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Metallophytes of serpentine and calamine soils – their unique ecophysiology and potential for phytoremediation

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Abstract

For years, metallophytes of both natural and human-influenced metalliferous soils have focussed considerable attention due to their unique appearance and ability to colonise often extremely harsh habitats. A majority of metal-contaminated areas comprise serpentine (ultramafic, rich in Ni, Cr, and Co) and calamine (rich in Zn, Pb, and Cd) soils hosting characteristic serpentine and calamine flora, which is the focus of this review. Through microevolution, the plants inhabiting metalliferous habitats have developed a range of intriguing adaptive traits, demonstrated as characteristic morphological, behavioural, and physiological alterations that enable them to avoid and/or tolerate metal toxicity. The mechanisms responsible for protection of the plant cell from metals entering the protoplast as well as for detoxification of toxic metal ions inside the cell by chelation, vacuolar sequestration, and exclusion from the protoplast are reviewed. These mechanisms have resulted in highly specialised plants able to hyperaccumulate or avoid metals in the shoots. Potential applications of both kinds of metallophytes in rehabilitation and phytoremediation of metal-polluted sites are briefly discussed. Moreover, other beneficial applications of metal-rich plant biomass are mentioned, e.g. as a bio-ore for precious metal recovery (phytomining, agromining), a by-product for eco-catalyst production, or a natural source of micronutrients that are essential for human diet and health (biofortification). The need of active protection of metalliferous sites and conservation of metallophyte biodiversity is pointed out.

Key words: excluders, (hyper)accumulators, metal tolerance, metalliferous soils, (micro)evolutionary changes, organic acids, phytochelatins, phytoextraction, phytostabilization

Introduction

Heavy metals are present in all soils due to the mineral composition of the lithosphere. The background metal concentrations considered as natural in soils vary depending on the element content and geochemistry of the underlying local rocks and oscillate in a range from 0.1 to 150 mg kg⁻¹ (e.g. for cobalt, Co; arsenic, As; cadmium, Cd; copper, Cu; nickel, Ni; chromium, Cr; lead, Pb; zinc, Zn; in increasing order) (Kabata-Pendias & Mukherjee, 2007) or from 200 to 2000 mg kg⁻¹ (for manganese, Mn) (Reeves, 2006). However, due to natural processes or anthropogenic activity, these concentrations may become several thousand-fold higher, thereby significantly influencing the structure of ecosystems and the development of vegetation (Baker et al., 2010).

Although some soils may be completely bare and devoid of vegetation due to their extreme phytotoxicity, spontaneous development of metal tolerant flora has been observed in many cases (Tordoff et al., 2000; Wójcik et al., 2014). Naturally occurring metalliferous soils are known to foster unique assemblages of vegetation; however, human-influenced metalliferous habitats may also support development of similar types of metal tolerant plant populations (Baumbach & Hellwig, 2007; Szarek-Łukaszewska, 2009; Wierzbicka & Rostański, 2002). The latter habitats are sometimes species-poor. For instance, monocultures of a few grass species only, including *Agrostis stolonifera*, *Agrostis capillaris*, or *Agropyron repens* are found in a close vicinity of some metal smelters in Poland, England, Belgium and the Netherlands (Brej, 1998; Dueck et al., 1984; Vangronsveld et al., 1996). In some newly heaped deposits, sparse vegetation patches dominated by grassland and ruderal vascular plants usually connected with shallow soils and dry and warm habitats are found (Szarek-Łukaszewska, 2009; Wójcik et al., 2014). However, the longer the process of colonisation of metalliferous substrates, the bigger the number of plant species is usually established there.

Many metalliferous sites are characterised not only by a remarkable diversity of higher plant species (several dozens or hundreds of species) but they also harbour a range of protected, rare, or threatened taxa (Baker et al., 2010; Jędrzejczyk-Korycińska, 2009). Additionally, such habitats provide niches for bryophytes, lichens, and a plethora of animal species, mainly insects, allowing them to persist and produce offspring.

Due to specific and harsh environmental conditions, plant communities developing on metalliferous sites represent a paradigmatic case of edaphic uniqueness that provides outstanding opportunities and model systems for ecological, physiological, and evolutionary studies of adaptation and speciation. Moreover, they constitute an excellent reservoir of species for degraded land rehabilitation or phytoremediation. It is thus not surprising that metallicolous plants have fascinated many botanists for a long time and have been an object of studies across the Earth's continents.

Natural and anthropogenic metalliferous sites

Metalliferous sites may be classified by the origin of metal contamination or by the mineral composition of the substrate. Baker et al. (2010) distinguished three types of metalliferous habitats based on the genesis of metal enrichment. 'Primary sites' have arisen naturally due to metal ore outcropping or weathering without any anthropogenic impact, creating the so-called metalliferous or orogenic soils. Such habitats are extremely rare today and are scattered as relics of the Late Glacial epoch e.g. in Central Europe, in the Pyrenees, the Alps and the Balkan region. Almost all of these habitats were destroyed or modified by mining activities dating back to the Bronze Age and intensified in the Middle Ages up to present time. In addition to soil forming processes, other natural phenomena, such as volcanic eruptions and associated gaseous fumaroles or hot-spring activity, may significantly

contribute to local heavy metal contamination (Kabata-Pendias & Mukherjee, 2007; Nagajyoti et al., 2010). The two other metalliferous habitats distinguished by Baker et al. (2010) are created by anthropogenic activity. The 'secondary sites', resulting from ore mining and processing, deposition of spoil and slag heaps, or other derived soil alterations, originally replaced the primary sites but afterwards they covered large areas located often at big distances from the primary sites. It is estimated that approximately 1% of the global land surface has been influenced by mining activities (Prach & Tolvanen, 2016). Finally, the 'tertiary metalliferous sites' originate by an input of metals usually in a non-metal enriched environment due to atmospheric deposition of metal pollutants or by alluvial deposition of metal-rich substrates in river floodplains or riverbanks. Important sources of metals to these habitats include all kinds of industrial emissions, e.g. from metal smelting, burning of fossil fuels, incineration of municipal wastes, production of batteries and other metal products, and ceramic and chemical industries. Also agricultural practices such as application of fertilizers and pesticides as well as sewage sludge and municipal waste disposal, transport and urbanisation have a significant input into metal pollution (Ali et al., 2013; Kabata-Pendias & Mukherjee, 2007; Nagajyoti et al., 2010).

With regard to the mineral composition, both naturally and secondarily metal-enriched soils can be classified as serpentine, calamine, seleniferous, or copper/cobalt-containing soils. Serpentine (ultramafic) soils are derived from ferromagnesian-rich mantle rocks and are characterised by anomalous Ni, Cr, and Co concentrations, accompanied by high concentrations of iron (Fe) and magnesium (Mg) (more than 70%) and low concentrations of calcium (Ca), phosphorus (P) and potassium (K) (Kruckberg, 2002; Martin & Coughtrey, 1982). Such soils have often a blue-green hue from serpentinite minerals (like lizardite, antigorite, or chrysotile) and are the most abundantly represented metalliferous soils on all continents. Huge areas of ultramafic soils of tens of hundreds of square kilometres are found

e.g. in New Caledonia, Cuba, or Turkey (Reeves, 2006). The vegetation inhabiting such soils is called 'serpentine/ultramafic flora', and the component species are called 'serpentinophytes'. Calamine soils, with their specific 'calamine flora', derived naturally from rock outcrops with galena (PbS), zinc blende (ZnS), and other calamine deposits (non-sulphide Zn minerals), are enriched mainly with Zn, Pb, and Cd, sometimes with a significant admixture of sulphur and/or copper (Reeves, 2006; Tordoff et al., 2000). The richest and the most intensively studied patches of calamine vegetation are found in a few countries in Europe (Belgium, France, Germany, Poland) and in South Asia (China, Iran, Afghanistan, Pakistan) (Bothe, 2011; Ghaderian et al., 2007; Wierzbicka & Rostański, 2002). Soils rich in Cu and/or Co develop from argillites and dolomites containing sulphides of these metals; they occur mainly in Central Africa, in the DR Congo and Zambia (Reeves, 2006). Seleniferous soils (bearing more than 0.5 mg Se kg⁻¹) are derived from various Se-rich rock types, e.g. from Cretaceous shales, and are found in Australia, southern Asia, Ireland, and the USA (Dhillon & Dhillon, 2001). Due to the abundance of natural and human-influenced ultramafic and calamine soils, serpentine and calamine vegetation has been the subject of the most intense studies and will be the focus of the present review.

