additives in removing Cd and Zn from the flue gas. In the control, the total recoveries of Cd and Zn were only 11.2 and 23.8%, respectively, at the end. The additives Al_2O_3, CaO, and kaolin increased the recovery of Cd from the bottom ash and fly ash, and of Zn from the fly ash. During incineration of *S. plumbizincicola*, recoveries of Zn, Cd, and Pb mainly decreased with the elevated temperatures occurring in the bottom ash and increased in the fly ash, thus following the typical volatilization behaviour of heavy metals (Zhong et al. 2015). Nearly 80% of the Cd was present in the fly ash. Major proportions of Pb (>95%) and Zn (>90%) remained in the bottom ash at temperatures of 350–650 °C. When the temperature was above 650 °C, most of the Zn and Pb were found in the fly ash. Recoveries of Cd, Zn, and Pb from flue gas were lower than 0.05%. The melting point was the key factor determining the recoveries of these metals. Addition of elemental sulfur and sulfide increased the retention of Cd and Zn in the bottom ash through the formation of sulfides under the local reducing environment of the furnace. In contrast, the presence of sulfur in the forms of Na_2SO_3 and Na_2SO_4 had little effect on heavy metal volatilization. Chlorine compounds did not increase this volatilization or partitioning into the fly ash during incineration, because most of the chlorine was present as KCl. The Cd and Zn were found to occur as pure metals, oxides, and carbonates; silicate compounds containing these metals developed under low-temperature conditions during the incineration process.

Heavy-metal recovery rates with increasing airflow rate from fly ash were higher than those from bottom ash, which indicates that at higher temperatures both the oxidizing atmosphere and reducing conditions favoured the transfer of Cd, Zn, and Pb to the fly ash phase (Zhong et al. 2015). However, more Pb and Cd were found in the bottom ash under reducing conditions (air flow rate <0.75 L min^{-1}) than under oxidizing conditions, whereas more Zn was found in the bottom ash under oxidizing conditions at 850 °C. When air excess ranged from 0.2 to 2 at a temperature of 850 °C, the equilibrium distributions of heavy metals showed that sulfur compounds are predominate in the vapour of heavy metals under reducing conditions, relative to oxidizing conditions. Zinc is found as Zn, ZnO, and ZnS when the air excess quotient is <0.6, whereas only ZnO was produced when this quotient is >0.6. Cadmium and CdS are found under reducing conditions (air excess quotient <1), whereas CdO and Cd are found when the air excess quotient is >0.2.

3.3 Characteristics of Pollutant Emissions During Incineration

As shown in Table 3, incineration of *S. plumbizincicola* biomass produced high total PAH concentrations (35,420 µg m^{-3}) in a laboratory scale, entrained flow-tube furnace of the control treatment, including 86% of PAHs found as 3-ring and 13.9% as 4-ring congeners (Wu et al. 2013b). Most 3-ring PAHs were present in the gas phase emissions, whereas the PAHs with higher ring numbers were associated with fly ash. Total PAH emissions decreased with increasing temperature during incineration of *S. plumbizincicola*, especially in the bottom ash. With increasing temperature, the toxic equivalent quantity (TEQ) of total PAHs showed increasing trends in both the flue gas and fly ash and decreasing trends in the bottom ash. High concentrations of PAHs existed in the flue gas. Over 99% of total PAHs were removed from the flue gas when activated carbon was used as the adsorbent. When silica was used as the bed material, the 4-ring PAHs decreased, and the 3-ring PAHs increased.

The concentrations of polychlorinated dibenzofurans (PCDFs) in flue gas were 131 pg m^{-3} and 107 TEQ pg m^{-3}. These values were lower than the Chinese emission standard (500 TEQ pg m^{-3}), but higher than the emission limit (92 pg TEQ m^{-3}) set for waste incinerators in many countries (Wu et al. 2013b). The OCDD had the highest concentration in PCDD/F, at 25.4 pg m^{-3}.

During the incineration of *S. plumbizincicola* the concentration of CO (>1000 mg m^{-3}) in flue gas was >10 times the Chinese Emission Standard (GB18484–2001) and the NO_x emission was about 500 mg m^{-3} (Table 4). With increasing temperature, the CO emission decreased but the NO_x emission increased. Additives can change the release of CO and NO_x. The additive CaO significantly increased emissions of both CO and NO_x. When the temperature was >850 °C, the concentrations of CO and NO_x in flue gas were higher than the Chinese Emission Standard.

Table 3 PAH concentrations measured in flue gas [µg m^{-3} at 11% O_2] (Wu et al. 2013b)

Treatment	Control	Activated carbon	SiO_2 + Activated carbon
Naphthalene	4918	52.8	58.6
∑PAHs with 2 rings	4918	52.8	58.6
∑PAHs with 3 rings	30,451	90.8	289
∑PAHs with 4 rings	92.6	8.34	2.48
∑PAHs with 5 rings	ND	ND	ND
∑PAHs with 6 rings	ND	ND	ND
∑PAHs	35,420	152	350
TEQ concentration	35.8	0.40	0.39

Table 4 NO_x, SO_2, CO, and HCl concentrations measured in flue gas (mg m^{-3} at 11% O_2) (Wu et al. 2013b)

Treatment	CO	SO_2	NO	NO_2	N_2O	NO_x	NH_3	HCl	HCN
Control	1009	6.99	536	17.6	0.003	553	15.1	1.62	3.95
Kaolin	2444	0.08	426	34.2	1.43	461	38.2	0	5.16
CaO	2578	0.03	677	25.1	0.12	702	35.9	2.54	4.64
Al_2O_3	1854	0.66	493	19.3	0.06	512	31.5	2.19	5.68

Using kaolin as an additive gave the lowest concentration of NO_x, 461 mg m^{-3}, which is lower than 500 mg m^{-3} (the Chinese Emission Standard). Using Al_2O_3 also decreased NO_x in flue gas. SO_2 and HCl in flue gas were much lower than their respective Emission Standards (GB18484–2001).

3.4 Pyrolysis Products of *Sedum plumbizincicola*

The Thermal Gravity (TG) Analysis curves also show that the mass percentages of bottom ash relative to original biomass after incineration generally decreased with increasing temperature in the horizontal tube furnace (Zhong et al. 2015). Weight losses of *S. plumbizincicola* were 3.56–9.51, 49.8–50.8 and 73.9–81.2% at three temperature steps of <170 °C, 170–400 °C and 400–800 °C during the pyrolysis process.

During the pyrolysis of *S. plumbizincicola* the tar yield increased to 6.34 wt% at 650 °C and decreased to 3.19 wt% at 750 °C. The bio-oil yield increased from 22 wt% at 450 °C to 31.7 wt% at 650 °C and decreased to 15.9 wt% at 750 °C. Char yield decreased from 32.4 wt% at 450 °C to 22.5 wt% at 750 °C. The gas yield remained nearly constant up to 650 °C, then increased significantly at 750 °C. The main components of the pyrolysis oils were acids (at 450 °C) that decreased with increasing pyrolysis temperature. Pyrolysis at 650 °C led to the highest yield of alkanes with low-oxygen compounds being found in the bio-oil.

