

Evolution of metal tolerance in higher plants

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Abstract

Evolution of tolerance to one or more metals enables plant species to colonise naturally metal-enriched soils. In the phylogeny of angiosperms, the evolution of high metal tolerance is not homogeneously distributed over taxonomic groups and shows differences not only within a taxonomic group, but even among populations of the same species. Anthropogenic metal emissions have pressed plants from non-metalliferous soils to evolve *de novo* metal tolerances that are predominantly realised in grasses. In this review, information is presented on the evolution of metal tolerance in angiosperms, its time scale and its physiological consequences.

Keywords: adaptation, compartmentalisation, metal transporter, mycorrhiza, nutrient demand, phylogeny

1 Introduction

During the evolution of angiosperms (Magnoliophyta), 19 chemical elements have been selected for the basic metabolism. Some elements are required at high amounts, i.e. the macronutrients C, H, O, Ca, K, Mg, N, P, and S, and others at moderate to low amounts, i.e. the micronutrients B, Cl, Co, Cu, Fe, Mn, Mo, Na, Ni, and Zn. In addition, Si, defined as beneficial element, is necessary for the maintenance of plant structures in some plant groups. On metal-enriched soils, there may be an imbalance of a plant's requirement and supply. Plants are immobile and can only slightly escape with their roots from adverse soil conditions in a small patch within the local site. Therefore plants have to modify their physiological processes to the environment in which they have germinated. For the survival of a local population for more than one generation, favourable metabolic characters have to be passed to the next generation modifying the genetic structure of the local population. From the physiological point of view, the persistence of a plant population in a specific environment is based on a combination of requirements of an array of chemical elements for the basic metabolism (macronutrients, micronutrients, and beneficial elements; MARSCHNER 1995) and/or of tolerances to a surplus of essential and metabolically non-essential elements (LARCHER 2003). In this contribution, I will focus on physiological requirements and tolerances in the evolution of angiosperms in time and space.

2 Metal requirements as the result of evolution

Metals are required by plants in a wide range of concentrations. During the evolution of angiosperms, the metal requirements are strongly steered by the demands of physiological processes in different organelles, cells, tissues and whole plants. At the cellular level, the function of the cell and the presence of specific organelles determine the metal amount. In cell organelles, the latter is relatively stable among all angiosperms. For example, in photosynthetic active cells the metal demand of the chloroplasts varies from 15 % of the total cell Zn in the carbonic anhydrase (HEWITT 1983) to nearly 50 % of the total cell Cu in the plastocyanin (HEWITT 1983; LOKEMA and VOOIJIS 1986). In mitochondria, however, Cu-enzymes are already satisfied with 3 to 6 % of total cell Cu (PENG *et al.* 2005), and Fe-enzymes with 2 to 4 % of total cell Fe (HEWITT 1983). At the moment, there are no biological concepts, which can explain the evolution of these demands.

At upscaling from the cell to a plant organ, the evolution of morphological biodiversity has resulted in quite different metal demands. Even a tiny plant part such as the pollen grain can show a high variability in species-specific and metal-specific concentrations which range per mg of dry pollen for Cu from 1.5 to 20 µg, for Mn from 17 to 112 µg, for Zn from 30 to 250 µg, and for Fe from 60 to 9560 µg (STANLEY and LINSKENS 1974). Exemplified for leaves of forest trees, the range of leaf metal concentration varies in a species-specific manner with a factor of 2 for Fe, 3 for Cu and Zn, and factor of 9 for Mn (BAUMEISTER and ERNST 1978). The demand is dependent on species and genera, as the high Zn demand of birches (ERNST and NELISSEN 2006), but it is often strongly modified by external factors such as metal and water supply.

At the whole plant level, a plant's metal requirement is defined by the ratio of the different plant parts within a plant, by the development stage, by the turnover rate of plant organs, and by the life history. In the future, genomic scale profiling of nutrients in plants, called "ionomics" (LAHNER *et al.* 2003) will contribute to a further understanding of metal requirements of plant species and will hopefully help to elucidate the processes that have governed the evolution of metal physiology (SALT 2004; VREUGDENHIL *et al.* 2004).

Primary metabolism alone, however, does not guarantee the survival of a plant, but other adaptations are necessary for the interaction with other organisms of its environment. The defence against herbivores and pathogens has driven the evolution of additional metal requirements. This may be achieved by metal accumulation up to toxic levels (BOYD and MARTENS 1998a), but the matter is still under debate (TOLRA *et al.* 2001; NORET *et al.* 2005). Such an accumulation can be specific for taxa within a plant family. An example is the Zn accumulation of *Betula*-species as compared with the low ones of co-occurring tree species of the same family (*Carpinus* and *Corylus*; ERNST 2004). The metal supply by the environment in interaction with all other nutrients will determine a plant's performance, increasing from deficient performance at short supply to an optimum at sufficient supply and decreasing with surplus supply in a range from toxicity up to death (Fig. 1).

If a species colonises a new habitat with a too high or a too low metal supply, modification through growth and adaptation through reproduction have to build up a new internal metal balance, called homeostasis (CHAT 1999). Adaptation, and hence evolution, of the metabolism can be realised either by enhancing the population's tolerance to higher metal levels (Fig. 1) or by stimulating the uptake or physiological efficiency in the case of short supply (Fig. 1).

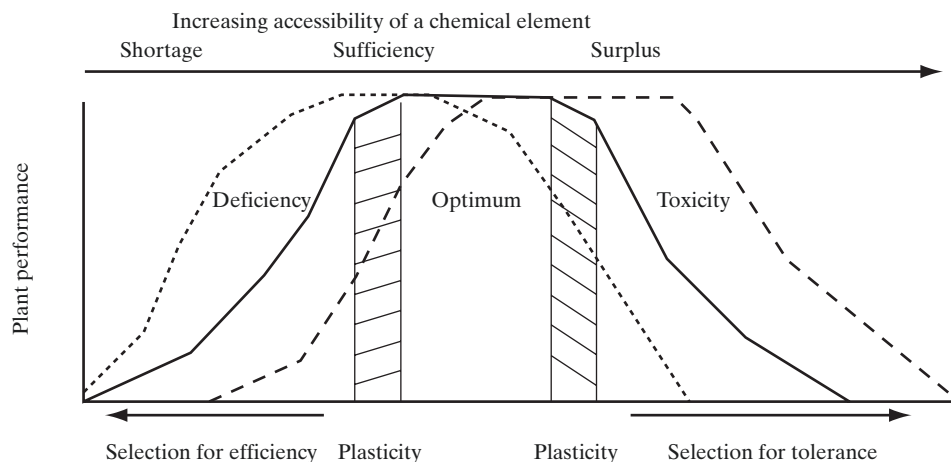


Fig. 1. Direction of the evolution to manage shortage or surplus of a chemical element in the environment. The hatched zone is the range of phenotypic plasticity of individuals. The dotted line gives the performance after the selection process in the population that has resulted in an enhanced efficiency in the range of shortage, e.g. iron on calcareous soils. The dashed line shows the shift to improved performance after the evolution of tolerance to increased metal levels, e.g. zinc on zinc-enriched soils. Modified after ERNST (1996).