Both the mineral composition and the level and origin of metal contamination determine the development and composition of vegetation in metalliferous habitats. However, not only high metal concentrations but also other unfavourable habitat conditions seriously hinder the development of vegetation. At primary sites, metal surplus is often accompanied by low nutrient availability (Baker et al., 2010). Anthropogenically influenced sites are usually characterised additionally by depleted organic matter concentrations, severe nutrient (especially phosphorous) deficiency, low water retention capacity, strong insolation and wind, acidification, poorly developed soil structure, and sometimes by steep slopes (Dechamps et al., 2008; Szarek-Lukaszewska, 2009; Wierzbicka & Rostański, 2002; Wójcik et al., 2014).

Metallophytes

Plants that are adapted to live on metal-enriched soils and able to survive and reproduce there without suffering from toxicity are termed ‘metallophytes’ (Baker et al., 2010; Whiting et al., 2004). In general, metallophytes can be classified as obligate (absolute, strict or eu-) metallophytes, i.e. species found exclusively on metalliferous soils, or facultative (pseudo-) metallophytes, comprising species occurring both on metalliferous and non-metalliferous, ‘normal’ soils (Baker et al., 2010; Kruckeberg, 2002; Whiting et al., 2004). There are also plants existing solely on non-metalliferous soils; such species are designated as ‘obligate non-metallophytes’.

Obligate metallophytes are usually endemic to specific ecological niches provided by metalliferous soils with a well-defined metal composition. Due to their strict dependence upon occurrence of these metals in the substrate, such species have often been used as geobotanical indicators in mineral exploration (Brooks, 1998; Martin & Coughtrey, 1982). Classic examples of geobotanical indicators, successfully used for prospecting Cu deposits, are the “copper flowers” of Katanga Province of Congo (formerly Shaba Province in Zaire) – *Becium homblei* and *Haumaniastrum katangense* (Brooks et al., 1992). In general, the serpentine flora is globally the richest in endemic metallophytes with over 1000 higher plant species described to date (Kruckeberg, 2002; Pollard et al., 2014). Some genera are particularly rich in ultramafic obligate endemics, such as *Buxus* and *Leucocroton* (Euphorbiaceae) in the Caribbean region, *Phyllanthus* and *Psychotria* (Rubiaceae) in New Caledonia and the Caribbean region, *Homalium* (Salicaceae), *Xylosma* (Salicaceae), *Hybanthus* and *Rinorea* (Violaceae) in New Caledonia and tropical SE Asia, *Turnera* in South America, and *Alyssum*, *Odontarrhena*, and *Noccaea* (Brassicaceae) and *Centaurea* (Asteraceae) in the Euro-

Mediterranean and Irano-Turanian regions. *Clarkia* and *Epilobium* (Onagraceae), *Linum* (Linaceae), and *Streptanthus* (Brassicaceae) include several obligate endemics in western North America (Kruckeberg, 2002; Safford et al., 2005). In New Caledonia, i.e. the major hot-spot of serpentine plant diversity in the world, endemic genera associated with ultramafic soils represent more than 37 % of all the endemic genera, (39% of the indigenous flora), many of which are monotypic (Isnard et al., 2016; Kruckeberg, 2002). A similar percentage is also found on Cuba, the other major centre of diversity of ultramafic flora at the global scale (32–33% of all endemic genera; Borhidi, 1991; Kruckeberg, 2002). The Balkans are the major centre of the diversity of serpentine plants in Europe, with approximately 335 taxa of which 123 are obligate specialists of both neo- or paleo-endemic type (Stevanović et al., 2003), among which the two monotypic genera *Halacsya* and *Paramoltkia* of family Boraginaceae (Cecchi & Selvi, 2009; Coppi et al., 2014).

In contrast to serpentinophytes, species endemic to calamine soils are in fact a rare phenomenon. *Viola lutea* subsp. *calaminaria* or *Viola lutea* subsp. *westphalica* are examples of obligate metallophytes associated with this type of soil in Belgium, the Netherlands, and Germany (Bizoux et al., 2004; Bothe, 2011).

Facultative metallophytes are by far more numerous and widely taxonomically distributed in comparison with obligate metallophytes. They are usually recruited from adjacent non-metalliferous environments and, though often not distinguishable at even the varietal rank, they develop metal-tolerant genotypes or ecotypes in response to the presence of specific metals in the soil. They occur, as species, in various metalliferous and non-metalliferous soil types but when adapted to the metalliferous substrate they become dependent on the presence of metals. The most frequently studied examples of facultative metallophytes on calamine soils are: *Noccaea* (formerly *Thlaspi*) *caerulescens*, *Noccaea goesingense*, *Arabidopsis* (formerly *Cardaminopsis*) *halleri*, *Silene vulgaris*, *Minuartia verna*

s.l., *Armeria maritima* (Bothe, 2011; Pollard et al., 2014 and references therein). Some authors distinguish a term ‘pseudometallophytes’ or ‘bodenvag plants’ (‘soil wanderers’) within facultative metallophytes to refer specifically to the group of plants indifferent to the parent rock material, moderately tolerant to heavy metals, but never dependent on their occurrence (Baker et al., 2010; Kruckeberg, 2002). Such plants belong to common monocot and dicot species with a wide geographic distribution range and often accompany metallophyte vegetation. Calamine pseudometallophytes are represented, for instance, by *Agrostis capillaris*, *Agrostis stolonifera*, *Deschampsia caespitosa*, *Festuca ovina* (*halleri*), *Festuca rubra*, *Holcus lanatus*, *Phragmites australis*, *Achillea millefolium*, *Campanula rotundifolia*, *Plantago lanceolata*, *Ranunculus acris*, *Rumex acetosella*, *Thymus pulegioides*, and *Viola tricolor* (Baker et al., 2010; Bothe, 2011; Wójcik et al., 2014). Examples of bodenvags on serpentine soils include *Achillea lanulosa*, *Gilia capitata*, *Senecio pauperculus*, *Fragaria virginiana*, *Prunella vulgaris*, or *Pinus contorta* (Kruckeberg, 2002). However, the term ‘pseudometallophytes’ is often indiscriminately used as synonymous with ‘facultative metallophytes’. Even less popular and rather awkward is the term ‘incidental pseudometallophytes’ (Pollard et al., 2014) referring to ‘associated non-metal tolerant’ species (Baker et al., 2010) used for some weeds and ruderal plants appearing sporadically and showing reduced vigour in metalliferous habitats.

(Micro)evolutionary dynamics of metallophytes

Species constituting plant communities on metalliferous soils have adapted to the adverse environmental conditions through (micro)evolutionary processes and often represent genetically altered ecotypes. Normally, evolution of metal tolerance is achieved in plants over thousands or even millions of years (Whiting et al., 2004). However, under strong selection

pressure imposed by extreme environmental conditions, it may evolve in a relatively short time of 40–150 years or even within less than a decade, i.e. in only a few generations (Ernst, 2006). The degree of specialization of the metal resistance trait is governed by the length of exposure to metals. At anthropogenically created metalliferous sites, e.g. spoil heaps or areas around metal smelters usually inhabited by pseudometallophytes, a limited level of metal tolerance is developed after a few years of metal emergence at the site. In plants facing the presence of mineral deposits at natural metal outcrops for centuries, more specialized mechanisms of tolerance are built up, resulting in their full dependence on metals (obligate metallophytes).

Many reports provide evidence that metal tolerance has evolved independently in different taxonomically unrelated plant affinities (within over 34 different plant families) and in geographically distant conspecific populations, being thus a clear-cut example of convergence (Bizoux et al., 2008). Moreover, colonisation of metalliferous habitats can take place through multiple independent events from non-metallicolous populations. For example, Patterson and Givnish (2003) showed that serpentine tolerance in *Calochortus* had evolved seven times independently across mountainous ranges in California and Oregon, while in *Streptanthus* this ability originated four to five times (Cacho et al., 2014). A further remarkable case of polyphyletic origin with multiple events of adaptation to ultramafic substrates is provided by the genus *Onosma* (Cecchi et al., 2011) or *Odontarrhena*, until recently included in *Alyssum*, where serpentine and nickel hyperaccumulator endemics from southern Europe were scattered in three main evolutionary lineages without geographical cohesion (Cecchi et al., 2010, 2013).