During the pyrolysis process of *S. plumbizincicola*, kinetic analysis shows that the value of activation energy changed from 146.4 to 232.4 kJ mol^{-1} and that the frequency factor value changed greatly from 1.34×10^{11} to 8.99×10^{15} s^{-1} (Zhong et al. 2016). Heavy metals in the gas were not detected. At temperatures of 450–750 °C more than 43.6% of Zn remained in the char, whereas 54.4% of Zn was found in bio-oil at 750 °C. More than 87.6% of Cd was vaporized during pyrolysis and was detected in the bio-oil. By comparison, mass balances of heavy metals under pyrolysis conditions were higher than those under incineration conditions (Lu et al. 2012). The Zn content in flue gas increased with increasing temperature but the Cu and Cd contents fluctuated in an entrained flow-tube furnace. In a horizontal tube furnace, incineration favoured the volatilization of Cd in contrast to pyrolysis in flue gas. The percentages of heavy metals in bottom ash after pyrolysis were 3.5, 2.7 and 2.3 times greater than those after incineration, especially for Cd, Pb, and Zn, which indicates that the oxidizing atmosphere favours transfer of these metals to the gaseous phase.

4 Conclusions

Sedum plumbizincicola, originally discovered in Zhejiang Province, east China, is a new *Sedum* species and is documented as a Cd-Zn hyperaccumulator. In an area of Pb-Zn mining, the shoots of *S. plumbizincicola* accumulated up to 1470 mg kg^{-1} Cd and 14,600 mg kg^{-1} Zn. In hydroponic experiments, the shoot accumulated 7010 mg kg^{-1} Cd and 18,400 mg kg^{-1} Zn. Spatial distribution analysis showed that the preferential sequestration of Zn in stem and leaf (and to a lesser extent, Cd in stem) into the epidermis, is an important mechanism for hyperaccumulation and detoxification in *S. plumbizincicola*. Nevertheless, the parenchyma cells, e.g. mesophyll in the leaf, cortex, and pith in the stem, play more important roles in the storage and detoxification of Cd than does Zn in *S. plumbizincicola*. Based on the establishment of a genetic transformation system, *Sedum* species can be used as a new model plant for elucidating the molecular mechanisms of Cd and Zn hyperaccumulation by gene RNA interference, CRISPR/Cas9, and overexpression approaches; both *SpHMA1* and *SpHMA3* are important for Cd hypertolerance in *S. plumbizincicola*. Rhizosphere acidification and specific bacterial community function can play important roles in metal uptake by *S. plumbizincicola.* Suitable agronomic measures including cultivation management, intercropping with other plant species, and nutrient management can enhance Cd and Zn phytoextraction efficiency by *S. plumbizincicola*. Intercropping and a rotating system involving *S. plumbizincicola* with other crops may realize the combined benefits of phytoremediation of metal-polluted soil and simultaneously safe production of food; however, activation of soil Cd by *S. plumbizincicola* roots and following effects on crops should be studied further. Repeated pot phytoextraction results indicated that soil properties such as pH and metal concentrations affect growth and efficiency of metal removal. The removal efficiencies of Cd and Zn were high for relatively acidic polluted soils. During repeated phytoextraction, different available metal pools control metal desorption, and acid-soluble, reducible, and oxidizable fractions can be removed by the hyperaccumulator. Field assessment results confirm that phytoextraction using *S. plumbizincicola* is a promising technique for the remediation of slightly Cd-polluted soils without the need to halt normal agricultural production. Finally, fresh biomass after harvest can be cut and crushed in order to reduce volume and be dried quickly, and incineration and pyrolysis may be employed for the disposal and energy-reuse of *S. plumbizincicola* biomass.

Acknowledgements The production of this chapter was supported by the National Natural Science Foundation of China (41325003), and the Chinese High Technology Research and Development Programme (2012AA06A204 and 2006AA10Z406). We thank Alan J. M. Baker and Antony van der Ent for their helpful comments on the manuscript.

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Element Case Studies: Rare Earth Elements

Chang Liu, Ming Yuan, Wen-Shen Liu,
Mei-Na Guo, Hong-Xiang Zheng,
Hermine Huot, Bastien Jally, Ye-Tao Tang,
Baptiste Laubie, Marie-Odile Simonnot,
Jean Louis Morel, and Rong-Liang Qiu

Abstract

The growing demand for strategic resources such as rare earth elements (REEs) for development of modern technologies has spurred an increase in mining activities and consequently a release of REEs into the environment, posing a potential threat to human health. Phytoextraction or phytomining, regarded as an in situ and low-cost means to use the growth and harvest of hyperaccumulator plants that take up high concentrations of metals in their shoots, allowing metal removal from contaminated soil (phytoextraction) or commercial production of high value metals (phytomining). In this chapter, we review the discovery of REE hyperaccumulators worldwide, focusing on the fern species *Dicranopteris linearis* (synonym *D. dichotoma*) that preferentially takes up light REEs and *Phytolacca americana* that preferentially incorporates heavy REEs. Although less well understood, mechanisms of REE uptake, translocation, and distribution in hyperaccumulator plants are discussed. Finally, we analyze the challenge of improving REE yield by phytomining, and taking *D. linearis* and *P. americana* as examples, estimate the phytomining potential for REEs using these species based on their biomass production, REE concentrations in the ash and current market prices for REEs.

1 Introduction

Rare earth elements (REEs), which include 15 lanthanides and yttrium and have similar chemical and geochemical properties, make up a special group of heavy metals studied in the research fields of environmental toxicology and

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C. Liu · M. Yuan · W.-S. Liu · M.-N. Guo ·
H.-X. Zheng · H. Huot · B. Jally · Y.-T. Tang ·
J. L. Morel · R.-L. Qiu
School of Environmental Science and Engineering,
Sun Yat-sen University, Guangzhou 510275,
People's Republic of China

Y.-T. Tang (✉) · R.-L. Qiu (✉)
Guangdong Provincial Key Laboratory of
Environmental Pollution Control and Remediation
Technology, Guangdong Provincial Engineering
Research Center for Heavy Metal Contaminated Soil
Remediation, Sun Yat-sen University, Guangzhou
510275, People's Republic of China
e-mail: eestyt@mail.sysu.edu.cn

R.-L. Qiu
e-mail: eesqrl@mail.sysu.edu.cn

B. Jally · B. Laubie · M.-O. Simonnot
Laboratoire Réactions et Génie des Procédés
(LRGP), Université de Lorraine-CNRS, UPR 3349,
1 Rue Grandville BP20451, 54001 Nancy Cédex,
France

C. Liu · M.-N. Guo · J. L. Morel
Laboratoire Sols et Environnement,
INRAE-Université de Lorraine, 2 avenue de la Forêt
de Haye, TSA 40602, 54518, Vandoeuvre-lès Nancy
Cédex, France

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_24

plant nutrition. According to their atomic mass and effective ionic radius, REEs can be divided into light REEs (LREEs: La, Ce, Pr, Nd, Pm, Sm, Eu) and heavy REEs (HREEs: Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu and Y) (Tyler 2004). In recent decades, rapid industrialization and urbanization have spurred increased use of REEs in modern green technologies, such as high-strength magnets, electric vehicles, and medical devices (e.g. Long et al. 2010). In addition, REE microfertilizers have been widely used in agricultural activities in China for decades, because application of REEs at low levels is thought to benefit both yield and quality of crops (Diatloff et al. 1999; Hong et al. 2000; Pang et al. 2002; Redling 2006). The growing global demand for these elements has triggered an increase in mining activities and subsequent release of REE residues into the environment, leading to locally raised REE contamination in waters, sediments, and soils (Kulaksiz and Bau 2011; Gonzalez et al. 2015; Liu et al. 2019a). High concentrations of REE may induce adverse effects on microorganisms, invertebrates, and plants (Hu et al. 2004; Chen et al. 2002; Feng et al. 2014; Fujita et al. 2015) and pose a potential threat to human health (Haley 1991; Hirano and Suzuki 1996). It has been reported that long-term exposure to REEs may cause damage to the circulatory, immunologic, intelligent, and nervous systems of human beings (Zhu et al. 1996, 1997a, b; Zhang et al. 2000). Consequently, control and remediation of water and soil systems polluted by REEs have become a serious environmental concern.