3 Genetics and physiology of metal tolerance

3.1 Avoidance and excluders of metal surplus

As soon as a species colonises a metal-enriched soil, it has to adapt its metabolism to the new situation. This demands a new regulation of metal homeostasis and detoxification of metal surplus. LEVITT (1972) has separated metal resistance into two reaction modes, avoidance and tolerance. Roots are the plant organs that are first exposed to a surplus of metals in the soil solution. If metal concentrations are very inhomogeneously distributed, roots may explore less metal-enriched soil patches (WHITING *et al.* 2000). However, in a highly and homogeneously metal-enriched soil, such avoidance will not help. For those metals that are micronutrients, full avoidance will also cause metal deficiency.

The evolution of metal uptake mechanisms for non-functional metals cannot be tailored so closely because their chemical characteristics or the metal ion size are often too similar to essential metals. Examples are Cd uptake via a low affinity system of one of the Ca transporters (PERFUS-BARBECH *et al.* 2002) and/or via the high affinity uptake system of the Fe transporter IRT1 (KORSHUNOVA *et al.* 1999), and the uptake of arsenate by phosphate transporters (MEHARG and MACNAIR 1991). One option to diminish the uptake of such elements is a down-regulation of transporter activities. Such a mechanism has evolved in populations of several grass species growing on As-enriched soils. As-tolerant plants are down-regulating the high affinity phosphate transporter to diminish arsenate uptake (MEHARG and MACNAIR 1991, 1992). Restriction of Cu uptake by a Cu-tolerant population of *Silene vulgaris* (LOLKEMA *et al.* 1984) may also be based on such a down-regulation of Cu-transporters. Most species with different sensitivities to a surplus of Cu and Zn, however, take up metals to a similar degree (HARMENS *et al.* 1993; ASSUNÇÃO 2003). Another option of avoidance is the change of the bioavailability in the rhizosphere by

exudation of protons or organic acids (KINRAIDE *et al.* 2005). Yet another type of avoidance is related to symbiosis with mycorrhizal fungi. The latter can extend their hyphae over several tens of meters outside the rooting zone and transfer other types and amounts of chemical elements to the plant (DAHLBERG 1997). These fungi, mostly having a higher metal tolerance than angiosperms, may enhance metal resistance of an angiosperm by changing metal speciation or by restricting the transfer of the metal into the plant. One example is *Viola calaminaria*, a characteristic plant species of the heavy metal vegetation in Western Europe (ERNST 1974). Zinc violets have low Zn tolerance (RUTHER 1967), but their symbiosis with one to four arbuscular-mycorrhizal fungi diminishes the Zn flux into the plant (HILDEBRANDT *et al.* 1999; TONIN *et al.* 2001), but can not prevent the accumulation of Zn in the plant (ERNST 1974). In general, however, the avoidance concept is not applicable to metal responses of plants growing in metal-enriched soils.

Differences in the internal metal allocation between roots and shoots were taken by BAKER (1981) to categorise plants as excluders, indicators and tolerators. Excluders are those plant species that retain most of the metals in their roots and restrict metal transfer into the shoots (LOLKEMA *et al.* 1984). But exclusion it is not an avoidance at the whole plant level *sensu* LEVITT (1972), because the uptake by the root has to meet a plant's demand for (1) the primary metabolic processes, and (2) the defence function. Consequently, the only option for survival on metal-enriched soils is evolution of tolerance mechanisms. Soil patches with very high metal concentrations, so-called "hot spots", are usually not covered by any vegetation (ERNST 1974; SHEWRY *et al.* 1979) indicating the limits of evolution of tolerances to metals and to accompanying environmental constraints.

3.2 The genetics of metal tolerance

In contrast to a metal-sensitive plant species (= non-metallophytes), a metal-tolerant plant species (= metallophytes) maintains good performance, because it can cope with higher plant-internal metal levels due to genetic changes (= adaptation). It is accepted that metal-specific tolerances have independently evolved several times in different species from local non-tolerant ancestral populations (CHAT *et al.* 2000; PAUWELS *et al.* 2005). It is speculated that the genes for metal tolerance are pre-existing at a low frequency in non-tolerant populations of some plant species (GARTSIDE and MCNEILLY 1974; BRADSHAW and MCNEILLY 1981; MACNAIR 1987). When sown on metalliferous soils, seed collections of non-metallophytes can germinate, but usually seedlings do not produce survivors due their low metal tolerance. There is now sufficient evidence that the exclusion of some species from mine areas is because of their inability to evolve sufficiently metal tolerant mutants. Therefore, a successful colonisation of highly metal-enriched soils demands a *de novo* and *in situ* evolution of metal tolerance.

In metalliferous soils, there are gradients of plant-available metal levels, which are reflected in a gradient of metal tolerant individuals (GRIES 1966; CHAT *et al.* 1996; CHAT and VOOIJIS 1997; SMITH and MACNAIR 1998; ASSUNÇÃO 2003; ZHA *et al.* 2004; BRATTELER 2005). In reports of metal tolerance of plant species, populations, and genotypes, the existence of such different degrees of metal tolerance is insufficiently recognised. When the expression of enzymes is manipulated by genetic engineering, the metal tolerance of transgenic plants is only enhanced by a factor of two (ZHU *et al.* 1999; BENNETT *et al.* 2003; POMPONI *et al.* 2006). This enhancement is insufficient to grow and establish a population on metalliferous soils. In this review, I will use only the term "metal tolerance" for plants that can survive and contribute to the establishment of population on metal-enriched soils, i.e. being metallophytes.

The type of a metal tolerance test is decisive for the interpretation of the degree of metal tolerance. Root growth is more rapidly inhibited by heavy metals than are other parts of the plant. Therefore short-term tests for metal tolerance mostly compare root elongation growth of suspected tolerant plants with non-tolerant ones in a solution with one enhanced or a steadily increasing level of the metal under question (WILKINS 1957; SCHAT and TEN BOOKUM 1992; MACNAIR *et al.* 1993). Seldomly, the tolerance of leaf cells, shoot cells, and pollen is tested (GRIES 1966; RUTHER 1967; SEARCY and MULCAHY 1985). Short-term tests are insufficient for the evaluation of metal tolerances of a plant, because survival of seedlings (GARTSIDE and MCNEILLY 1974; ERNST *et al.* 2000) does not ensure survival at later vegetative state, and does not guarantee reproduction (ERNST *et al.* 2000), as emphasised earlier by SIBLY and CALOW (1989).