Rapid development of molecular techniques in the last few decades has provided excellent tools to study evolutionary changes in metalliferous habitats. Besides selection pressure, the mutation rate, reproductive success, gene flow from neighbouring populations,

and successive colonisation events contribute to evolutionary processes (Babst-Kostecka et al., 2014; Bickham et al., 2000). Both epigenetic processes and DNA changes may be the source of genetic divergence (Abratowska et al., 2012; Dresler et al., 2015 and references therein). Abratowska et al. (2012) pointed out that, more than the type of the substrate, geographic isolation had a crucial influence on the genetic structure of calamine populations of *Armeria maritima*; a similar effect may not be excluded in *Dianthus carthusianorum* (Wójcik et al., 2013) and *Echium vulgare* (Dresler et al., 2015). Similarly, Nyberg Berglund & Westerbergh (2001) showed that serpentine populations of *Cerastium alpinum* in Scandinavia are genetically more similar to non-serpentine populations within the same geographic region than with distant serpentine populations. Gustafson et al. (2003) found close genetic relationships between serpentine and non-serpentine populations of *Cerastium velutinum* from North America and similar evidence was provided by Mengoni et al. (2006) in a comparison of serpentine and limestone populations of *Onosma echioides* in Italy. On the other hand, in their study of several calamine and non-metallicolous populations of *Biscutella laevigata*, Babst-Kostecka et al. (2014) showed that edaphic conditions and heavy metal exposition rather than geographic distance determined genetic divergence. Evidence for ecotypic differentiation on serpentine soil has been reported in numerous transplanting and cultivation experiments (Burgess et al., 2015; Chaturanga et al., 2015; O'Dell & Rajakaruna, 2011; Wright & Stanton 2011), suggesting that the directional selection driven by the substrate can overcome the homogenization of populations by gene flow.

It is commonly accepted that strong selection pressure leads to reduced population size and genetic diversity. Accordingly, decreased genetic variability has been found in several metallicolous populations of *Deschampsia cespitosa* (Bush & Barrett, 1993), *Armeria maritima* (Vekemans & Lefèbvre, 1997), *Silene paradoxa* (Mengoni et al., 2001), *Sedum alfredii* (Deng et al., 2007), and *Biscutella laevigata* (Babst-Kostecka et al., 2014). However,

in populations of *Viola calaminaria* and *Dianthus carthusianorum*, the level of genetic diversity was similar in metallicolous and non-metallicolous populations (Bizoux et al., 2008; Wójcik et al., 2013). Moreover, other studies showed higher genetic diversity within the metallicolous compared to non-metallicolous populations of *Viola tricolor* (Słomka et al., 2011b) or *Echium vulgare* (Dresler et al., 2015). The conflicting results obtained in the aforementioned studies might be related to the use of different molecular markers (PCR-based fingerprinting) (Dresler et al., 2015). However, they may also result from high heterogeneity of metalliferous sites in terms of different ecological factors and, therefore, genotypic variation and flexibility within a population may be crucial to survival.

Pre-adaptive and concomitant traits

Besides evolving metal tolerance, plants growing on metal-enriched substrates were co-selected for tolerance to other adverse site conditions. Therefore, many metallophytes are characterised by specific adaptations ensuring a high degree of resistance to drought and nutrient deficit or an ability to grow on a loose substrate (Baker et al., 2010). Pre-existing adaptation, being most likely a plesiomorphic trait, seems a crucial prerequisite for the evolution of specialized ecotypes and/or taxa (Meyer et al., 2016). Which morphological and/or physiological features convey pre-adaptation is still not well understood, but drought resistance is undoubtedly a major trait due to the extremely xeric nature of most metalliferous environments (Brady et al., 2005; Proctor, 1999). Actually, drought resistance has been experimentally documented in several metallophytes, e.g. in serpentine *Mimulus sp.* (Hughes et al., 2001) and *Cerastium sp.* (Nyberg Berglund et al., 2004) as well as in calamine *Silene vulgaris* or *Biscutella laevigata* (Wierzbicka & Rostański, 2002). Recently, Armbruster (2014) commented that pre-existing tolerance to open habitats, by themselves ecologically

stressful, can explain the multiple origins of serpentine-soil endemism in Californian genera such as *Collinsia* and *Streptanthus*, and this trait is thought to have evolved before adaptation to serpentine soils (Cacho and Strauss, 2014). In fact, many metallophytes are sensitive to shade (Szarek-Lukaszewska, 2009). One of the most shade-sensitive species *Minuartia verna* disappears during succession of arborescent vegetation (Baker et al., 2010 and references therein). Therefore, as a means of active protection of unique calamine metallophytes inhabiting over 130 years old Zn-Pb waste spoil in Bolesław, Poland, as well as similar sites elsewhere, trees and shrubs spontaneously spreading there are being removed (Jędrzejczyk-Korycińska et al., 2014).

Due to their complex adaptive nature, metallophytes are usually restricted to metalliferous soils. This may be explained by the fact that they require relatively high levels of metals for optimal growth and reproduction or because they are poor competitors on “normal” substrates in respect to herbivores and pathogens (Armbruster, 2014; Rascio & Navari-Izzo, 2011). Their competitive weakness on non-metalliferous soils may also result from a high energy cost incurred to development of adaptation mechanisms related to metal transport, storage, and detoxification (Baker et al., 2010) or with their adaptation to drought or nutrient deficiency.

Strategies of metal accumulation

There is clear evidence that different plant species take up different metals to varying degrees, even when grown on the same soil. Moreover, the propensity of a species to accumulate one metal does not necessarily imply that other metals will also be accumulated in superior concentrations and that the metals will be similarly distributed over roots and shoots. Plants growing on metalliferous soils may exclude, indicate, or accumulate metallic elements

in their shoots, being thus classified as 'excluders', 'indicators' or '(hyper)accumulators' (Baker, 1981).

A majority of metallophytes are 'excluders' since they are able to restrict either the entry of metals into the roots, or their transport to the shoots over a wide range of soil metal concentrations (Wójcik et al., 2014). The shoot/soil metal concentration coefficient in such plants is always lower than unity. Metal excluders retain and detoxify most of the metals in the roots; however, when the control mechanisms break down, unrestricted metal transport to the shoots occurs, which is usually deleterious to the plant. The majority of serpentinophytes adapted to elevated nickel soil concentrations restrict element accumulation in their leaves and consequently show lower nickel concentrations in their tissues than non-serpentine conspecifics when grown in the presence of the metal (Nagy and Proctor, 1997; Pakdaman et al., 2013). This is also the case for numerous calamine metallophytes, including *Silene vulgaris* (Mohtadi et al., 2012) or *Dianthus carthusianorum* (Wójcik et al. 2015b).

In 'indicators', shoot metal concentrations reflect soil metal concentrations (shoot/soil metal concentration coefficient = 1). Therefore, such plants have been proposed as biomonitors for the assessment of environment pollution (Mertens et al., 2005). In practice, the situation is more complicated since most metalliferous sites exhibit polymetallic contamination and, to date, no plant species are known to respond with a strong positive correlation between soil and shoot metal concentration to a wide range of elements.

Metal 'accumulators' are characterised by an efficient metal uptake and translocation to the shoots without showing any toxicity symptoms. The shoot/root and shoot/soil metal concentration ratios are higher than unity, although these criteria may sometimes be misleading in recognising such plants, as explained by Reeves (2006). Some accumulators, referred to as hyperaccumulators, have an intriguing strategy to concentrate elements in leaves at levels that are at least an order of magnitude higher than in other metallophytes from

metalliferous soils or a few orders of magnitude higher than in ordinary plants from non-metal enriched soils (Pollard et al., 2014; Rascio & Navari-Izzo, 2011). The threshold values of metal concentrations designating a plant as a hyperaccumulator have changed since the early reports defining hyperaccumulators and vary depending on the metal (Baker & Brooks, 1989; Reeves, 2006). The threshold criteria proposed currently are 100 mg for Cd, Se, and Tl; 300 mg for Co, Cr, and Cu; 1000 mg for Ni, Pb, and As; 3000 mg for Zn, and 10 000 mg for Mn determined in kg of dry leaf tissue of a plant growing in a natural habitat (van der Ent et al., 2013; Pollard et al., 2014). Based on these criteria, the innate ability to hyperaccumulate trace elements has been documented in approximately 500 species and is primarily attributed to metallophytes of serpentine soils accumulating nickel (over 90% of all hyperaccumulators identified so far; Pollard et al., 2014). Interestingly, the highest ever-recorded metal concentration present in a plant tissue (26%) is exactly for nickel, specifically in the latex of the *Sebertia acuminata* tree which is endemic to serpentine soils in New Caledonia (Sagner et al., 1998). Only approximately 50 species have been recognised as hyperaccumulators of other metals (Rascio & Navari-Izzo, 2011). *Noccaea caerulescens* is probably the best-known hyperaccumulator of Zn, Cd, or Ni, and together with *Arabidopsis halleri*, a Zn/Cd hyperaccumulator, used as a model species in studies on metal homeostasis and detoxification (Assunção et al., 2003; Meyer & Verbruggen, 2012). A majority of hyperaccumulators (approximately 85-90%) are obligate metallophytes; however, some of them, including *Noccaea caerulescens* and *Arabidopsis halleri*, also occur on normal, non-metalliferous soils.