Phytoremediation is considered a cost-effective and environmentally acceptable process that employs plants to achieve remediation of polluted soil (e.g. Chaney et al. 1997). Among several phytoremediation techniques, phytoextraction or phytomining uses the growth and harvesting of hyperaccumulator plants that take up high concentrations of metals in shoots, allowing their removal from a contaminated site or commercial production of high-value metals (Chaney et al. 1997; van der Ent et al. 2015). The effectiveness of phytomining depends ultimately on the amount of metals that accumulate in the harvestable biomass per year; some plants (e.g. *Odontarrhena chalcidica,* a Ni-hyperaccumulator) meet both the requirements of high biomass yield and metal hyperaccumulation. In addition, a prerequisite of feasible phytoextraction or phytomining is gaining a comprehensive understanding of how targeted metals are assimilated and translocated to the above-ground part of hyperaccumulator plants. Some REEs (e.g. Nd and Y) are becoming the highest supply risk around the world (US Department of Energy 2011). Therefore, recovering REEs from REE-contaminated soils or mine tailings, or from low-grade REE deposits is a promising alternative. To date, the hyperaccumulators of heavy metals such as Ni, Zn or Cd have been intensively studied, but less attention has been paid to mechanisms of REE hyperaccumulation and the potential for REE phytomining.

2 REE Pollution in Soil and Their Bioavailability

The average concentrations of REEs (sum concentration of the all REEs) in Chinese soil and Earth's crust are 177 and 189 μg g^{-1}, respectively (Wei et al. 1991; Liang et al. 2005). However, the soils from some REE mining sites and surrounding areas in China can reach extremely high concentrations of REE (Li et al. 2013a; Liang et al. 2014; Wang and Liang 2015). For example, the exploitation of ion-adsorption REE deposits in South China has resulted in an area of >100 km^2 mine tailings (Guo et al. 2014) that have total REE concentrations of 300–1200 μg g^{-1} (Li et al. 2013a; Liu et al. 2019a). In another REE mine at Baotou City in Inner Mongolia, North China, concentrations of La and Ce in the soil near the REE tailings dam are as high as 11,100 and 23,600 μg g^{-1}, respectively, which are 340 and 481 times greater than the average REE concentrations of local soils (Guo et al. 2013). Moreover, the extensive use of Gd-based contrast agents for magnetic resonance imaging in hospitals also induces REE contaminations in some rivers in Europe and the USA, a problem that may further contaminate the surrounding farmland soils (Kulaksiz and Bau 2011; Hatje et al. 2016). Soil contaminated by REEs has become a serious environmental concern in China and some other countries.

Bioavailability of REE in soil is a critical factor that determines the amounts of these metals that can be accumulated by plants. The REE bioavailability in the soil is generally quite low, although these can be solubilized into soil solution under acid conditions (Khan et al. 2016). Organic matter (OM) has varying effects on the bioavailability of REE, depending on soil properties, REEs species, and OM properties. On the one hand, the presence of organic matter decreases REE bioavailability by adsorption and alkalization; on the other, it could solubilize the insoluble REE into the soil solution by complexation (Tang and Johannesson 2010; Davranche et al. 2011). Other factors such as phosphate precipitation-dissolution, adsorption-desorption on minerals, and oxidation-reduction of Fe-/Mn- oxides, may also influence the bioavailability of REE (see review by Migaszewski and Gałuszka 2015). All of the REEs show trivalent valences, except Ce (III and IV) and Eu (II and III), among which Ce is sensitive to redox conditions. Ce (III) can be easily oxidized to CeO_2 (IV) in the soil, whereas Eu (II) is able to substitute for Ca^{2+}, Na^+ and Sr^{2+} in primary soil minerals, i.e. feldspars (Laveuf and Cornu 2009). Therefore, both Ce and Eu may be less bioavailable than their lanthanide neighbours and hence show negative anomalies in plants (Miao et al. 2011).

In a preliminary survey of ion-adsorption REE mine tailings in Ganzhou, Jiangxi Province, South China, we have found that the available fraction (extracted with 0.01 mM $CaCl_2$) accounted for 11–13% of the total REEs (unpublished data). These proportions are similar to that measured in tailings (14.9%, using the Tessier extraction procedure) collected in Xinfeng county, southern Jiangxi Province (Wen et al. 2013). Because of the large areas covered by REE tailings in parts of Jiangxi, as mentioned above, the relatively high availability of REEs in the tailings soils suggests the potential for agromining using REE hyperaccumulator plants. However, in general, the actual bioavailability is strongly affected by site-specific properties such as pH, and by contents of adsorbing phases including clay, organic matter, and oxides (Groenenberg et al. 2010). Thus, one should evaluate the changes of REE speciation and availability induced by the reclamation practices before attempting agromining. Organic amendments are used to tackle the limiting factors (e.g. high bulk density, lack of organic matter, nutrients and clays) for successful plant colonization on mine tailings (Liu et al. 2015; Nkrumah et al. 2016). A pot experiment showed that the addition of organic material or biochar can significantly decrease the REE concentrations in soil solution, leading to lower REE concentrations in the shoots of *Phytolacca americana* grown on REE ion-adsorption mine tailings (Liu et al. 2020a). In addition, plant-induced processes involved in the mobilization of nutrients such as Fe or P could also influence the bioavailability of REE (e.g. Nakamaru 2006; Brioschi et al. 2013; Wiche and Heilmeier 2016). The role of rhizospheric processes in the mobilization and transport of REEs in hyperaccumulators is still poorly studied. For instance, the pot experiment revealed that planting with *P. americana* induced a soil acidification, which could be linked with strategies developed by the plant for the acquisition of P (Liu et al. 2020a).

3 REEs Hyperaccumulator Plants

The threshold concentration for REE hyperaccumulators is notionally defined as 1000 $\mu g\ g^{-1}$ in dry biomass of the aerial parts (Wei et al. 2006). This criterion is the same as that used for other trace metals (Co, Cu, Ni, Pb, Sb), metalloids (As), and nonmetals (Se), typically two or three orders of magnitude greater than that for normal plants (e.g. van der Ent et al. 2013). Moreover, the bioaccumulation factor (BF), which is the ratio of REE concentration in shoots to that in soil, is generally required to be greater than one, thus being indicative of a high ability of soil-to-plant REE transfer (Krzciuk and Galuszka 2015). To date, approximately 21 plant species have been reported through field investigations to accumulate or hyperaccumulate

REEs; these species occur across 11 families and 11 genera, with the greatest numbers being within ferns (Table 1).