Metal tolerance segregates in crosses between plants from metalliferous and normal soil. Depending on the tolerance level of the parent plants used to generate segregating populations, one or two major genes determine Cd, Cu, Ni, and Zn tolerance of *Silene vulgaris* (BROKER 1963; SCHAT *et al.* 1993, 1996; BRATTELER 2005), in Cu tolerance of *Mimulus guttatus* (MACNAIR 1993), and in Zn tolerance of *Arabidopsis halleri* (BERT *et al.* 2003). In the case of Cu tolerance some minor genes (=“modifiers”) are involved enhancing the effect of the major gene(s) and increase the metal tolerance, at least in Cu-tolerant populations of *Mimulus guttatus* and *Silene vulgaris* (SMITH and MACNAIR 1998; SCHAT and TEN BOOKUM 1992). The few major genes steering metal tolerance indicate that the specific toxicity of a metal surplus allows only a few effective biochemical adaptations (SCHAT and VERKLEIJ 1998).

PRAT (1934) was the first who detected the genetic adaptation of plant populations to metal surplus. *Melandrium rubrum* (= *Silene dioica*) from a population growing on the Cu mine at Piesky was more Cu-tolerant than plants from a “normal” soil. Nearly 20 years later, BRADSHAW (1952) and BAUMEISTER (1954) took up this research approach that is still ongoing. On soils with more than one metal in surplus, each metal is under the control of specific genes as shown for Pb and Zn tolerance in *Festuca ovina* (BROWN and BRINKMANN 1992) and Cd and Zn tolerance in *Silene vulgaris* (SCHAT *et al.* 1996).

The genetics of metal tolerances is investigated for the following metals and plant species: As in grasses (MACNAIR *et al.* 1992), Cd in *Arabidopsis halleri* (BERT *et al.* 2003), Cd, Cu, Ni, and Zn in *Silene vulgaris* (SCHAT and TEN BOOKUM 1992; SCHAT *et al.* 1996; BRATTELER 2005), Cd, Ni and Zn in *Thlaspi caerulescens* (ASSUN²AO *et al.* 2003a, b; DUBOIS *et al.* 2003; FREROT *et al.* 2003; ZHA *et al.* 2004), Cu in *Silene paradoxa* (MENGONI *et al.* 2000), and Cu and Ni tolerance in *Mimulus guttatus* (MACNAIR 1983; TILSTONE and MACNAIR 1997).

3.3 The physiological mechanisms of metal tolerance

Despite independent evolution of metal tolerances within angiosperm groups, their metal-specific physiological regulations are nearly identical (ERNST *et al.* 1992). (1) At the cellular level, protection of physiologically active sites in the cell is achieved by a rapid cellular compartmentalisation of the metal surplus, especially into the vacuole (Fig. 2). The original compartmentalisation concept (ERNST 1969, 1974) has received much support during the past decade. The role of metal-binding metabolites in the cytosol is elaborated for Cd- and Cu-tolerant plants (DE KNECHT *et al.* 1994; VAN HOOF *et al.* 2001; MENGONI *et al.* 2003). The removal of surplus metals from the cytosol and their transport across the tonoplast are accelerated in metal-tolerant plants (VERKLEIJ *et al.* 1998; ASSUN²AO *et al.* 2001; DRAGER *et al.* 2004). Metal-tolerant enzymes in the cytosol were not developed in metal-tolerant plants (MATHYS 1975).

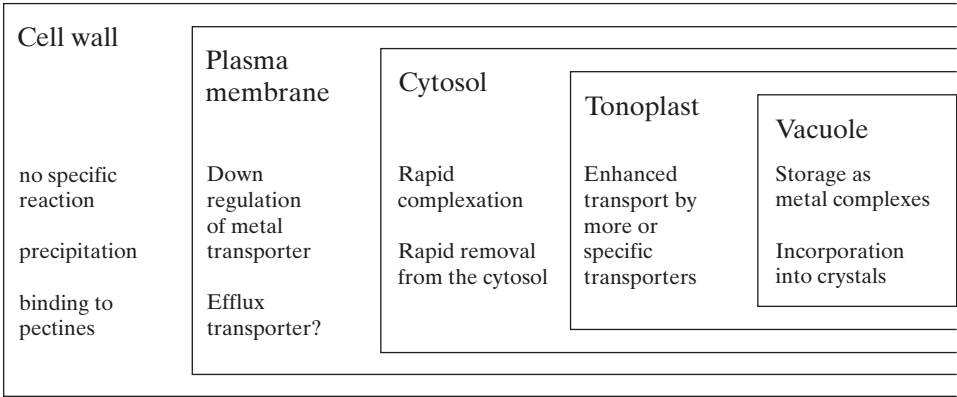


Fig. 2. General physiological processes in metal tolerant plants at the cellular level. No specific processes are described for the cell wall of metal-tolerant plants.

(2) At the tissue level, metal surplus is allocated to metabolically less active tissues (CHARDONNENS *et al.* 1999; BROADHURST *et al.* 2004), such as epidermal cells with the exception of guard cells (VAZQUEZ *et al.* 1992; HEATH *et al.* 1997; PSARAS *et al.* 2000). The consequence of tolerance to a metabolically essential metal may be a higher metabolic demand for that element. A rapid compartmentalisation may hamper an adequate metal supply to the cellular demands, if a metal-tolerant plant is experimentally grown on a soil with a low supply. Such a shift in demand was shown in a comparison of photosynthesis in Zn-sensitive and Zn-tolerant plants of *Silene vulgaris* (= *S. cucubalus* = *S. inflata*) by BAUMEISTER and BURGHARDT (1956) and later by in situ studies of enzyme activities (MATHYS 1975). (3) At the whole plant level, metal surplus is highly allocated to deciduous organs at the time of senescence, and a limited metal amount is allocated into seeds (ERNST 1974; ERNST *et al.* 2000; BHATIA *et al.* 2003). The seed as a metal-poor niche in metal-enriched plants is a welcome target to seed predating insects that can severely decrease seed production and population rejuvenation (ERNST *et al.* 1990). At the very moment, the molecular biological approaches are fine-tuning and deepening metabolic and genetic processes governing metal tolerances (PLESSL *et al.* 2005; ASSUN²AO *et al.* 2006; RIGOLA *et al.* 2006; WEBER *et al.* 2006).