Several hypotheses to explain metal hyperaccumulation have been proposed (Boyd, 2007). There is evidence that extreme metal concentrations in the leaves protect plants against herbivores (Zn) and pathogens (Ni). Accumulated metals may also be used as allelopathics against nearby competitors or serve as osmolytes in drought resistance. Finally, extraordinary metal concentrations may be a means of efficient metal tolerance and disposal mechanisms;

alternatively, they may result from accidental metal uptake, which seems rather improbable in view of the large amount of metals accumulated without imposing toxicity symptoms.

Specific characteristics of metallophytes

As already mentioned, metallophytes endemic to metalliferous sites may be considered as geobotanical indicators of mineral deposits. However, not only the presence of particular species but also floristic composition (assemblages of species) or some characteristic physiological or morphological changes (plant appearance) may be indicative of soil metal enrichment or potential mineral deposits. Typically, the vegetation of highly mineralised areas appears to be sparse and stunted, relative to surrounding areas, and is usually devoid of trees. Since heavy metals accumulate gradually in cells, it seems that short living herbs can cope better with the metal toxicity than long-lived plants (shrubs and trees) (Dickinson et al., 1991). However, shortening of the life cycle is apparently not an adaptive strategy on serpentine soils where perenniality seems an advantageous trait, though this lacks clear explanatory hypotheses (Selvi, 2007).

Morphological variations

Many plant species exhibit abnormal appearance when growing on metal enriched substrates. In comparison with individuals of the same species from unpolluted areas, they are characterised, among others, by reduction in stature (shrubiness of arborescent species; dwarfism and plagiotropism in herbaceous species) although sometimes gigantisms may occur. Additionally, increased root system, xeromorphic foliage (with stenophylly, glaucescence), chlorosis or purpling of leaves, changes in the colour and pattern of flowers,

and abnormally shaped fruits can be observed (Kruckeberg, 2002; Martin & Coughtrey, 1982; Wierzbicka & Rostański, 2002). In the case of serpentinophytes, such xerophytic and stunted appearance has been termed the “serpentine syndrome” or ‘serpentinomorphoses’ (Menezes de Sequeira & Pinto da Silva, 1992). Reduced growth is a typical symptom on metalliferous substrates. Metalicolous plants are smaller, have more numerous but thinner and lower/shorter or sometimes trailing shoots, less numerous, smaller, and narrower leaves. This was observed, for instance, in calamine ecotypes of *Silene vulgaris*, *Dianthus carthusianorum*, or *Arabidopsis halleri* in Poland (Bothe, 2011; Fiałkiewicz & Rostański, 2006; Wierzbicka & Rostański, 2002; Wójcik et al., 2013) and serpentine ecotypes of *Trifolium sp.* or *Jasione crispa* in Portugal (Menezes de Sequeira & Pinto da Silva, 1992). A significant increase in the plant size, manifested by e.g. greater rosette diameter, larger stem and rosette leaves, or numerous lateral stem branches in metalicolous individuals of *Arabidopsis* (synonym *Cardaminopsis*) *arenosa* (Rostański et al., 2005) is a rather rare phenomenon among metallophytes.

Plants occurring on metalliferous substrates exhibit various root morphology in terms of root length, depth, and surface area. In general, no special root size or architecture distinguishes metalicolous from non-metalicolous plants. Different root systems have been described in metallophytes, from short, thin, marginally branched primary roots through abundant fine root bundles to long and thick primary roots with or without well-developed side roots (Alford et al., 2010; Bothe, 2011; Menezes de Sequeira & Pinto da Silva, 1992). However, what is striking at a comparison of individuals from non-contaminated and metalliferous soils, the latter often produce deeper roots and dense root hairs, probably as an adaptation to the dry substrate rather than the presence of metals (Menezes de Sequeira & Pinto da Silva, 1992; Wierzbicka & Rostański, 2002). Chlorosis of leaves is a very common symptom in plants from metal-enriched areas, although it may also result from accompanying

deficiency of essential nutrients, e.g. nitrogen or iron in the soil. Similarly, the frequently observed anthocyanin accumulation manifested as intense purple/violet stem and leaf discolorations may at least partially be due to phosphorous deficiency on calamine and serpentine soils (Kabata-Pendias & Mukherjee, 2007; Menezes de Sequeira & Pinto da Silva, 1992). Variation in the colour of flowers has also been reported in plants exposed to heavy metals. For instance, *Epilobium angustifolium* has rose-purple coloured flowers which only exceptionally are white or pink; however, populations associated with uranium deposits in Canada or Alaska are distinguished by almost pure white sepals and petals (Martin & Coughtrey, 1982).

Behavioural variations

Alterations in the rhythm of growth and flowering periods are other common symptoms found in plants growing in metalliferous habitats (Martin & Coughtrey, 1982; Wierzbicka & Rostański, 2002). To increase a species chance of survival, many metallophytes tend to shorten their vegetative phase and increase fertility by earlier and longer flowering, producing more flowers per plant, and delivering more seeds. A short life cycle and large reproductive effort at the expense of individual parental investment is typical for the “r” strategy, where the ability to reproduce quickly and abundantly is crucial to survive under strong environmental pressure. Such behaviour was observed, among others, in calamine populations of *Silene vulgaris*, *Dianthus carthusianorum* (Wierzbicka & Rostański, 2002), *Arabidopsis arenosa* (Przedpelska-Wasowicz & Wasowicz, 2013), or *Minuartia verna* (Martin & Coughtrey, 1982).

Most plants avoid root proliferation in metal-enriched soil niches. However, some hyperaccumulating plant ecotypes, e.g. these of *Noccaea caerulecsens* (Dechamps et al.,

2008; Whiting et al., 2000) or *Sedum alfredii* (Li et al., 2005) respond positively to trace elements in the soil by directing their root growth towards spots/patches with high metal content and increasing root biomass, length, and hair root number while reaching them. This is, however, not a species-specific feature since other less metal-tolerant ecotypes of the same species do not show such foraging behaviour.

Physiological variations

Intraspecific variation in the degree of metal tolerance has been found in most studied metallophytes (and specifically in facultative metallophytes) and the level of the tolerance has been consistently higher in ecotypes/populations growing at high metal concentrations in the substrate (Dresler et al., 2014; Schat et al., 2002; Wierzbicka & Rostański, 2002; Wójcik et al., 2015b). To identify mechanisms responsible for enhanced metal tolerance, comparative methods are frequently employed in hydroponic experiments where natural metallicolous versus non-metallicolous ecotypes or mutants/transgenic versus wild type plants are exposed to acute short-term single metal stress. Although such experiments are useful for fast screening of metal resistance and provide valuable information on the molecular and genetic background, they may not accurately reflect the response of plants grown in metalliferous habitats under chronic poly-metallic stress (Wójcik et al., 2015b). Therefore, a greater emphasis should be placed on mimicking more complex metal-plant-microorganism interactions in field trials or soil experiments to understand fully the complex nature of adaptation to metalliferous habitats.

Mechanisms of metal uptake, translocation, and tolerance/detoxification

Some metals, such as Zn, Cu, Fe, Mn, Mo, Co, Ni, and V are required in minute quantities for plant growth and development; however, excessive amounts of these elements can become harmful to organisms. Other heavy metals or metalloids, such as Pb, Cd, Hg, or As do not have any beneficial effects on plants and they become toxic already at relatively low concentrations in the growth medium (Ernst et al., 2008; Singh et al., 2016). The level of the metal concentration at which toxicity symptoms appear (or adverse effects are recorded) strongly depends on the plant species and the mechanisms of metal tolerance developed.

The physiological mechanisms and genetic background of metal uptake, transport, sequestration, and homeostasis in metal accumulating and non-accumulating metallophytes have been extensively studied, yet are still not well understood/elucidated (Singh et al., 2016). Enhanced metal tolerance results from changes in the metal homeostasis network virtually shared by all higher plants. The differences between tolerant and non-tolerant plants might possibly rely on different expression of genes found in plants, both hyperaccumulators and excluders, as already reported for Zn and Cd by Verbruggen et al. (2009). Indeed, the mechanisms of metal tolerance in non-accumulators seem to be under the control of relatively few genes (Schat & Vooijs, 1997), in contrast to hyperaccumulators, where the genetic background is much more complex (Pollard et al., 2002; van de Mortel et al., 2006).

Ernst et al. (2008) distinguished three degrees of plant metal tolerance: hypotolerance, basal tolerance, and hypertolerance. In fact, metal hypotolerant (hypersensitive) plants do not occur in nature; this term refers to mutants and transgenic plants with increased metal sensitivity in comparison with wild type plants. A great majority of plants, namely all pseudometallophytes, exhibit a basic level of metal tolerance (constitutive tolerance), which enables them to live on non-metalliferous soils but also to cope with low, non-toxic concentrations of metals alternatively appearing in the environment (Wójcik et al., 2015b). Basal metal tolerance is specific for various metals, plant species, and ecotypes and is a

starting point to evolve adaptive metal tolerance or hypertolerance in species or ecotypes growing and coping on highly metal-enriched soils (Ernst, 2006). A number of studies have demonstrated that plants develop enhanced tolerance specifically to the metal actually present in surplus in their natural environment; to all other metals, they have a basic level of tolerance. However, some cases of co-tolerance have also been reported (Colzi et al., 2014; Gonnelli et al., 2001; Remon et al., 2007; Schat & Vooijs, 1997).