The discovery of high REE accumulation in plants can be traced to the 1930s. In studies of mutual dependence of the soil-plant composition, Robinson and Scribner (1938) and Robinson (1943) found, quite by accident, that hickory (*Carya cathayensis*) leaves contained detectable REEs. The REE concentrations in the hickory leaves ranged from 3–2300 μg g^{-1} dry weight (DW), with the highest level found in plants that had grown on soils derived from granite and gneiss. Moreover, the ash of these leaves contained more than 2.5 wt% REEs. Later, high concentrations of REE in dry leaves (e.g. >1000 μg g^{-1}) were reported in a number of plant species including *Carya tomentosa* (1350 μg g^{-1} in ash; Thomas 2011), *Dicranopteris linearis* (3358 μg g^{-1}; Wang et al. 1997; Fig. 1a and b), *Blechnum orientale* (1022 μg g^{-1}; Xiao et al. 2003), *Pronephrium simplex* (1230 μg g^{-1}; Lai et al. 2005), and *Pronephrium triphyllum* (1027 μg g^{-1}; Xue 2009). Despite lower capacity for REE accumulation, *Phytolacca americana*, a high-biomass plant that is naturalized worldwide (Fig. 1c), accumulated REEs up to 623 μg g^{-1} in dry leaves, although plant REE concentrations varied substantially among sampling sites (Ichihashi et al. 1992). Recently, our field survey observed a large presence of *P. americana* in an area with ion-adsorption REE mine sites in south Jiangxi Province, where the highest concentration of

Table 1 Rare earth element (REE) concentrations in the shoots of potential accumulator plants (mg kg^{-1} dry matter)

Family(1)	Species	REE concentration	Location	Reference
Gleicheniaceae	*Dicranopteris linearis*	Up to 7000(2)	China	Shan et al. (2003)
	Dicranopteris strigose	12; BF > 1(3)	Japan	Ozaki et al. (2000)
Juglandaceae	*Carya cathayensis*	2300(2)	America	Robinson (1943)
	Carya tomentosa	136(2)		Thomas (1975)
Thelypteridaceae	*Pronephrium simplex*	1230(2)	China	Lai et al. (2005)
	Pronephrium triphyllum	1030(2)		Xue (2009)
Phytolaccaceae	*Phytolacca americana*	1040(2)		Yuan et al. (2019)
Blechnaceae	*Blechnum orientale*	1020(2)		Xiao et al. (2003)
	Woodwardia japonica	367(2)		
Lindsaeaceae	*Stenoloma chusana*	725(2)		
Athyriaceae	*Athyrium yokoscence*	202(2)	Japan	Ichihashi et al. (1991)
Dryopteridaceae	*Dryopteris erythrosora*	32; BF > 1(3)		Ozaki et al. (2000)
	Dryopteris fuscipes	10; BF > 1(3)		
Aspleniaceae	*Asplenium filipes*	25; BF > 1(3)		
	Asplenium hondoense	14; BF > 1(3)		
	Asplenium ruprechtii	40; BF > 1(3)		
	Asplenium ritoense	12; BF > 1(3)		
	Asplenium subnomale	14; BF > 1(3)		
	Asplenium trichomanes	21; BF > 1(3)		
Blechnaceae	*Blechnum niponicum*	9.7; BF > 1(3)		
Adiantaceae	*Adiantum monochlamys*	11; BF > 1(3)		

The table is adapted from Wei et al. (2006) with minor modifications. *(1)* The La concentration in *Glochidion triandrum* (Euphorbiaceae) leaf is ≤ 1 mg kg^{-1}, thus not a REEs accumulator and is removed from the figure; *(2)* means the total concentrations of the sum of 16 rare earth elements; *(3)* means the La concentration in the shoot, BF means bioaccumulation factor (i.e. La in the shoot/La in the soil)

Fig. 1 **a** *Dicranopteris linearis*, a natural perennial fern species widely distributed in southern China, is a LREE hyperaccumulator plant around REEs mining sites of Ganzhou, Jiangxi Province; **b** *D. linearis* grows on ion-adsorption REE mine tailing; **c** *Phytolacca americana* L., a high-biomass herbaceous plant which prefers to accumulate high HREE, could colonize and complete its life history on REEs mine tailings of Ganzhou, Jiangxi Province; and **d** *P. americana* grows on ion-adsorption REE mine tailings amended with organic materials

REEs reached 1040 μg g^{-1} in leaves. *P. americana* exhibited a preferential accumulation of LREEs during soil-to-root assimilation, and a preferential accumulation of HREEs during stem-to-leaf translocation (Yuan et al. 2018). Moreover, other potential hyperaccumulator species have been reported (Table 1), all of which are ferns. For example, Ozaki et al. (2000) reported that many pteridophyte species enriched in La and Ce belong to genera such as *Polystichum* and *Dryopetris* in the Dryopteridaceae, *Diplazium* in the Woodsiaceae, and *Asplenium* in the Aspleniaceae. A recent pot experiment showed that additional fern species are likely to accumulate REEs (Grosjean et al. 2019a). The reason why ferns accumulate high concentrations of REE in shoots remains uncertain. One possibility is that this process may render ferns to better adapt to changing environments (Ozaki et al. 2000).

Among the reported REE hyperaccumulator plants (Table 1), a majority of studies has focused on *D. linearis*, a fern species widely distributed in sub-tropical and tropical climatic zones in the Old World and Pacific region (Fig. 1a and b). The concentration and

Table 2 Information of sampling sites and concentration and characteristic parameters of REEs in samples of *Dicranopteris linearis*

Study area	Soil type	∑REEs in leaf	∑REEs in soil	BFs	LREE/HREE	δCe	δEu	Referencece
Fujian, China	REE mine	3260	414	7.88				Li et al. (1992)
Guangxi, China	Non-mining area	1910	15	130				
Shikoku Island, Japan	Non-mining area	412						Ichihashi et al. (1992)
Guangdong, China	Gold deposit	1960	277	7	159	0.67	0.49	Miao et al. (2011)
Jiangxi, China	Background area	424	159	3	77.8	5.98	0.43	
	Background area	225	93.6	2	109	11	0.52	
	LREE-enriched mine	1660	1030	2	18.4	0.14	0.23	Wei et al. (2001)
	LREE-enriched mine	22,710	1220	2	9.47	0.54	0.23	
	REE Mine where both LREEs and HREEs were enriched	1410	342	4	5.01	0.57	0.32	
	HREE-enriched mine	977	195	5	2.20	0.13	0.13	
	Non-mining area	1120	15	75	40.8	4.20	0.25	
	LREEs mine	2650	331	8	6.78	0.26	0.40	Wang et al. (2005)
	HREEs mine	2090	207	10	6.90	0.24	0.05	
	Non-mining area	1500	–	–	24.3	0.51	0.40	
Fujian, China	REE mine tailings	2190	202	11	–	–	–	Li et al. (2013a)
	Non-mining area	351	499	0.7	–	–	–	
Jiangxi, China	REE mine tailings and Non-mining area	Young: 479	–	–	–	–	–	Liu et al. (2019b)
		Middle: 1560						
		Mature: 2750						