3.4 Metal accumulation

Plants on metal-enriched soils often accumulate metals to very high levels. This accumulation combines two components, the intrinsic demand of a plant's metabolism, and the impact of the external metal supply on metal uptake with its consequences for the storage in roots and for the translocation process from roots to shoots. Genetically, metal accumulation is independent of metal tolerance (ASSUN²AO *et al.* 2006), and under regulation of a small number of other genes (MACNAIR *et al.* 1999). Therefore, it is not possible to conclude that a plant with an increased metal concentration in leaves is also metal tolerant. Metal concentration in leaves per se can only be taken as an indication of a plant's potential tolerance to that metal, but not as evidence of tolerance itself. Nonmetallicolous populations of *Thlaspi caerulescens* accumulate similar high zinc levels (above 10000 mg Zn kg⁻¹ d.m.) on metal-poor soils than metalliferous populations on highly zinc-enriched soils (MOLITOR

et al. 2004), but their Zn-tolerance is low (ASSUN²AO 2003). This independence of tolerance from accumulation is present in *Arabidopsis halleri* for Cd and Zn (MACNAIR *et al.* 1999; BERT *et al.* 2002) and in *Thlaspi caerulescens* for Zn (ESCARRE *et al.* 2000; ASSUN²AO *et al.* 2001). F2 data from interecotype crosses between metalliferous and nonmetalliferous populations of *Thlaspi caerulescens*, however, suggest that Zn accumulation and Zn tolerance may be co-selected in metalliferous populations, but being ruled by counteracting processes in nonmetalliferous populations (FREROT *et al.* 2005).

Based on the level of metals in aboveground plant parts, BROOKS *et al.* (1979) have distinguished plants as either accumulators or as “hyperaccumulators”. The latter group is characterised by arbitrarily defined metal concentration per kg dry leaves: for Cd >100 mg, for Co, Cu and Ni >5000 mg and for Mn and Zn >10000 mg (BROOKS 1998). The evolutionary background of the population-specific and metal-specific accumulation patterns (ASSUN²AO *et al.* 2003b) is still unknown.

3.5 Metal efficiency and its risk

On highly mineralised soils, plants are not only confronted with a surplus of one or more metals, but also with a shortage of another metal or of macronutrients (ERNST 1974). At a too low supply (shortage), plants evolve enhanced efficiency in the case of essential elements such as iron (Fig. 1). Too low iron availability can be overcome by a change of the chemistry of the rhizosphere, which can be realised by acidification of the rhizosphere via proton exudation and/or secretion of iron reductants, called Strategy I (MARSCHNER 1995). An increased efficiency may also be achieved by stimulation of the expression of high-affinity metal transporters, e.g., the iron transporter IRT. The IRT gene is only expressed in plant species originating from calcareous soil, when grown at low Fe supply, but not in those originating from acid soils with generally sufficient Fe levels. Such a difference in response is also shown for *Thlaspi caerulescens* ecotype Ganges from a calcareous metal-enriched soil in comparison with the ecotype Prayon from acid shales (LOMBI *et al.* 2002), highly expressing the IRT gene at a low Fe supply in the nutrient solution, resulting in enhanced uptake of Cd (LOMBI *et al.* 2002). Due to the low specificity of IRT (KORSHUNOVA *et al.* 1999), other metals in surplus may be transferred into the plants risking toxification, as shown for the enhanced Cd uptake of *T. caerulescens* ecotype Ganges when grown at Fe-limited conditions (LOMBI *et al.* 2002). Chlorosis of highly metal tolerant plants such as *Arabidopsis halleri* on metal-enriched soils (ERNST 1974) indicates that neither enhancement of the metal transporter nor internal retranslocation can change competition between iron and zinc in this Cd/Zn-tolerant herb. Evolution of metal efficiency has also strong limits.

In addition, grasses use a chelation strategy (Strategy II) for primary acquisition of Fe from the soil. On metal-enriched calcareous soils, grasses may be deficient in Fe (GRIES and RUNGE 1995). Then a concerted action of several genes occurs. Increased expression of NAAT-genes stimulate the synthesis of nicotianamine aminotransferase (NAAT), one of the enzymes for the biosynthesis of phytosiderophores, i.e. non-proteinogenic amino acids (TAKAHASHI *et al.* 1999). The excreted phytosiderophore forms stable Fe(III)-chelates in soil. A second gene (YS1), encoding a Fe-phytosiderophore transporter (Yellow Stripe 1), regulates the uptake of Fe-phytosiderophore chelates, and thus Fe supply (ROBERTS *et al.* 2004). Besides Fe, phytosiderophores mobilise also Cd, Cu, Mn, and Zn (ROMHELD 1991; SHENKER *et al.* 2001), and may enhance the uptake of these elements up to toxic concentrations in even metal-tolerant grasses (ERNST 1996). This impact of phytosiderophores on the metal supply of metal-tolerant grasses, mostly living in symbiosis with arbuscular mycorrhizal fungi (IETSWAART *et al.* 1992), however, has not thoroughly been investigated.

3.6 Costs of metal tolerance

As mentioned above, metal-enriched soils are demanding multimetal-tolerant plants. Such plants are characterised by very low growth rates and very restricted reproduction, as shown for the As-, Cd-, Cu-, Pb- and Zn-tolerant population of *Silene vulgaris* on the Bronze-Age smelting site near Langelsheim/Germany (ERNST *et al.* 2000). It seems conceivable that diminished plant and seed production may indicate metabolic costs of metal tolerances (ERNST 1983; WILSON 1988). HALDANE (1954) stated that there are costs of natural selection for new beneficial alleles. Considering the costs for the maintenance of tolerance mechanisms at the cellular level, the synthesis of complexing agents in the cytosol, e.g. phytochelatins for detoxifying As and Cd and metallothioneins for detoxifying Cu, withdraw N, S, and energy for their synthesis from the primary metabolism. A further cost is ATP consumption for the transfer of metals across the tonoplast (VERKLEIJ *et al.* 1998). At the whole plant level, the transfer of metals from root to shoot, and their allocation to the different tissues and cell types demand further energy investments of the metal-tolerant plant.