Avoidance or increased metal uptake

Only metal ions that are present in the form of soluble components in the soil solution or those that are easily solubilized, e.g. by root-mediated acidification of the rhizosphere, are available for plant uptake. Root exudates comprise the first line of defence against metal uptake. Many plants, predominantly monocots, excrete into the soil phytosiderophores, which chelate metal ions forming large complexes not taken up by the root cells. Exclusively, grass species use this strategy for acquisition of iron since they possess membrane transporters for Fe(III)-phytosiderophore complexes (Zhao & McGrath, 2009). Similarly, some carboxylic acids, including malate, citrate, or oxalate as well as amino acids, e.g. histidine, excreted by roots bind metal ions in the soil solution thus preventing uptake thereof. This phenomenon is well documented and is crucial in the case of Al tolerance (Watanabe & Osaki, 2002); it has also been shown to reduce Ni uptake by *Thlaspi arvense* (Salt et al., 2000). Alternatively, root exudates may selectively increase metal uptake by plants (Luo et al., 2008; Mench & Martin, 1991). However, in metal hyperaccumulators, such as Zn accumulating *Noccaea caerulescens* (Zhao et al., 2001) or Ni accumulating *Noccaea goesingense* (Salt et al., 2000), no increase in secretion of specific metal-chelating compounds has been reported.

Restricted metal uptake may also result from their binding to extracellular carbohydrates, such as callose or mucilage (Siedlecka et al., 2001). Furthermore, root cell walls play a key role in preventing toxic metal intake into the cytosol by immobilization thereof in ligno-polysaccharide (cellulose, hemicelluloses, pectins) matrix. Depending on the kind of metal, its concentration, and the plant species and organ, the cell wall can retain a great majority of the metal taken up, also as a sink for metals excluded from the protoplast (Krzesłowska, 2011). It is also noteworthy that the cell wall is actively modified under metal exposure mainly by increasing the level of all compounds and particularly the low-methyl esterified fraction of pectins in order to increase its thickness and metal binding capacity (Krzesłowska, 2011). An interesting phenomenon has recently been found in *Noccaea caerulescens* with its additional peri-endodermal layer of cells (not found in its metal-sensitive relative *Thlaspi arvense*) characterised by irregularly thickened and lignin-impregnated cell walls (Zelko et al., 2008). Whether these cells act as an additional physical barrier for metal immobilization in extracellular metal movement across the root should still be elucidated.

The plasma membrane plays an important role in preventing or reducing metal uptake into the cell. The difference in the metal uptake and accumulation rate between excluders and hyperaccumulators results from different expression levels of the same plasma membrane transporters, although the existence of transporters with different kinetic properties cannot be excluded. Plants possess various families of transporters on the plasma membrane and tonoplast and these have been extensively reviewed recently (Guerinot, 2000; Haydon & Cobbett, 2007; Krämer et al., 2007; Manara, 2012; Singh et al., 2016). They belong mainly to the ZIP (Zinc-regulated transporter Iron-regulated transporter Protein), CDF (Cation Diffusion Facilitator also known as MTP, Metal Tolerance Protein), NRAMP (Natural Resistance Associated Macrophage Protein), and P-type ATPase (also known as HMAs,

Heavy Metal ATPases) protein families of transporters (Krämer et al., 2007; Singh et al., 2016). Many metal transporters exhibit low ion selectivity and, in addition to essential elements, they may competitively transport non-essential and toxic ions with similar oxidation states and ionic radii (Alford et al., 2010). However, in the Ganges ecotype of *Noccaea caerulea*, existence of a transporter in the root cell plasma membrane specific for Cd and independent on Zn transport has been suggested (Lombi et al., 2001). The entrance of Ni into the root cells seems to be mediated by a low-affinity uptake system with a high preference for Zn over Ni in *Noccaea caerulea* (Assunção et al., 2001), with genes *ZNT1*, *ZNT2* (zinc-regulated transporter belonging to ZIP family), *NRAMP3*, and *NRAMP4* showing specific up-regulation in this plant grown in the presence of Ni (Visioli et al., 2014). Moreover, also IRT1 (iron-regulated transporter of ZIP family) and ZIP10 (ZRT, IRT-like protein) proteins have been indicated as candidates for Ni accumulation in the same plant (Halimaa et al., 2014). Enhanced Zn uptake by the Zn hyperaccumulators *Noccaea caerulea* and *Arabidopsis halleri* is attributed to the constitutive overexpression of several genes for ZIP transporters (e.g. *ZNT1*, *ZNT2* and *ZIP6*, *ZIP9*, respectively), which in non-hyperaccumulating relatives are expressed only under Zn deficiency (Assunção et al., 2001). Recently it has been reported that rapid membrane voltage changes and diminution of the membrane depolarization may be the cause of higher metal tolerance of *Arabidopsis halleri* and *Arabidopsis arenosa* in comparison with *Arabidopsis thaliana* (Singh et al., 2016).

Root-to-shoot metal translocation

There is a huge inter- and intraspecies diversity in metal distribution within a plant. For instance, serpentine populations can show higher root-to-shoot translocation of Ni than non-serpentine ones (Chaturanga et al., 2015; Gonnelli et al., 2001). On the other hand,

when grown in hydroponics, a metal sensitive ecotype of *Dianthus carthusianorum* translocated more Zn and Pb to the shoots than a metal tolerant one; however, the tendency was just opposite in plants cultivated on polluted soil (Wójcik et al., 2015b). In fact, a majority of plants inhabiting metalliferous habitats preferentially retain metals in the roots, whereas efficient root-to-shoot translocation is typical for hyperaccumulators. This implies more efficient cytoplasmic detoxification and vacuolar sequestration of metal ions in the root cells of excluders, while in hyperaccumulators more metals are available for xylem loading due to either a low sequestration rate in the vacuole or efficient efflux out of the vacuole at the root level (Assunção et al., 2003, Rascio & Navari-Izzo, 2011). Indeed, Lasat et al. (2000) and Yang et al. (2006) found much lower amounts of Zn accumulated in the vacuole of root cells and faster Zn efflux from the vacuole in Zn hyperaccumulators *Noccaea caerulescens* and *Sedum alfredii* compared with their non-accumulating relatives/accessions.

Several types of transporters have been recognised to be involved in metal transport to the xylem (Singh et al., 2016). It is now evident that the P_{1B}-type ATPases HMA4 and HMA2 play a crucial role in Zn and Cd ion transport from the pericycle cells to the xylem vessels (Wong and Cobbett, 2009). In *Noccaea caerulescens*, the Ni-nicotianamine complex seems to be transported by proteins YSL3 (yellow stripe like) for the vascular loading and translocation (Gendre et al., 2007; Halimaa et al., 2014).

Metal ions loaded into the xylem are transported to the shoots in the form of complexes with various chelators, primarily with organic acids and amino acids or their derivatives (Haydon & Cobbett, 2007) but also as free hydrated cations (Krämer et al., 1996). Malate was supposed to be involved in Ni translocation in the hyperaccumulator *Stackhousia tryonii* (Bhatia et al., 2005), whereas citrate and other organic acids complexed almost all Ni in the latex of *Sebertia acuminata* (Callahan et al., 2008). Histidine and nicotianamine were reported as the major xylem chelators for Ni in the Ni-hyperaccumulating *Alyssum* (Krämer et

al., 1996) and *Noccaea caerulea* (Mari et al., 2006), respectively. The same compounds have been found to complex Zn, Cd, Pb, Mn, Cu, etc. for long distance transport (Álvarez-Fernández et al., 2014). It should also be noted that metals may be distributed within the plant via the phloem, being largely complexed with the same ligands. In the case of Cd, Cd-phytochelatin complexes have been found both in xylem and phloem saps (Álvarez-Fernández et al., 2014).

Metal tolerance mechanisms

Rapid removal of free metal ions from metabolically active cell compartments to prevent physiological damage is at the core of metal tolerance mechanisms. This can be achieved by metal chelation in the cytoplasm by a variety of ligands, metal sequestration in the vacuole, or exclusion from the protoplast. It cannot be definitely declared that these processes are metal-specific; the same mechanisms are usually employed to cope with different metals, although their efficiency is different from metal to metal, species to species, and even between the ecotypes/populations of a given species. They also depend on the metal concentration in the growth medium and time of exposure (acute or chronic stress) (Wójcik et al., 2015b).