∑REEs: the sum of 16 rare earth elements
BFs (bio-concentration factor): the ratio of the concentration in leaf of a plant to the concentration in the soil
LREE/HREE: the ratios of the sum concentrations of light rare earth elements (LREEs, La to Eu) to heavy rare earth elements (HREEs, Gd to Lu)
δCe: δCe = Ce/(0.5Pr + 0.5La), the value of Ce, La and Pr are chondrite-normalized
δEu: δEu = Eu/(0.5Sm + 0.5Gd), the value of Eu, Sm and Gd are chondrite-normalized

fractionation parameters of REEs in samples of *D. linearis* from different sampling sites reported in the literature are summarized in Table 2. Total REE concentrations of *D. linearis* fronds differed significantly among sampling sites, ranging from 225–3260 μg g^{-1}; bioconcentration factors (BFs) of all plant samples were mostly greater than one, indicating its ability to accumulate and transport REEs, especially LREEs. Furthermore, strong negative Eu anomalies were observed in all *D. linearis* samples. Positive Ce anomalies occurred in the shoots of *D. linearis* that grew on background sites and unmined area, whereas negative Ce anomalies were present on REE or gold mining areas in South China. Cerium in *D. linearis* from background sites and unmined area showed the greatest ability for transportation from the underground to above-ground biomass (Wei et al. 2001; Wang et al. 2005; Miao et al. 2011; Li et al. 2013b).

4 Mechanisms of REE (Hyper) Accumulation

4.1 REE Uptake

Previous studies have reported that REE uptake is related to the available REE fractionation patterns (e.g. ratios of different REEs) in the soil. In the naturally grown REE hyperaccumulator *D. linearis*, Wei et al. (2001) found that the fractionation pattern of soluble REEs in the soil A layer was very similar to that in roots and that; Ce anomalies in *D. linearis* organs were like those of the soluble REEs in the soil A layer. The negative Ce anomaly present in this plant was more obvious than that in the soil. Rare earth elements in soils generally occur in trivalent state except Eu and Ce. Under oxic conditions, Ce is typically present in tetravalent form (Ce^{4+}) that is more likely to precipitate by a hydrolytic reaction, making Ce difficult to accumulate in plants. Therefore, this phenomenon shows that the distribution characteristics of soil-soluble REEs may directly influence REE uptake by plants.

Through electron microscopic and X-ray microanalyses of *D. linearis*, concentrations of LREEs were observed in the cell walls, intercellular space, plasmalemma, vesicles, and vacuoles of the root endodermis and stele cells, but not in the Casparian strip of the adventitious roots. Moreover, LREE deposits were observed in the phloem and xylem of the rhizome (Shan et al. 2003). These results suggest that in *D. linearis*, both symplastic and apoplastic transport mechanisms for REE co-exist (Shan et al. 2003). Because of the similar ionic radii and physiochemical properties of trivalent REEs and Ca^{2+}, the Ca^{2+} channel is considered to be a specific pathway for the uptake of REEs. It was demonstrated that La^{3+} might enter into root cells via the membrane and intracellular Ca^{2+} channels; La also participated in signal transduction networks via Calmodulin (CaM) (Liu et al. 2012). However, Yuan et al. (2017) found that only 23.9% of the REE concentrations in the roots of *P. americana* were decreased by the treatment of Verapamil (a Ca^{2+} ion inhibitor), indicating that REE uptake by hyperaccumulators may rely on other transporters. In a hydroponic experiment, it was determined that apart from Ca, REEs may enter through the cell membrane of barley (*Hordeum vulgare*) via the K^+ and Na^+ channels (Han et al. 2005). Yuan et al. (2017) found that the concentrations of REE in *P. americana* can be diminished by treatment with Al^{3+}, especially in the case of HREEs because the ratio of $\sum LREE/\sum HREE$ in roots showed a significant increase, which indicates that the HREEs may share pathways with Al^{3+} in *P. americana*. Moreover, a recent study reported that the accumulation of REEs in *P. americana* may be associated with Mn, an element also hyperaccumulated by this plant (Grosjean et al. 2019b). These results suggest that the REE uptake by hyperaccumulators may relate to the uptake of K, Na, Ca, Mn and Al. However, the physiological, molecular, and biochemical mechanisms of REE uptake by hyperaccumulators are still not fully understood.

In addition, low-molecular amino acids and organic acids enhance the uptake and sequestration of LREEs in *D. linearis*, although the enhancement mechanism of each acid is different. In the soil-root system, organic acids (e.g. citric and malic acids) increased LREE desorption from soil, and thus enhanced the uptake of LREEs into the soil solution by fern roots; histidine may promote the sequestration of LREEs in cells by forming complexes with LREEs (Shan et al. 2003). Extraneous additions of aspartic acid, asparagine, histidine, and glutamic acid in a hydroponic solution enhanced La absorption in tomato and *P. americana*, compared with La treatment alone (Wu et al. 2013). Therefore, aspartic acid and asparagine could potentially promote the phytoextraction of La from contaminated soils when these compounds are used as chelators.

4.2 REE Translocation

Through field investigation, Wei et al. (2001) compared the accumulation coefficient of the soil-plant system and found that REE transport was efficient from the soil solution of the surface soil layers to roots, and from petioles to lamina, but was more difficult from stems to petioles. The most conspicuous fractionation of LREEs from HREEs appeared during the translocation of REEs from stem to petiole. In addition, Shan et al. (2003) found a modest increase of LREEs in fern fronds amended with histidine and proposed that free histidine may be involved in the uptake and translocation of LREEs from roots to fronds, and in the sequestration of LREEs in fern fronds. In another study using tomato, it was reported that aspartic acid, asparagine, histidine, and glutamic acid may be related to xylem long-distance transport of La and Y in tomato (Wu et al. 2009). In addition, citric acid may also play an important role in the REE translocation process. In the xylem sap of *P. americana*, Yuan et al. (2017) found that citric acid can ligate with most of the REEs, particularly HREEs. Similarly, in wheat, HREE enrichment was found in leaves, being ascribed to the formation of stronger organic complexes of HREEs than LREEs within the xylem solution, thus suggesting that organic acids are involved in the long-distance transport of REEs, especially HREEs (Ding et al. 2007). All in all, amino acids (e.g. histidine) and small organic acids (e.g. citric) may facilitate the translocation of LREEs and HREEs, respectively, in hyperaccumulator plants. However, whether these compounds can form complexes with REEs and facilitate the translocation of REEs still needs direct evidence and further investigation.

4.3 REE Distribution

The distribution of REEs among the main organs of vascular plants differs considerably. However, roots typically have higher concentrations than other plant organs. Numerous studies have shown the decrease of REE concentrations in the order roots > leaves > stems, in a variety of crops such as wheat and maize (Xu et al. 2002; Ding et al. 2007). Conversely, REE hyperaccumulators exhibited decreasing REE concentrations in the order leaves > roots > stems > petioles (Wei et al. 2001). Furthermore, the total amount of REEs within different subcellular fractions of the *D. linearis* leaves were cell walls > organelles > the ‘soluble’ fraction (i.e. cytosol and vacuoles) > cell membranes (Wei et al. 2005). In the leaves of *D. linearis*, REEs are preferentially localized at some physiologically inactive cellular sites and tissues. In a recent study using Synchrotron Radiation-based X-ray Fluorescence, we found that LREEs ($\sum$La + Ce) are mainly localized at the necrotic tissues and epidermis of pinnae, whereas less is distributed within veins (Liu et al. 2020b). Organic acid seems to play an important role in REE sequestration within *D. linearis*, based on the high concentrations of histidine identified in the leaf cells (Shan et al. 2003). However, Liu et al. (2019b) found similar extraction patterns of Al/REEs in *D. linearis* pinnae to that of Al_2SiO_5 and confirmed the existence of phytoliths (Al) deposits in the protoplast and apoplast of the pinna cells, indicating that REEs are probably present in the form of REE-Si complexes in this species.