It is obvious that metal-tolerant plants produce less biomass compared to their non-metal-tolerant ancestors, but seldomly if grown under favourable environmental conditions. But it is more likely that the diminished production is the result of adaptation to other environmental conditions, such as low water and nutrient supply (see below). This diminished biomass is an integration of all costs related to survival on metal-enriched soils, indicating an investment of up to 20 % (ERNST 1983; HARPER *et al.* 1997), and also including “ecological” costs for adaptation to other environmental constraints of metal-enriched soils such as the low-water holding capacity and poor nutrient supply. The latter constraints change the morphology and physiology among populations independent of the genetics of metal tolerance (BRADSHAW 1952; SCHWANITZ und HAHN 1954; BROKER 1963; ERNST *et al.* 1990; SCHAT *et al.* 1997; BRATTELER 2005). On dry Cu-mine tailings, Cu-tolerant plants of *Silene vulgaris* have a constitutively 5- to 6-fold higher proline level (SCHAT *et al.* 1997). In experiments, this higher structural level of proline in the Cu-tolerant plants is not inducible by increasing Cu-concentrations. It is an adaptation to a low structural water supply of these Cu shales. This proline synthesis demands for one ATP and two NADPH+H⁺ to be spent in the four-step process of proline synthesis. The enhanced proline level is consuming 5 to 6 $\mu\text{mol N g}^{-1}$ dry leaf which is only 0.2 to 0.4 % of the total N amount in leaves of metal-tolerant plants (ERNST *et al.* 2000).

Most of the morphological traits, such as leaf area, leaf shape, hairiness, and stunted growth, are under separate genetic control (BROKER 1963; BRATTELER 2005). The inheritance of morphological traits, by taxonomist described as character of metal tolerant plants such as *Minuartia verna* subsp. *hercynica*, *Silene vulgaris* subsp. *humilis*, and *Thlaspi caerulescens* subsp. *calaminare* (HAEUPLER and SCHONFELDER 1988; OBERDORFER 1994), may be ensured by linkage between metal tolerance and the morphological traits (BRATTELER 2005), but they do not reflect the response to metal surplus.

At the moment, the available information does not allow the calculation of full costs, but it is conceivable that the costs of metal-tolerance are low as compared to the accompanying ecological costs to adverse edaphic conditions of heavy metal-enriched soils and to defense compounds against herbivores.

4 Evolution of metal tolerance: the time period

4.1 The naturally metal-enriched soils

Mineralisation of bedrock and its weathering are very inhomogeneous processes in space, and chemical composition of ores results in a patchy, spatially restricted distribution of soils enriched with one or many metals. Only the weathering of siliceous rocks (granites, sandstone) has resulted in huge areas with a surplus of manganese so that many plant species of siliceous, mostly acidic soils are manganese tolerant. In environments, where vegetation development was not disrupted by glaciation or desertification, the permanence of metal-enriched soils over long geological periods of millions of years principally gave ample time for plant species to evolve metal tolerances (WILD 1978; ERNST 2000). This is exemplified in Central Africa with soils highly mineralised with Co, Cu, Ni, and to a lesser degree, with Zn. The metalliferous vegetation is a grass savannah with metal-tolerant herbs, grasses, and sedges, whereas trees are absent (DUVIGNEAUD and DENAEYER-DE SMET 1963). Trees of the Miombo woodlands are obviously not able to evolve high tolerances to these metals so that there are relatively sharp borderlines between metal-enriched and “normal” soils (Fig. 3). Only on moderately metal-enriched soils, some tree species have evolved slight metal tolerance (ERNST 1972). Trees growing on nickeliferous soils in New Caledonia and Cuba accumulate high Ni concentrations in their leaves (BOYD and JAFFRE 2001; REEVES *et al.* 1996), but no experimental evidence is yet available to demonstrate nickel tolerance of these trees.



Fig. 3. On the copper-enriched soils at Swambo copper hills (Zaïre) lives a copper-tolerant vegetation that is perhaps over one million of years old. This vegetation is composed of herbaceous and graminaceous plant species originally derived from plants from non metal-enriched soils of the surroundings. One of the endemic species on copper soils is the herb *Haumanniastrum robertii* (Lamiaceae) in a grassland with Cu-tolerant ecotypes of the herb *Becium centrali-africanum* (Lamiaceae) and evenly tolerant perennial grass *Loudetia simplex*. Tree species from the adjacent Miombo woodland (*Albizia adianthifolia*, *Baphia bequaertii*, *Ekebergia benguelensis*) have not evolved copper tolerance and are only present on soils with low metal concentrations. The small shrubs in the transient zone are *Xerophytia*-species (Velloziaceae) on stony soil. In the background, a further treeless area with heavy metal vegetation can be seen.

In the Northern Hemisphere, extensive glaciation has restricted the time for the evolution of metal tolerance. Since the last glacial epoch, about 12000 years were available for this selection process. In addition, most metal-enriched soils were disturbed by mining activities sometimes for thousands of years so that plants were confronted with additional selection factors (Fig. 4). As in the tropics of Africa, trees are absent on highly metal-enriched soils in the Northern Hemisphere. Only *Betula pendula*, *B. pubescens* and some *Salix* species were able to develop moderate Cu-, Ni- or Zn-tolerance (BROWN and WILKINS 1985; UTRIAINEN *et al.* 1997). BONE and FARRES (2001) have calculated the rate of the evolution of metal-tolerance to be in the order of 40 to 1000 years, but under novel and high selection pressure such as metal fall-out in the vicinity of metal smelters, it can be realised within less than a decade (ERNST 1999).



Fig. 4. A former Cd-Zn-enriched hill was destroyed by mining activities in the “Bleikuhle” (= lead grove) at Blankenrode (Germany). The remaining metal-enriched soils at the bottom of the ore body and on the right slope still maintain a metalliferous vegetation with the herbs *Arabidopsis halleri*, *Minuartia verna*, *Silene vulgaris*, the endemic *Viola guestphalica*, and the grasses *Agrostis capillaris* and *Festuca ophiolitica* (ERNST 1974). The shrubs in the background are growing on metal-poor soil. The site is now under nature protection.

4.2 Anthropogenic metal-enriched soils

When smelters and other metal-emitting industries were established in areas without metal-enriched soils, metal-tolerant genotypes had to be selected from plant species in the surrounding non-metalliferous vegetation. This selection process followed the same rules as that on naturally metal-enriched soils, however, on a sharp time scale. If the metal concentration is very high and the selection pressure very strong, then the time for the establishment of a metal-tolerant population can be as short as four years, but it demands several decades at a lower selection pressure (Table 1). Selection of metal tolerances in man-made metal-enriched soil is highest in perennial grasses, due to their symbiosis with arbuscular mycorrhizal fungi (except on soils highly enriched with Cu; GRIFFIOEN 1994; GRIFFIOEN *et al.* 1994), and to the low metal-translocation from roots to shoots inherent in grasses (ERNST 1974). Pollen transfer from plants from non-metal enriched soils may endanger the

persistence of a plant's adaptation to the local environment. However, the permanent strong selection of local offspring helps to conserve metal-tolerant genotypes (MCNEILLY 1968).