Metal binding ligands

The production of metal binding ligands to detoxify and facilitate transport and storage of metals is crucial to cope with the metal surplus. Up to date, no specific metabolites have been detected in metal-tolerant ecotypes. The most important ligands comprise thiol peptides (phytochelatins, PC; metallothioneins, MT; glutathione, GSH), organic acids (malate, citrate),

amino acids (histidine, proline), and phytate (Anjum et al., 2015; Manara, 2012; Rauser, 1999). Metallothioneins are cysteine-rich metal binding peptides that have hardly been isolated from plants although their genes are abundant and expressed (or even overexpressed in some metallophytes, including *Noccaea caerulescens*) upon metal exposure virtually in all plant tissues (Cobbett & Goldsbrough, 2002; Manara, 2012). At present, however, there is no direct evidence in favour of their role in metal tolerance or hyperaccumulation. Glutathione is the most abundant thiol compound in plants serving a broad range of biochemical functions, including its role in metal detoxification and tolerance (Wójcik & Tukiendorf, 2011; Jozefczak et al., 2012). It is a rather poor metal chelator and its role in enhanced, adaptive metal tolerance but not basal metal tolerance is rather doubtful (Ernst et al., 2008; Schat et al., 2002; Wójcik et al., 2015b); therefore, it will not be further addressed to in this paper.

Phytochelatin are the best-characterized metal chelators (Cobbett, 2000; Cobbett & Goldsbrough, 2002; Pal & Rai, 2010). These thiol peptides are synthesized from GSH and have a general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n=2-11$, usually 2-5). They chelate metal ions, and this mechanism is the best recognised for Cd ions, through thiol groups of cysteine to form so-called LMW (low molecular weight) PC-metal complexes in the cytoplasm for further transport thereof to the vacuole (Ernst et al., 2008; Pal & Rai, 2010; Rauser, 1999). This transport is mediated by ATP-dependent ABC transporters in the tonoplast. Once in the vacuole, LMW PC-metal complexes incorporate acid labile sulphur (S^{2-}), yielding HMW (high molecular weight) PC-metal complexes with higher stability under acidic vacuolar conditions (Wójcik, 2009). Phytochelatin synthesis is rapidly activated by different heavy metal ions, mainly Cd but also Cu, Zn, Pb, Ag, Au, and Hg, although stable *in vivo* complexes were only detected with Cd, Cu, and Au (Cobbett and Goldsbrough, 2002). In addition to PCs found in all plants studied so far, PC homologues varying in the kind of the carboxy terminal amino acid (β Ala, Ser, Glu, Gln instead of Gly) or its absence have been distinguished in

some plant species (Rauser, 1999; Siedlecka et al., 2001); however, their role in metal detoxification and tolerance is not well recognised.

Similarly, contrasting evidence has been reported for the role of PCs in metal tolerance of metallophytes. Many studies, including these on mutants, transgenic plants or based on other modifications of the PC synthesis pathway, have confirmed that these peptides are the main factor for basal tolerance, especially Cd tolerance (Verbruggen et al., 2009; Wójcik & Tukiendorf, 2011). There are also a few examples of higher PC accumulation in metal-tolerant versus non-metal-tolerant populations, e.g. in *Echium vulgare* or *Dittrichia viscosa* (Dresler et al., 2014; Fernández et al., 2014). Nevertheless, in a great majority of studies, more intense PC synthesis was found in non-metal adapted populations in comparison with tolerant ones, both within calamine metal hyperaccumulators, e.g. *Sedum alfredii* or *Noccaea caerulescens*, and non-accumulators, among others in *Silene vulgaris* or *Dianthus carthusianorum* (Schat et al., 2002; Sun et al., 2007; Wójcik et al., 2015b). Further evidence against the role of PC in adaptive metal tolerance is provided by the fact that hardly ever have these peptides been detected in plants growing in metal-enriched soils, either in controlled experiments or under natural conditions (Ernst et al., 2008 and references therein; Wójcik et al., 2005, 2015b). This is most probably related to the high metabolic costs of sulphur assimilation necessary for their synthesis (Ernst et al., 2008; Rascio & Navari-Izzo, 2011).

Thiol peptides have a role in homeostasis and detoxification of metal ions with high affinity to thiol groups, such as Cd, Cu, Hg, Ag, and As; detoxification of other metals, e.g. Ni or Zn, is more related to hydroxyl and amino groups (Rauser, 1999; Ernst et al., 2008). Organic acids, such as malate, citrate, and oxalate, chelate metal ions in the cytosol but mainly participate in storage thereof in the vacuole (Haydon & Cobbett, 2007; Wójcik, 2009). Citrate has a higher capacity for metal ions than the other organic acids and exhibits strong affinity for Ni and Cd (Anjum et al., 2015), whereas malate was proposed as the main ligand

for Zn (Mathys, 1977). However, the existence of both citrate and malate complexes with all these metal ions was confirmed *in planta* (Krämer et al., 2000; Sarret et al., 2002). In the case of the increase in the root mitochondrial synthesis of malic and citric acids, Agrawal et al. (2013) reported a fast and transient influx of nickel in mitochondria in *Alyssum murale* prior to sequestration thereof in vacuoles. In turn, in the case of metal storage in cell walls, Halimaa et al. (2014) showed that the Ni-malate complex was transported to the apoplast by malate anion channel ALMT12 (aluminium-activated malate transporter) of the root stele plasma membrane.

Although higher constitutive levels of organic acids have been reported in some hyperaccumulators and non-accumulating metal tolerant metallophytes (Assunção et al., 2003; Wójcik et al., 2015b), a clear relationship between metal accumulation and the level of organic acids has not been established. A positive correlation between metal treatment and the concentrations of these compounds was found in *Arabidopsis halleri* (Zhao et al., 2000), *Noccaea caerulescens* (Wójcik et al., 2006), and Ni-tolerant accessions of *Arabidopsis thaliana* (Agrawal et al., 2012). On the other hand, increasing Zn, Pb, or Cd concentrations did not affect malate or citrate concentrations in metalicolous and non-metallicolous ecotypes of *Dianthus carthusianorum* (Wójcik et al., 2015a, b; Wójcik & Tukiendorf, 2014), *Silene vulgaris* (Harmens et al., 1994), *Armeria maritima* (Olko et al., 2008), or *Echium vulgare* (Dresler et al., 2014). Similarly, Ni did not affect malate concentrations in Ni-susceptible accessions of *Arabidopsis thaliana* (Agrawal et al., 2012). Probably the constitutively high tissue content of organic acids, which are involved, besides metal detoxification, in a plethora of physiological functions, may be sufficient for effective chelation of the increasing concentrations of metals. Nonetheless, their role in hypertolerance and hyperaccumulation is questionable (Callahan et al., 2006, Verbruggen et al., 2009).

Amino acids (histidine, proline) and derivatives (nicotianamine) have also been reported to chelate metal ions in the cytoplasm before loading thereof to the xylem. Histidine is considered the most important ligand of this group of compounds, especially for Ni detoxification, translocation, tolerance, and hyperaccumulation (Callahan et al., 2006). Complexation of Ni to histidine has been suggested to play a role in preventing metal entrapment in root cell vacuoles and in keeping the metal in the cytosol in a detoxified form available to be released into the xylem in *Alyssum* species and *Noccaea caerulescens* (Krämer et al., 1996; Kerkeb & Krämer, 2003; Richau et al., 2009). For generation of such a large pool of histidine, the gene for the first step of its biosynthesis encoding an ATP-phosphoribosyl transferase was reported to be constitutively overexpressed in the roots of the Ni hyperaccumulator *Alyssum lesbiacum* (Ingle et al., 2005). In contrast, increased histidine concentrations in response to Ni do not appear to be a feature of the Ni hyperaccumulators *Noccaea goesingense* (Persans et al., 1999) and *Noccaea caerulescens* (Richau et al., 2009). Another ligand involved in the Ni and Zn chelation in the cytosol in their path toward the xylem is nicotianamine, showing metal-triggered accumulation in roots in *N. caerulescens* and *Arabidopsis halleri* (Mari et al., 2006; Weber et al., 2004). Nicotianamine also chelates Zn, Cu, and Fe for storage thereof in the vacuole (Manara, 2012).