Although REEs have little phytotoxicity to plants at low concentrations, these metals may influence the physiological activities of plants. In naturally grown *D. linearis*, La and Ce were found to be bound to chlorophyll a (Zhao et al. 1999; Wei et al. 2005); and REEs might replace or partly replace Mg in chlorophyll (Wei et al. 2004). The role of REE-bound chlorophylls in photosynthesis cannot be neglected. Moreover, REE detoxification by proteins is likely one of the mechanisms against physiology toxicity by REEs (Wang et al. 2003).

5 Potential for Agromining of REEs

Phytomining or agromining is a technology that uses hyperaccumulator plants to take up heavy metals and recover them from the biomass for resource cycling. Phytomining technology has been applied in Ni phytoextraction by using the Ni hyperaccumulator *O. chalcidica* (Zhang et al. 2014; van der Ent et al. 2015). Economic feasibility of phytomining depends on market price for the element, annual yield per unit area (biomass produced and contained amount of target element), and availability of surface areas enriched in the element (van der Ent et al. 2015). Current (2020) prices, in US dollars per tonne, are high to very high for Pr_2O_3 ($47,000), Nd_2O_3 ($43,000), Eu_2O_3 ($31,000), Gd_2O_3 ($25,000), Tb_2O_3 ($610,000), Dy_2O_3 ($278,000), Ho_2O_3 ($54,000), Er_2O_3 ($23,000), Yb_2O_3 ($16,000), Lu_2O_3 ($6,200,000), and Sc_2O_3 ($1,268,000), but relatively low for La_2O_3 ($1800), Ce_2O_3 ($2000), Sm_2O_3 ($2000), and Y_2O_3 ($3000). These prices are from the website of the Association of China Rare Earth Industry (http://www.ac-rei.org.cn/portal.php) and were converted from renminbi (RMB) into US$ as of 20 March 2020.

The success of phytomining depends largely on the yield of target metals, namely the product of the concentrations of target metals in the shoots by the quantity of biomass. REE ion-adsorption mine tailings in South China have soil physico-chemical properties (e.g. lack of nutrients, organic matter and clays) unfavorable to the growth of most plants. Therefore, it is necessary to add soil amendments before planting. However, as mentioned above, amendments such as organic materials (OM) and biochar (BC) reduced soil REE availability, causing negative impacts on phytoextraction, even though these amendments can significantly increase plant biomass. We compared the effects of different dosages (1 and 5%) of OM and BC on the REE yield of *P. americana* grown on REE-rich ion-adsorption mine tailings, finding that the highest REE yield was obtained using 1% BC treatment (Liu et al. 2020a). Based on this study, we concluded that amendments with a low dosage of BC could be a potential means to optimize REE phytomining by *P. americana*. However, how to balance the increase of plant biomass and high REE concentrations in plant shoots depends on the type of hyperaccumulating plants, the nature of substrates and reclamation practices, all of which require more research.

Except for *P. americana*, *D. linearis* is also appropriate for REE phytoextraction. *D. linearis* is a native and pioneer fern that shows high tolerance to the unfavored soil properties of the ion-adsorption REE mine tailings. Based on our field survey with *D. linearis* collected at mine tailings in Ganzhou, Jiangxi Province, we could expect to harvest 12–15 t of dry biomass of shoots per ha containing 0.2 wt% REEs, yielding 24–30 kg REEs ha^{-1} (unpublished data).

In the same way as for Ni phytomining, several methods can be explored to recover REE from ferns. The first stage is combustion, required to remove organic matter and obtain a concentrated bio-ore (Table 3). Alternatively, the REE contained in the plant can be recovered by direct biomass leaching. Processes should, however, be adapted to the specificity of the hyperaccumulating plant(s), and especially to their high contents of Si, Al and Mn in *D. linearis* and *P. americana*. Recently, an eco-friendly method of vacuum pyrolysis condensation was proposed for disposing the biomass of *D. linearis* in order to concentrate REEs in the residua ash. It was found that La had the highest concentration in *D. linearis* samples (929 mg kg^{-1}) and in residual ash (2964 mg kg^{-1}), and that the total REE concentration in residual ashes reached 6160 mg kg^{-1} (Qin et al. 2019). Hence, the residual ashes, having higher REE concentration, could be considered as a rich REE bio-ore that could further be extracted by leaching, chemical precipitation, and other techniques. Moreover, chemical-resource ethylene oxide was detected in the pyrolytic products derived from the REE-rich biomass of *D. linearis*, and no hazardous wastes were produced during the process (Qin et al. 2019). However, the residual ashes mainly contain amorphous phases, which make effective acidic leaching nearly impossible (Laubie et al. 2016). The presence of Si in *D. linearis* leads to the formation of a glass, stabilizing REEs. Other processes are currently in development, e.g. direct extraction (Chour et al. 2020). An original process relies on an enhanced ion exchange leaching step carried out in 0.5 M nitric acid

Table 3 REEs concentration of *Dicranopteris linearis* collected from Ganzhou, Jiangxi province

REE	REEs concentration in dry plant	REEs concentration in ash (500 °C, 2 h)	REEs concentration in ash is converted into that in dry plant	Recovery rate (%)
La	983	7575	893	91
Ce	180	1421	167	93
Pr	167	1310	154	92
Nd	504	4069	480	95
Sm	65.9	527	62.1	94
Eu	9.21	72.4	8.52	93
Gd	43.5	348	41	94
Tb	4.73	36.4	4.29	91
Dy	17.7	134	15.7	89
Ho	2.49	18.7	2.20	89
Er	4.98	40.7	4.80	96
Tm	0.50	4.80	0.57	114
Yb	3.23	23.5	2.77	86
Lu	0.50	4.05	0.48	96
Y	45.8	371	43.7	95
Sc	0	2.02	0.24	–
∑REEs	2032	15,956	1881	93

solution. Once the ion exchange resin is transferred into a column, REE purification is carried out by percolating successively three solutions through the resin bed: two washing steps, with water and 0.75 M nitric acid to remove competing ions, and one elution step using 3 M nitric acid. These operating conditions led to 81.4% REEs purity and 78% recovery (Chour et al. 2018).

6 Conclusions

The widespread use of REEs in modern technologies have in some areas resulted in REE pollution in soils and caused serious problems for human health. Phytoextraction is a feasible means to decrease the harmful effects of REEs in soil, and thus has great potential as a method for in situ, low-cost remediation. Some plants like *D. linearis* and *P. americana* have been proved to possess a high accumulation potential for REEs, and hence a potential for REE phytomining. The uptake of REEs by hyperaccumulator plant roots may be related to K, Na and Ca ion channels, and to Al and Mn transporters. Low molecular weight organic acids and amino acids likely contribute to REE translocation. In the shoots, REEs are preferentially localized within some physiologically inactive tissues. Co-deposition of Si with REEs and Al may be a mechanism for dealing with the high concentrations of REEs and Al in *D. linearis* fronds. The above factors can also result in different fractionation characteristics among different plant organs. However, additional studies are needed using a combination of plant physiological, molecular biological, and geochemical tools in order to better understand specific mechanisms of REE fractionation and accumulation in plants. The large scale of agro-mining practices, and the potential extraction and purification of REEs from these hyperaccumulators, still remain as major challenges.