The presence of metal-tolerant plant species in the surroundings of metal-processing industries is not necessarily due to local selection, but may also be the result of introduction of seeds from already metal-tolerant plants together with ores. Such an example is the introduction of a Cd/Zn-tolerant population of *Thlaspi caerulescens* and *Festuca ophiolitica* to the Cd/Zn-smelter site at Prayon/Belgium perhaps 100 years ago (DENAYER-DE SMET and DUVIGNEAUD 1974). It added already metal-tolerant species to the vegetation with locally selected Zn-tolerant populations of *Agrostis capillaris* and *Avenella flexuosa* (*Deschampsia flexuosa*) (DUVIGNEAUD and JORTAY 1987).

Table 1. Evolution of metal tolerance in populations of plant species growing on soils affected by high metal emissions surrounded by soils with normal metal levels. Exposure period refers to the time between the start of the emission and the first analysis of the metal tolerance of plants (modified from ERNST 1999).

Metal	Metal source	Plant species	Exposure period (years)	Reference
Cu	Cu refinery	<i>Agrostis stolonifera</i>	4–70	WU <i>et al.</i> (1975)
	Cu smelter	<i>Agropyron repens</i>	45	BREJ (1998)
	Cu refinery	<i>Festuca rubra</i>	70	WONG (1982)
Zn	Cd/Zn smelter	<i>Agrostis capillaris</i>	5	ERNST <i>et al.</i> (1974)
	electricity pylons	<i>Avenella flexuosa</i>	20	COULAUD and MCNEILLY (1992)
	galvanized wire	<i>Agrostis canina</i>	22	BRADSHAW <i>et al.</i> (1965)
	electricity pylons	<i>Agrostis capillaris</i>	32	AL-HIYALY <i>et al.</i> (1988)
	electricity pylons	<i>Agrostis stolonifera</i>	32	AL-HIYALY <i>et al.</i> (1990)
	electricity pylons	<i>Festuca ovina</i>	32	AL-HIYALY <i>et al.</i> (1990)
	Zn/Cd-smelter	<i>Agrostis capillaris</i>	< 80	DUECK <i>et al.</i> (1984)

5 Evolution of metal tolerances: the phylogeny

5.1 Macro-evolution at the level of higher taxonomic ranks

Metal-enriched substrates are geologically old and occur worldwide. The macro-evolution of angiosperms started 140 million years ago, but not all taxonomic groups were able to evolve metal-tolerance (ERNST 2000). Potential for the evolution of high metal tolerances is not homogeneously distributed across the phylogeny of angiosperms and shows differences within a taxonomic group between continents. High tolerances to Cd, Co, Cu, Ni, and Zn have evolved in the orders Brassicales, Caryophyllales, Plumbaginales and Poales around the world, whereas tolerances to Co, Cu and Ni are found in Asterales, Commelinales, Cyperales, Ericales, Fabales, Lamiales and Liliales only in the Southern Hemisphere. The Campanulales, Ranunculales, Saxifragales, Scrophulales and Solanales have only developed moderate metal tolerances. A low potential for the evolution of tolerance to Cd, Pb, and Zn is present in the order Fagales. In the Rosales only *Sedum alfredii* has evolved a high tolerance to Cd and Zn in China (YANG *et al.* 2004). Malpighiales including Violaes have moderate degrees of Cd and Zn tolerance in Europe (RUTHER 1967; JEDRZEJCZYK *et al.* 2002), which is often associated with symbiosis with arbuscular-mycorrhizal fungi (HILDEBRANDT *et al.* 1999; TONIN *et al.* 2001). In Australia and Indonesia species of Malpighiales have also evolved Ni tolerance (SEVERNE and BROOKS 1972; REEVES 2003).

5.2 Macro-evolution at the level of plant families

At the family level, evolution of metal tolerance did not take place in each tribe of a given family, as shown for the family Caryophyllaceae (ERNST 2001) and here exemplified for the family Brassicaceae (Fig. 5). Only a few tribes were able to evolve tolerance to one or more metals. In the tribe Alysseae, many *Alyssum* and *Bornmuellera* species tolerate high nickel levels (BROOKS and RADFORD 1978; REEVES *et al.* 1983). Several genera among the Lepidieae tolerate a surplus of one or more metals, such as Ni in *Cochlearia aucheri* (REEVES and ADIGUZEL 2004), Tl in *Iberis intermedia* (BROOKS *et al.* 1999) and a high diversity in metal tolerance (Cd, Cu, Ni, Pb, Zn) in *Thlaspi* species (Fig. 6: ERNST 1965, 1968; OUZOUNIDOU 1995; BOYD and MARTENS 1998b; FREEMAN *et al.* 2004; SHEN *et al.* 1997; MIZUNO *et al.* 2005; VOGEL-MIKUS *et al.* 2005). *Arabidopsis halleri* is the most famous example of metal tolerant species in the Sisymbrieae (ERNST 1968; KUPPER *et al.* 2000; MACNAIR 2002; BERT *et al.* 2003; BECHER *et al.* 2004; DRAGER *et al.* 2004; PAUWELS *et al.* 2005; WEBER *et al.* 2006). In the tribe Hesperideae, there is only *Malcolmia fruticulosa* that tolerates levels of more than 1000 mg Zn kg⁻¹ d.m. on mine tailings in Lavrion/Greece (ERNST 1974). In the tribe Streptanthae, *Streptanthus polygaloides* is the best-known nickel tolerator (JHEE *et al.* 2005). Tribes with distribution centres in Central and South America, i.e. Cremolobeae, Romanschulzieae, and Schizopetaleae, fit in the general poverty of metal tolerant plant species of these parts of the world. The metal-poor soils of the Kerguelen do also not demand for metal tolerance of species in the tribe Pringleae. More obvious is the lack of evolution of metal tolerances in the tribes Arabideae and Brassiceae. In the latter tribe, *Brassica juncea* has a high capacity for metal accumulation in short-time experiment (4–8 d), but its high metal sensitivity, especially in long-term experiments (SCHAFFER *et al.* 1997; ZHU *et al.* 1999), shows its inadequacy for effective phytoremediation, although highly advised as phytoextractor of heavy metals from contaminated soils (SALT *et al.* 1996). In *Brassica oleracea*, there are a number of cultivars with high Tl accumulation (KURZ *et al.* 1999), but none of them colonised Tl-enriched soils.