Metal exclusion from the protoplast and plant organism

Compartmentalization of metals in the vacuole is a key mechanism to remove excess metal ions from the cytosol and seems to be crucial for metal tolerance. Indeed, the vacuole, which in plants may account for up to 90% of the cell volume, is generally considered the main metal storage compartment in plant cells (Wójcik, 2009). A number of tonoplast transporters are responsible for metal transport into the vacuole, both in the form of free metal

ions and in complexes with ligands (Wójcik, 2009; Manara, 2012). The enhanced ability of Ni vacuolar storage in shoots seems to be mediated by high expression of *MTP1* (metal transport protein of the CDF family) in *Noccaea goesingense* (Persans et al., 2001). This protein appears to be involved also in the Ni efflux from the cytoplasm to the cell wall (Kim et al., 2004). Another tonoplast located metal transporter, i.e. a ferroportin transporter PgIREG1, showed higher expression in *Psychotria gabriellae* and was suggested as a candidate for Ni tolerance and hyperaccumulation by Merlot et al. (2014). Zinc and Cd sequestration into the shoot vacuole is probably mediated by transporters belonging to tonoplast P_{1B}-ATPase and CAX (cation exchanger) families, respectively, whose genes are overexpressed in the Zn/Cd hyperaccumulators *Noccaea caerulescens* and *Arabidopsis halleri*, probably contributing to their hypertolerance (Rascio & Navari-Izzo, 2011; Wójcik, 2009 and references therein).

Active metal efflux from the cell is well documented in bacterial and animal cells but only a few plasma membrane efflux transporters have been recognised in plants so far (Manara, 2012). Based on sequence similarity to microbial and animal proteins, the most likely candidates for active metal ion pumping out of the cytoplasm are the P_{1B}-ATPases and the CDF families of transporters. Heavy metal ATPases (HMAs): HMA2 and HMA4 belonging to the first family export selectively Zn and Cd (Krämer et al., 2007). The AtPDR8 protein of the ABC transporter family localized in the plasma membrane of *Arabidopsis thaliana* root hairs and epidermal cells probably acts as an efflux pump for Cd and Pb, thus conferring tolerance to these metals (Kim et al., 2007).

When excluded from the protoplast, heavy metals may be removed from the organism by excretion or deposited in the cell wall, inside idioblasts, trichomes, or in some plant organs, the latter being often shed when overburdened with metals (Siedlecka et al., 2001). Active excretion of metals from leaf cells can occur via multicellular salt glands, e.g. in *Armeria maritima* ssp. *halleri* or via hydathodes, e.g. in *Minuartia verna*, when grown on

calamine soils (Olko et al., 2008). Nickel and Cd have also been reported to be excluded from stomatal cells (Broadhurst et al., 2004; Cosio et al., 2005). Many plants allocate metals in particular epidermal cells, including trichomes, and even in the cuticle (Asemaneh et al., 2006; Krämer et al., 1997; Mesjasz-Przybylowicz et al., 1994; Słomka et al., 2011a). In some halophytes, such leaf hairs are discarded when overloaded (Bothe, 2011); whether such behaviour is exhibited by metallophytes should still be elucidated. Instead, the phenomenon of seasonal intensive metal deposition in older leaves and other plant parts followed by shedding these organs is well documented. It occurs, among others, in Zn/Cd excluding *Armeria maritima*, *Minuartia verna*, *Anthyllis vulneraria* or *Biscutella laevigata*, in Cu excluding *Becium homblei*, and in Ni excluding *Indigofera setiflora* (Baker & Brooks, 1989; Bothe, 2011; Siedlecka et al., 2001).

If the defence mechanisms discussed above fail, the plant will suffer metal toxicity expressed as growth inhibition, chlorosis and necrosis, senescence, and ultimately death. These phytotoxicity symptoms may result from alterations in numerous physiological processes, such as photosynthesis, respiration, mineral nutrition, water relations, hormone balance, cell division, and elongation (Siedlecka et al., 2001; Singh et al., 2016). Another common consequence of metal toxicity is the enhanced production of reactive oxygen species (ROS) leading to oxidative stress resulting in lipid peroxidation, membrane dismantling, ion leakage, deterioration of macromolecules, and DNA-strand cleavage (Jozefczak et al., 2012). Therefore, a further defence mechanism adopted by metal-exposed plants relies on activation of the cell antioxidant system, including induction of enzymatic and non-enzymatic free radical scavengers (Jozefczak et al., 2012; Manara, 2012). Among the few studies performed on metalicolous versus non-metallicolous ecotypes, Gonnelli et al. (2001) reported that a serpentine population of *Silene paradoxa* seemed able to counteract Ni-induced oxidative

stress by limiting the metal inhibiting effect on peroxisomal H₂O₂ scavenging enzymes. This problem has been comprehensively analysed by [Hendrix et al., 2017 \(in this issue\)](#).

A better understanding of the processes involved in metal tolerance and adaptations to metalliferous habitats may be important as a basis for more targeted strategies for developing plants suitable for phytoremediation.

Potential of metallophytes for rehabilitation and phytoremediation

Metalliferous areas, and especially the recent ones of anthropogenic origin and usually devoid of vegetation, are a source of secondary metal pollution via water and wind erosion, posing a threat to adjacent ecosystems and human health (Tordoff et al., 2000; Wójcik et al., 2014). Some plant species occur spontaneously on degraded areas due to natural succession; however, this process is very slow and may last decades, often merely resulting in sparse patches of vegetation. Establishing the plant cover is one of the best ways to prevent metal migration and to increase the aesthetical value of degraded areas. To that end, it is important to use native species growing in the immediate neighbourhood or not far from the area to be reclaimed, because these plants are often more efficient in terms of survival, growth, and reproduction under environmental stress than plants introduced from other environments (Tordoff et al., 2000). Indigenous metallophytes may be used as a source of seedlings or seeds as well as fragments of natural turf with its soil, plants, microorganisms, mycorrhizas, seed bank etc. to increase the persistence of the plant cover and its biodiversity (Wójcik et al., 2014).

Various environmental approaches using metal tolerant plants are recommended, such as restoration, rehabilitation, or phytoremediation of metal-contaminated sites (Prach & Tolvanen, 2016; Whiting et al., 2004). The term 'restoration' refers to a process where the

recovery of a degraded or destroyed ecosystem to its pre-existing conditions is designed and prosecuted, which implies that only metallophytes originally present at the site can/should be used (Whiting et al., 2004). Since the metallophyte populations in most degraded mine sites have not been catalogued, such sites cannot be ecologically restored. On the other hand, 'rehabilitation' (or 'reclamation') means establishing a vegetative cover that is not representative of the pre-existing conditions and therefore any plant species able to tolerate the harsh site conditions can be exploited. Two approaches can be distinguished based on the level of human intervention to the environment. Assisted reclamation aims at speeding up the natural regeneration of ecosystems by improving the site conditions and/or introduction of target species, whereas technical reclamation often involves drastic restructuring of the habitat, including importing soil followed by sowing or planting some plants (Prach & Tolvanen, 2016). Vegetation can be introduced to a contaminated area for either phytostabilization or phytoextraction. Phytostabilization relies on stabilizing contaminants in the soil by plant imposed accumulation and precipitation of toxic elements in the rhizosphere or adsorption thereof on root surface. In contrast, phytoextraction assumes removal of metals and metalloids from soils through plants. Both approaches represent phytoremediation technologies and have been a subject of many reviews recently (Ali et al., 2013; Cundy et al., 2016; Vangronsveld et al., 2009). They will also be extensively addressed to in further chapters of this volume (Chaney and Baklanov, 2017 in this issue); therefore, they will be only briefly characterised here. The mode of management of metalliferous areas depends upon the level and area of pollution and the final purpose of the site utilization/exploitation.

Phytostabilization

There are plenty of species of metal-tolerant plants that may be considered for phytostabilization. These plants should be characterised by restricted metal uptake and/or transfer to the shoots (excluders) to avoid entry of metals into the food chain, dense rooting system, rapid growth to ensure adequate ground coverage, ease of establishment and maintenance under field conditions, and tolerance to high concentrations of metals and other adverse soil conditions (Vangronsveld et al., 2009; Whiting et al., 2004).