Acknowledgements This work was financially supported by the Key R&D Program of China of Jiangxi province (Grant No. 20192ACB70016); the Natural

Science Foundation of China (Nos. 41371315, 41225004, 41771343, and 21850410450); and the 111 project (Grant No. B18060); Fundamental Research Funds for the Central Universities (No. 15lgjc36); Science and Technology Planning Project of Guangdong Province, China (No.2016A020221013); and Science and Technology Transfer Program of Sun Yat-sen University. The International Joint Lab—ECOLAND is thanked for supporting this work. We also acknowledge the Sino-French Cai Yuanpei Programme (Grant No. 38896SC).

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Conclusions and Outlook for Agromining

Jean Louis Morel, Marie-Odile Simonnot,
Guillaume Echevarria, Antony van der Ent,
and Alan J. M. Baker

The technology of agromining has created a new era in the recovery of strategic metals from natural to secondary resources, including industrial wastes. During the last decades, hyperaccumulator plants have changed recognition from being merely a botanical curiosity to a prospect having tangible socio-economic and environmental applications. The knowledge base for this group of plant species, having unique properties capable of surviving and thriving in toxic and stressful environments, has increased greatly during the last 20 years, thanks to thorough investigations at various scales involving several disciplines including botany, ecology, ecophysiology, microbiology, soil science and agronomy. Processes and mechanisms that preside over the hyperaccumulation of toxic metals and metalloids in plants are now better understood. For example, the fate and biopathways of elements in plants is actively being investigated using powerful new explanatory techniques, such as synchrotron and microprobe analysis. In addition, new species having exceptional ability to accumulate metals have been discovered, thereby considerably increasing the number of known hyperaccumulators. In parallel, work conducted by agronomists and soil scientists has allowed the domestication of selected hyperaccumulator species and hence enabling large-scale implementation of agromining. This approach promotes a new form of agriculture, which could generate income for peoples in developing countries that live on agriculturally mediocre lands, such as those derived from ultramafic bedrock. It also provides new opportunities for the industrial sector through the recovery of strategic elements from contaminated soils and wastes, but also a benefit from metal resources that are produced in a different way with considerably reduced carbon and biodiversity footprints. Finally, the application of scientific knowledge in the chain of research and development, such as chemistry and chemical engineering, and the stimulation of pluri-disciplinary research programs, brings hope to the feasibility of manufacturing specialist products of high industrial interest from agromined bio-ore.

Phytoextraction is a branch of phytoremediation that specifically aims to remove toxic metals and metalloids from polluted media (e.g. soils, sediments, wastes) using hyperaccumulator plants. The biomass derived from phytoextraction operations is considered contaminated waste, but in phytomining it becomes the source

J. L. Morel (✉) · G. Echevarria · A. J. M. Baker
Laboratoire Sols et Environnement, Université de Lorraine-INRAE, 54000 Nancy, France
e-mail: Jean-Louis.Morel@univ-lorraine.fr

M.-O. Simonnot
LRGP, Université de Lorraine-CNRS, 54000 Nancy, France

G. Echevarria · A. van der Ent · A. J. M. Baker
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, QLD 4072, Australia

A. van der Ent et al. (eds.), *Agromining: Farming for Metals*, Mineral Resource Reviews,
https://doi.org/10.1007/978-3-030-58904-2_25

of valuable metals—a true bio-ore. Agromining encompasses the entire chain of processes that leads to the production of compounds of industrial and economic interest, by which agricultural systems are applied to metalliferous resources. Indeed, agromining is a combination of technologies that has opened a new sector aimed at producing strategic and valuable elements from agriculture and agroforestry. Agromining should not be considered a substitute for conventional mining processes: it will not replace current mining technologies for satisfying demands by the global market for metallic elements, if only by the economy of scale. However, agromining can exploit some mineral resources inaccessible to conventional mining because of the low concentration and dispersion of the elements of interest, and therefore represent a valuable complement (with a very low environmental impact) to the exploitation of natural ore deposits, while also allowing for the utilization of resources that are otherwise uneconomic for metallic elements, especially in industrial wastes, mine tailings, polluted soils, and sediments.

Apart from areas that host metallic deposits or are polluted (e.g. areas impacted by smelting or mining), there are numerous sources of secondary materials derived from industrial activities that may be used as matrices for agromining. As such, agromining adds to the circular economy and is another means for effective recovery of valuable elements from (industrial) wastes. Although Ni agromining has been successfully demonstrated at different scales (including field scale) on ultramafic soils, agromining is not confined to Ni as many other elements may be agromined. These include elements of industrial and economic interest such as Cd, Co, platinum group elements (PGEs), rare earth elements (REEs), and even Au, which are present at low concentrations in certain soils or mine tailings. Many of these elements can be recovered provided that the market and its fluctuations do not impair the economic value of the chain. Recovery of metallic elements of industrial interest, as well as minerals whose natural sources are increasingly depleted (e.g. P), are major challenges that agromining should carefully consider in the long term.

The performance of agromining is closely related to available resources of the target elements and to the ability of plants to grow and extract these elements from the resources. In particular, the extent of bioavailability of the target elements in soils is a major factor that determines the potential of a site for agromining. This knowledge on bioavailability is of high value for developing a means of manipulating soil metal availability (e.g. in the rhizosphere), and for promoting practices that ensure maximum transfer of elements to and within plants. However, plants remain the key ‘tool’ in agromining, and improvement of yields relies on knowledge of their physiology and ecophysiology, and on our capacity to produce efficient cultivars. Agromining is still at an early stage of development, wherein seeds used in this process are rarely derived from an appropriate selection of the best cultivars, and plant breeding and propagation of selected varieties is one of the main remaining challenges for future development and profitability. Therefore, attention should be drawn to both quantitative and qualitative performance of ‘metal crops’. Quantitatively, the doubling or even tripling of the quantities of extracted metal yields with selected plants and appropriate agronomy can be forecast. Qualitatively, the limited number of elements of interest that can be recovered could be increased; selection strategies should be oriented towards the most relevant elements, considering their intrinsic value as well as the value of the land where these elements can be potentially mined (e.g. susceptible and fragile ecosystems and organisms that are negatively affected by conventional mining activities). Despite the substantial research efforts demonstrated in this book, there is still insufficient knowledge on the botanical reservoir, phylogeny, physiology, ecophysiology and genetics of hyperaccumulation by plants. Molecular approaches for genomics, transcriptomics, and metabolomics should be encouraged in order to better understand the multiple mechanisms of hyperaccumulation phenomena in plants, and to make this unique property a means of sustainable mineral-resource management.