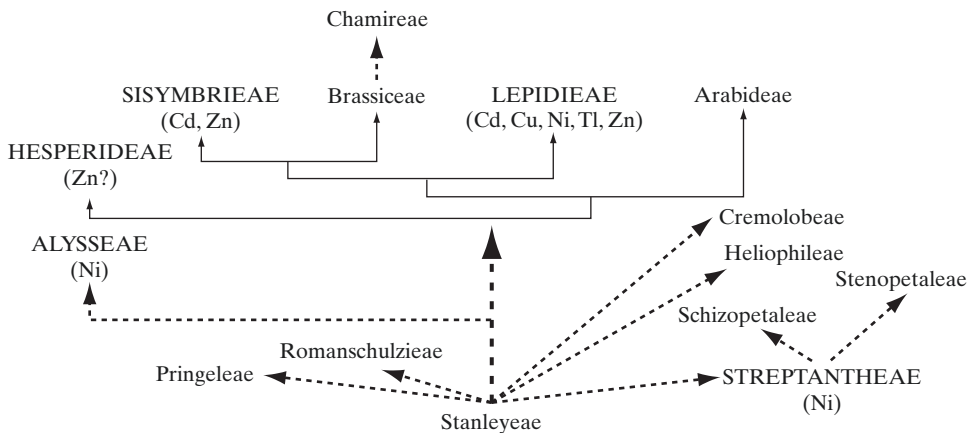


Fig. 5. Phylogenetic relationships among Brassicaceae and the evolutionary potential of metal tolerances. Tribes with metal tolerant species are given in capitals including the metals some species of the tribe are tolerant to. From the phylogenetic tree developed by JANCHEN (1942) are maintained those relationship of tribes (indicated by dashed lines) which were not investigated by KOCH *et al.* (2001) with the matK and chs genes (indicated by full lines). KOCH *et al.* (2001) and YANG *et al.* (1999) have confirmed that the tribes Arabideae, Brassiceae, Hesperideae, Lepideae, and Sisymbrieae, fit well with the approach developed by JANCHEN (1942).

5.3 Macro-evolution at the genus level

Even at the genus level, not all species have the ability to evolve metal tolerances, as exemplified in the genus *Silene* (ERNST 2001) and the genus *Thlaspi* (Fig. 6). The investigated annual species such as *Thlaspi arvense* and *Thlaspi perfoliatum* are metal-sensitive as most annual plant species including *Arabidopsis thaliana*, having different Zn uptake characteristics (LASAT *et al.* 1996) and different expression of Zn transporter genes at metal exposure (ASSUNÇÃO 2003). In contrast, perennial species are mostly tolerant to different metals, sometimes only to one metal (*Thlaspi praecox*), but also up to three metals simultaneously (*Thlaspi caerulescens*, *T. cepaeifolium*).

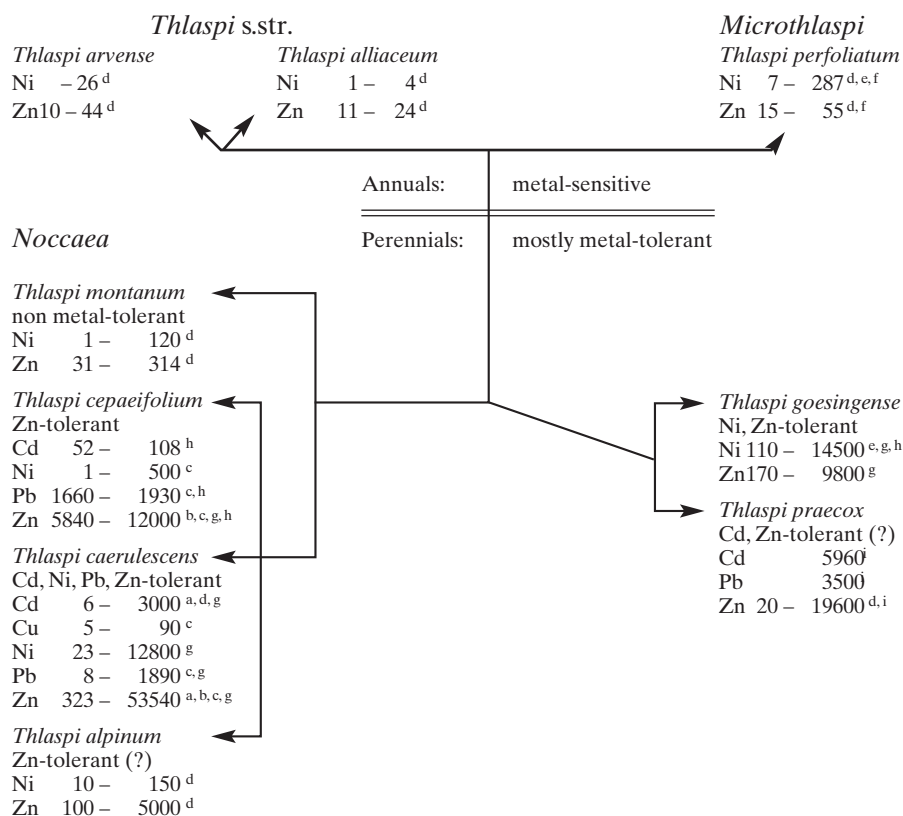


Fig. 6. Evolution in European *Thlaspi* taxa based on Rubisco isoelectric focussing patterns (MUMMENHOFF and ZUNK 1991) with regard to heavy metal resistance and heavy metal concentrations (mg kg⁻¹ d.m.) in leaves derived from ^aESCARRE *et al.* (2000), ^bERNST (1965), ^cERNST (1974), ^dREEVES and BROOKS (1983), ^eREEVES and BAKER (1984), ^fREEVES (1988), ^gREEVES *et al.* (2001), ^hWENZEL and JOCKWER (1999), ⁱVOGEL-MIKUS *et al.* (2005). Question marks indicate that tolerances have not been experimentally tested. The branching of the tree is based for all three subtribes on DNA data published in MUMMENHOFF *et al.* (1997), KOCH and MUMMENHOFF (2001) and KOCH and AL-SHEHBAB (2004). The branching within the subtribe Noccaea is primarily based on the Rubisco IEF pattern (MUMMENHOFF and ZUNK 1991), but modified and fine-tuned for *T. alpinum*, *T. caerulescens*, *T. cepaeifolium* and *T. montanum*, based on DNA data (MUMMENHOFF *et al.* 1997).