Metal-tolerant plant populations/ecotypes (mainly grasses but also some trees) have been commonly used to stabilize and revegetate waste spoils and other severely contaminated sites (Cundy et al., 2016; Johnson et al., 1994; Stuczyński et al., 2007; Szarek-Łukaszewska, 2009; Tordoff et al., 2000). Phytostabilization by grasses has been performed with success, among others, in Pb/Zn/Cd contaminated sites in Poland (Daniels et al., 1998; Stuczyński et al., 2007), Belgium (Vangronsveld et al., 1996), France (Cundy et al., 2016), USA (Li et al., 2000), and China (Shu et al., 2002). The most widely used grass species include *Agrostis capillaris*, *Festuca rubra*, *Festuca arundinacea*, *Poa pratensis*, *Vetiveria zizanioides*, and *Deschampsia cespitosa*. Zn/Pb mining waste deposits near Bukowno-Bolesław in Poland as well as other metalliferous remnants of mining and smelting activities elsewhere have been and are still being afforested by planting trees and shrubs, which yields stable woodlands characteristic for the landscape of these regions (Szarek-Łukaszewska, 2009). Trees that are most often introduced into reclaimed areas include pine (*Pinus sylvestris*), birch (*Betula pendula*), larch (*Larix decidua*), and black locust (*Robinia pseudoacacia*). To improve the vegetation capacity and increase the persistence of a plant cover, it is recommended to combine the introduced plants with some metal tolerant ecotypes of legume species, e.g. *Trifolium repens* or *Lotus corniculatus*, which can naturally maintain a nitrogen supply through symbiotic fixation. Successful attempts of such reclamation approaches have been reported e.g. in the Parc Pb/Zn mine in North Wales (Whiting et al., 2004) or in abandoned

metalliferous fluorspar dams in Derbyshire, UK (Turdoff et al., 2000). There are several examples of using metal-tolerant populations of herbaceous plants for revegetation of metal-contaminated sites. For instance, introduction of *Arabidopsis halleri* and *Armeria maritima* subsp. *halleri* originating from Central European calaminarian grassland into the Auby smelter area in France produced a positive effect (Bert et al., 2000; Dahmani-Müller et al., 2000). Seeds of native metallophytes were collected and successfully used for revegetation of Australian mineral wastes (Whiting et al., 2004). Similarly, seeds of a calamine population of *Biscutella laevigata* were used with a big success on another Zn-Pb spoil heap, in Upper Silesia, Poland creating a numerous and still increasing population just after four years (Rostański, 2014).

Phytoextraction

In contrast to organic pollutants, heavy metals do not undergo biodegradation and therefore persist in soils for thousands of years. For instance, Pb, one of the most toxic metals, has soil retention time of 150-5000 years and its high concentration may be maintained in the soil for 150 years (Singh et al., 2016). The only effective way of removal of metallic elements is extraction thereof using conventional physical or chemical methods (e.g. *in situ* vitrification, soil incineration, washing or flushing, but usually soil excavation and replacement - so called 'dig and dump'), which are usually very expensive and destructive to the soil ecosystem, or using phytoextraction (Ali et al., 2013; Vangronsveld et al., 2009). Phytoextraction represents a cost-effective, efficient, environment- and eco-friendly alternative to the conventional methods but is also the most challenging task among all phytoremediation approaches/technologies. The idea of phytoextraction is to concentrate high amounts of metals in aboveground plant parts that are subsequently harvested and incinerated

or processed in another way (Sas-Nowosielska et al., 2004). Therefore, ideally, it should combine hyperaccumulation with high biomass production; however, no such plant has been recognised so far. Hyperaccumulators are usually slow growing low-biomass plants, often with restricted and local occurrence, whilst fast growing and high-biomass plants are predominantly poor metal accumulators with a low to moderate metal tolerance level. Nevertheless, several successful applications of both kinds of plants for phytoextraction on a field scale have been noted. For instance, the Ni hyperaccumulators *Alyssum bertolonii* and *Strephanthus polygaloides* have been proposed for Ni phytoextraction (Li et al., 2003). Small-scale field trials showed a potential of some ecotype of the Zn/Cd hyperaccumulator *Noccaea caerulescens* for phytoextraction of Cd from polluted soils (Maxted et al., 2007; McGrath et al., 2006). On the other hand, high biomass plants such as willow (*Salix sp.*) (Janssen et al., 2015), poplar (*Populus sp.*) (Ruttens et al., 2011), maize (*Zea mays*) (Van Slycken et al., 2013), sunflower (*Helianthus annuus*) (Nehnevajova et al., 2009), *Brassica juncea* (Tlustoš et al., 2006), or *Jatropha curcas* (Papazoglou, 2014) appeared to be good candidates for decontamination of moderately polluted soils.

In phytoremediation treatment, only rarely can metal tolerant vegetation be established directly on the metalliferous substrate (like in Rostański, 2014). Such soils are often too phytotoxic and usually some physical or chemical modifications (mostly using some amendments) are applied prior to introduction of plants to improve soil fertility, render the soil less hostile and, depending on the aim, to decrease or increase metal bioavailability and accumulation for successful site management (Vangronsveld et al., 2009).

Phytoextraction efficiency of plants can also be substantially improved through natural cultivation for some desirable traits, mutagenesis, hybridization, or genetic engineering (Ali et al., 2013; Singh et al., 2016; Vangronsveld et al., 2009). However, due to the complex genetic background of mechanisms involved in metal uptake, translocation, and sequestration as well

as poor legal and societal acceptance of application of genetically modified plants, this strategy is still far from introduction in practice.

Successful phytoextraction requires decreasing the pollutant level to that complying with environmental regulations. From the practical point of view, phytoextraction is only feasible on low to moderately contaminated soils, like these at tertiary human-influenced sites. In the case of many severely metal-enriched soils influenced by industrial or mining activities, it would be very difficult or even impossible to carry out phytoextraction within a reasonable period; then, stabilization using metal-tolerant plants is a more logical alternative to initiate a healthy ecosystem (Whiting et al., 2004; Vangronsveld et al., 2009; Zhao & McGrath, 2009).

Other potential beneficial applications of metallophytes

In addition to the rehabilitation or phytoremediation of metal-contaminated areas, several other benefits from application of metallophytes can be mentioned. Metal-rich plant biomass from phytoextraction may be combusted to get energy and the remaining ash may be in some cases used as 'bio-ore' for recovery of precious metals (Ni, Tl, Au, Pd, Pt) in a process called phytomining or agromining (van der Ent et al., 2015; Chaney and Baklanov, 2017, this issue). The difference between these two applications is associated with on the type of soil used for obtaining plant biomass. Phytomining relates to degraded or mined lands being under the reclamation process, whereas agromining assumes using low-productivity agricultural metal-contaminated soils to generate economic profits to farmers. Up to date, phytomining has been commercially applied for Ni using *Alyssum murale* and *Alyssum corsicum* and it appeared to be more eco- and cost-effective than the conventional metal extraction methods with reference to many ores (Chaney et al., 2007).

Another novel and interesting way of processing with metal-rich plant biomass is to use it for eco-catalyst production (Escande et al., 2015). A polymetallic solid obtained after thermal and chemical treatment of plant material can serve as a catalyst or reagent in synthesis of valuable products for the fine chemical, e.g. fragrances and cosmetics, industry.

Metal hyperaccumulators may also have an alternative use – in biofortification (Clemens, 2016; Guerinot & Salt, 2001; Zhao & McGrath, 2009). Biofortification aims at production of crops enriched with trace elements that are essential for human diet in edible plant parts through cultivation, biotechnological approaches, or the use of appropriate fertilizers. Zinc, Se, Fe, and iodine (I) are the major target micronutrients since their deficiencies affect more than half of the world population.

Concluding remarks

Metallophytes are widespread throughout the world, forming an integral part of the biodiversity on metalliferous soils. Due to their amazing ecology and physiology as well as multiplicity of potential applications, there is a need to continue screening metalicolous vegetation for its ability to tolerate metals, both in terms of hyperaccumulation and exclusion. Severely contaminated sites bearing well-developed metal-resistant flora are peculiar ecological islands that should be protected as gene reservoirs. These include not only the natural outcrops or tracts of metalliferous areas (e.g. serpentine soils), being often a cradle for endemic vegetation, but also anthropogenically created metalliferous waste deposits (e.g. from mining and smelting of Zn and Pb ores) becoming niches for specific and unique vegetation. The latter artificial habitats, although often considered dull scars and eye-sores of the landscapes, may in fact represent very interesting biocenoses exceeding the pre-existing habitats with their biodiversity. Therefore, integrated efforts should be focused on protecting

the native environments of metallophytes, both of natural and human origin. Several strategies have been proposed for preserving the biodiversity of metallophytes: active protection of metalliferous habitats *in situ*, establishing collections of metallophytes in botanical gardens, arboreta, and seed or germplasm banks, etc. (Baker et al., 2010; Whiting et al., 2004).

Considerable attention has been given worldwide to the possibility of using metallophytes, either alone or in combination with microorganisms for rehabilitation or phytoremediation of contaminated sites. Moreover, exploitation of metallophytes as bio-ores coupled with bioenergy production or, alternatively, as a natural source of crucial micronutrients for improving human diet and public health is an attractive idea. However, scientific information on the mechanisms of adaptation to adverse environmental conditions is a basis for planning effective reclamation of contaminated sites using metallophytes or other ways of beneficial or even profitable utilization of metallophytes. Thus, it is of pivotal importance to understand the inter- and intra-species variation in mineral uptake, distribution, metabolism and tolerance, and the molecular mechanisms responsible for the process. Many details of these processes have already been presented; yet, there is still a lot to be elucidated and recognised, especially under natural site-specific field conditions.

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