Ultimately, agromining should be subjected to the same rules as conventional production systems such as major crops (e.g. wheat, rice, soybean). In food agriculture, production is driven by the market, which controls the demand in quantity and quality of products to be traded. This situation is similar to that for agromining, and decisions made by all actors along the chain of production (e.g. plant breeders, agronomists, farmers) are dependent on market needs. In addition, with growing awareness of the fragility of ecosystems, raised by the Millennium Ecosystem Assessment, and subsequently requested sustainable management, agromining should be equated with ecosystem conservation goals and hence follow the general trend of agricultural evolution. Sustainable development goals, as stated by the Strategic Plan for Biodiversity (2011–2020), should be one of the key motivations for agromining, carried out in such a way that it contributes significantly to the conservation and enhancement of biodiversity, in particular by using rare native or even endemic hyperaccumulator species. Indeed, agriculture is changing from intensive systems, often monospecific with high inputs (i.e. fertilizers, pesticides, energy, water), to more complex systems that rely heavily on soil biological functions. Within those agroecology and agroforestry systems, multi-functionality is sought in order to provide and optimize a wide range of ecosystem services. As a result, agromining could generate a wider range of services than only the provision of particular metals, including regulating services that lack negative impact on biodiversity, and positive outcomes for carbon neutrality (or carbon sinks), contamination attenuation, and erosion control. Rehabilitation of mine sites and the cultivation of ultramafic soils by agromining are suitable applications that promote this approach.

We believe that the ultramafic outcrops in many equatorial regions, especially South America, Southeast Asia, and the Pacific region, have tremendous potential for developing agromining of Ni. In these regions, agromining may provide opportunities for improving degraded land to make it suitable for other uses, by offering local communities a new source of income and hence a contribution to sustainable livelihoods. Agromining removes most of the Ni from the soil during the 10–30 years of typical operation, and as a result improves soil fertility making it suitable for other future land uses. Unlike the competition between food crops and biofuels on fertile soils, agromining does not replace food crop production, but is a temporal activity that improves soil quality sufficiently to allow food crop production in improved conditions after the metal resource has been extracted.

A mature and sustainable agromining system will fit both industrial and market demands for bio-sourced compounds and contribute to ecosystem services (e.g. no negative impact on and even improvement of biodiversity, reduction of soil run-off, carbon neutrality, etc.). It is rare in agricultural history to have such a new production system emerging almost *ex nihilo* and within such a short period of time. If research has made sufficient progress to ensure the transfer from concept to practical application, the concept must not mask the sophistication of the subject and the numerous questions that remain to be addressed in order to make this technology effective in the long term. The danger of overly rapid communication without sufficient proof of concept, thus raising false expectations, is often the main reason of failure when launching a new technology. Establishment of large-scale agromining must be based on sound scientific information, like for more traditional agricultural production systems. Furthermore, the application of a low-impact agromining at field scale, thus respecting the concepts of agroecology and agroforestry, appears to be a feasible and sustainable model of development both for disadvantaged regions and those areas that have suffered from excessive exploitation of natural resources (e.g. overlogging, land clearing). Finally, it is important to be aware that any chain of production has not only technical implications, but is mainly controlled by social and economic constraints that are major drivers of the chain, as well as regulatory constraints that may in some cases become limiting, e.g. the legal status of plants containing metals and of the bio-ore. However, progress is being made to give agromining an official legal status. In France, the recent Act on Circular Economy of 10 February 2020, now explicitly mentions agromining as a means to exploit secondary resources. Overall, we have a positive outlook on seeing the expanded development of agromining for several elements, including metals, metalloids, and other minerals of strategic value, at scale, carried out in various regions during the next decade.

In the last few years we have seen many indications that agromining is gaining momentum. In Europe, a milestone was reached with the creation of an active network of academic researchers collaborating to implement Ni agromining at the demonstration scale. This network included two large EU-funded projects started in 2016 that aimed to promote the agro-ecological revolution of agromining and the implementation of pilot-scale agromining demonstrations, both following the Agromine program funded by the French National Research Agency (ANR 2013–2018). The Agronickel project (ERA-NET FACCE Surplus), which involved scientists and companies from seven countries, created field demonstrations in Albania, Austria, Greece, and Spain. The project encompassed the full chain from producing 'metal crops' to metallurgical processing of bio-ores, ending with a Life Cycle Assessment. In China, agromining of REEs and Ni from land mined and degraded by industry is also progressing, adding to the international network on agromining. The recent discoveries reported in this book add to the global inventory of known hyperaccumulators to draw from for selecting suitable 'metal crops'.

The EU-funded 'LIFE Agromine' Project (Programme LIFE 2014–2020–LIFE/ENV/FR/000512) first established full-scale demonstration pilots of the entire agromining chain from field plots to produce bio-ore to the recovery of biomass energy and pure Ni salts. These plots were located in five European countries and established on several hectares of agricultural ultramafic soils, on land degraded by serpentinite quarrying and on Technosols derived from Ni-rich industrial wastes. Some of these areas have already been cropped for four years, and new standards for the cultivation of several Ni hyperaccumulator species have been established based on this unique experience of full-scale implementation with a strong emphasis on agroecological practices. The energy conversion of hyperaccumulator biomass was also optimized (energy recovery, gas emissions) as well as the processes needed to purify the Ni products as demonstrated by a semi-automated pilot. Altogether, the pilots have allowed for complete techno-economic and life cycle assessments. Stakeholders including farmers, the mining industry, energy producers, metal-waste producers, the metal industry and several countries were involved in events aimed at presenting the technology. This project is a landmark in agromining history and has proven the technical, environmental, and economic efficiency of agromining. It has also awakened strong interest from the press and media, especially because it involved several small and medium-sized enterprises (SMEs) that are currently implementing agromining.

Finally, several Ni mining companies have shown interest in using agromining to develop a new way of managing mine rehabilitation. This promising technology could help improve the quality of reconstructed ecosystems by using hyperaccumulator plants as a pioneer vegetation cover while also producing additional Ni from low-grade ores and mined lands. Industries that produce large quantities of metals and consequently thousands of tonnes of metal-rich waste are also interested in finding a more sustainable and circular way of managing the sourcing and recycling of valuable metals from their wastes. Metal and alloys producers have also shown great interest in agromining and a new means for sourcing low-carbon impact metals. It is now timely to set an official frame, and in particular a regulatory frame, for this new branch of metal production so that agromining can develop in a sustainable way and create value and job opportunities in the growing field of Green Economy.

On the other side of the world, in New Caledonia and Malaysia, and now in tropical America, the recent herbarium X-ray Fluorescence (XRF) screening of thousands of herbarium specimens has led to the discovery of hundreds of new Ni-hyperaccumulator species in less than five years. Only a very small proportion of these plants will show a favourable combination of characteristics to be useful as ‘metal crops’ in agromining, such as high accumulation <1 wt%), fast growth-rates, and adaptability to grow in exposed conditions on poor soils. However, this fast and exponential increase of new potential hyperaccumulator candidates opens new perspectives to metal agromining (new resources, new regions of the world, new elements to be agromined). We anticipate that extending herbarium XRF scanning to other regions of the world (especially in extremely resource- and plant species-rich countries such as Brazil, Cuba, South Africa, etc.) will undoubtedly yield a treasure trove of potential ‘metal crop’ candidate species, not only for Ni, but also for other elements such as Co, Mn, Zn, noble metals and REEs. Finally, we believe that agromining is a form of production of mineral commodities that heralds a new trend in the sustainable management of natural resources to meet the needs of modern technologies. Examples of using bio-sourced metals by industry are already available (e.g. the crystal art industry). More generally, agromining can be understood as a means of designing future mining/industrial production chains, in other words, a model test to build the foundation for a new era of sustainable human development.