5.4 Micro-evolution at the species level

At the level of species, micro-evolution can result in a selection of metal-tolerant ecotypes from metal-sensitive populations. Geographic isolation and restricted gene flow may isolate the metal-tolerant population to such a degree that local ecotypes or even endemic taxa can evolve. Such endemics on metalliferous soils are called metallophytes (KRUCKEBERG and KRUCKEBERG 1989). In the Northern Hemisphere, such endemics are scarce in areas deprived from vegetation during Pleistocene glaciation. They are restricted to the zinc violets (*Viola guesstphalica*, *Viola calaminaria*). Based on DNA sequences both endemics seems to have evolved from *Viola lutea* (HILDBRANDT *et al.* 2006), as postulated long ago by SCHULZ (1912). *V. lutea* was widespread in Central Europe after the last glacial period, but during postglacial climatic warming it was restricted to metal-enriched soils in lowland Central and Western Europe. This geographic isolation has obviously contributed to its micro-evolution. In areas with metal soil gradients, the maintenance of endemics is endangered by hybridisation with non-tolerant relatives, as demonstrated for the hybrids between *Viola guesstphalica* and *Viola arvensis* (KAKES 1977). In southern Europe with minor influence of glaciation, nickel-enriched soils bear a high number of endemics (VERGNANO GAMBI 1992), especially in the genus *Alyssum* (REEVES and ADIGUZEL 2004). Still more endemics are described from metal-enriched soils in Africa (DUVIGNEAUD and DENAEYER-DE SMET 1963). Recent taxonomic revisions, however, have shown that some of these endemics are nothing more than ecotypes of widespread species. For instance, the copper indicator *Becium homblei* is a Cu-tolerant population of the widespread *Becium centrali-africanum* (SEBALD 1988; ERNST 2000).

The genetic analysis of the microevolution of *Thlaspi caerulescens* shows that geographic isolation results in a certain degree of genetic distance among populations (Fig. 7), as also found for *Minuartia verna* on metal-enriched soils (VERKLEIJ *et al.* 1989). The Cd/Zn-tolerant populations of the Pennine (Malham, Brassington) in England has a greater genetic distance (0.193) than those on the Cd/Zn-enriched soils in Central and Western Europe (KOCH *et al.* 1998). The isolation of the British heavy-metal tolerant populations of *T. caerulescens* results in a genetic distance characteristic for the order of subspecies (AYALA 1975). The genetic distance between local populations of *T. caerulescens* in Central Europe is low and comparable to other plant species (RITLAND 1989). As in other plant species on non-metalliferous soils (LEDIG *et al.* 1997), also the genetic distance does not always coincide with the geographic distance. The populations from Silberberg and St. Jost (Germany), being isolated by 200 km, are genetically identical (NEI's genetic distance of zero) whereas a nearly similar geographic distance between metal-sensitive populations at Medebach and all metal-tolerant populations in the Breinig-Angleur region is essentially greater (0.133) (MUMMENHOFF *et al.* 1997; KOCH *et al.* 1998). The submediterranean population of *T. caerulescens* from Ganges differs in the V_{\max} - and K_m - values of Zn and Cd uptake from the Central European population from Prayon (COSIO *et al.* 2004), thus confirming differences in the expression of the ZTP gene from a calamine population and a serpentine population (ASSUNÇÃO *et al.* 2001). Such differences are indicative for micro-evolutionary processes as fine-tuning to the local soil metal levels.

Analysis of isozymes and DNA in all studies of phylogenies (SOLTIS *et al.* 1999) have in common that they are using neutral genetic markers; they are not suitable to estimate genetic distances of adaptive genes. Recent research to identify associations between molecular markers and the traits of Ni and Zn accumulation and tolerance in *Silene vulgaris* and *Thlaspi caerulescens* identified only two QTL for each metal (BRATTELER 2005; ASSUNÇÃO *et al.* 2006). The detected QTL (= quantitative trait loci analysis) in two Zn-tolerant populations of *T. caerulescens* did not co-localise with the ZNT1 gene steering Zn-accumulation

in this species. There was not detectable genetic variation for this locus being in agreement with the very similar mRNA expression of ZNT1 in these populations (ASSUN²AO *et al.* 2001).

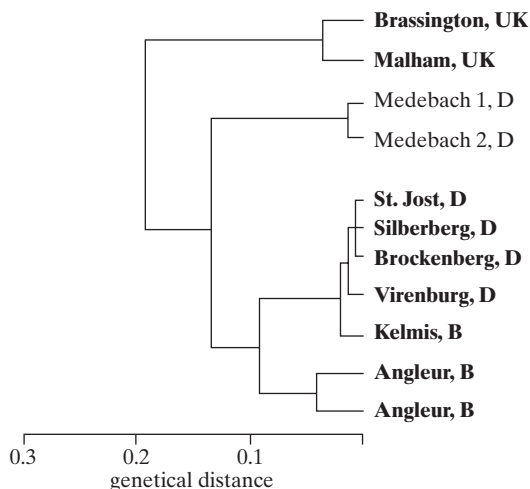


Fig. 7. Nei's genetical distance between populations of *Thlaspi caerulescens* in metalliferous (bold) and nonmetalliferous populations. Data from KOCH *et al.* (1998) after removal accessions from Botanical Gardens.

6 Conclusion and outlook

Evolution of metal tolerances depends on the exposure of a plant species to the metal level of the soils. It is restricted to some unrelated orders, families, and genera. Nevertheless, there is a great similarity in the involvement of one or two genes per metal controlling the specific metal tolerance and in its physiological mechanisms. They are all based on a shift of performance under high level of essential metals and on the compartmentalisation of the metals in physiological less active cells and tissues. Despite the evolutionary potential of metal tolerances, there are extremely multimetal-enriched soils without any plant growth. The absence of vegetation may indicate the limits of tolerance mechanisms, i.e., the inability of a cell to detoxify extreme metal amounts in time, but it may also indicate an insufficient metabolic answer to ecological restriction of metal-enriched soils such as low water capacity.

In the near future, recently developed techniques will speed up our knowledge of metal tolerance processes. Microarrays (BECHER *et al.* 2004; PLESSEL *et al.* 2005) may help to identify genes that are steering the physiological tolerance mechanisms and micro-PIXE studies (BRINGEZU *et al.* 1999; MESJASZ-PRZYBYLOWICZ *et al.* 1999) will facilitate metal localisation at the subcellular level.

Three attractive model species for the study of the genetics, physiology and ecology of metal tolerances are *Arabidopsis halleri* (BERT *et al.* 2002), *Silene vulgaris* (ERNST *et al.* 1990), and *Thlaspi caerulescens* (ASSUN²AO *et al.* 2003c). These three plant species have the advantage that they are nearly lacking a symbiosis with arbuscular-mycorrhizal fungi. Therefore the rhizodermis cells of the roots are in direct contact with the soil solution.

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