EQA – *Environmental quality / Qualité de l'Environnement / Qualità ambientale, 15(2014) 1-13*

THE " SERPENTINE SYNDROME"(H. JENNY, 1980): A PROXY FOR SOIL REMEDIATION

Claudio Bini [(1)*] , Laura Maleci [(2)]

[(1)] Department of Environmental Sciences, Informatics and Statistics,
Ca' Foscari University, Venice (Italy)
[(2)] Department of Biology, University of Florence (Italy)
* Corresponding author E.mail: bini@unive.it

Abstract

Serpentine soils have relatively high concentrations of PTEs (Co, Cr, Cu, Fe, Ni) but generally low amounts of major nutrients. They often bear a distinctive vegetation, and a frequently-used approach to understanding serpentine ecology and related environmental hazard has been the chemical analysis of soils and plants. In this paper we report past and current studies on serpentine soils and serpentinophytes. The serpentine vegetation differs from the conterminous non-serpentine areas, being often endemic, and showing macroscopic physionomical characters. Similarly, at microscopic level cytomorphological characteristics of the roots and variations in biochemical parameters were recorded in serpentinophytes. Light microscopy observations showed depressed mitotic activity in the meristematic zone, and consequent reduced root growth. The different tolerance mechanisms responsible for plant adaption to high concentrations of PTEs in serpentine soils can be related to the capacity of plants to limit metal uptake and translocation. The majority of serpentinophytes tend to limit metal absorption to roots: the cell wall constitutes a barrier against metal penetration inside plant tissues. Only a few species are able to accumulate metals in their aerial parts, acting a tolerance mechanism to very high metal concentrations. Serpentinophytes, therefore, could represent proxies for plants used in remediation of metal-contaminated soils and in phytomining as well.

Key words: *serpentine soils; serpentinophytes; metal uptake; phytoremediation*

Introduction

Serpentinite and its derived soils (serpentine soils) occupy only a very small part of the land surface of the earth (<1% according to Brooks, 1987), and are widespread around the world, in connection with ancient tectonic activity and ultramafic intrusions. In Italy, serpentinite outcrops occur in the metamorphic sequences in Central and Western Alps and in Calabria (Bonifacio et al, 2010; D'Amico et al., 2014) and as blocks in chaotic melanges (flysch) in the Northern and Central Apennines (Robinson et al., 1997). They have been widely studied from the geopetrological point of view (e.g. Bortolotti, 1974; Piccardo, 1983 and references therein), while contributions the their alteration and related soil genesis are rather scarce (Cortesogno et al., 1979; Bini et al., 1983, Minguzzi et al., 1985; Cecconi et al., 1990; Bonifacio et al., 1997; 2010; 2013; D'Amico and Previtali, 2012;

DOI: 10.6092/issn.2281-4485/4547

D'Amico et al., 2014). Serpentinite is frequently mined (produce of tailings, asbestos and the important minerals of Ni, Cr, Cu, Co and Mn), and soils are so infertile that few areas can be used for agriculture. The suggested causes of poor plant growth (what Hans Jenny called "the serpentine syndrome") have been identified so far as trace metal toxicity, low Ca/Mg quotient, high pH values and poor nutrient balance (Proctor and Woodel, 1975). This character is directly linked to the elemental composition of the parent rock. In addition, they have relatively high concentrations of Potentially Toxic Elements (PTEs; e.g. Co, Cr, Cu, Fe, Mn, Ni) but generally low amounts of major nutrients (Johnston and Proctor, 1981), with consequent limitation in plant growth. Element distribution in plants and soils depends upon element uptake and release back to soil with litter, a short-term biological cycle which induces an increase in available element concentration towards the top of the soil profile (Bonifacio et al., 2010). However, serpentine soils display a low litter mineralization rate because of the effect of heavy metals on microbial communities (Kazakou et al., 2008).

A frequently-used approach to understanding serpentine ecology and environmental hazard has been the chemical analysis of soils and plants. Long-term studies on serpentine soils and their vegetation provide results on metal concentrations, or on available fractions, of soil elements which counteract ecological conditions (Angelone et al., 1991; 1993). Among trace elements, Co, Cr, Cu and Ni are considered phytotoxic (Gonnelli and Renella, 2013); however, their available proportion is generally rather low, being these elements firmly bound to crystal lattices. Though Co is present in serpentine soils at quite high concentrations (up to 200 $mgkg^{-1}$, as reported by Lombini et al., 1998), the levels of EDTA-extractable Co are rather low (2-5 $mgkg^{-1}$), with a decrease from top to bottom, suggesting Co to be related to the organic matter cycle (biocycling). Although total Cr concentration is high in serpentine soils (up to 5000 $mgkg^{-1}$ according to Proctor and Woodel, 1975), being inherited from the parent material, the EDTA-extractable Cr shows very low and very homogeneously distributed concentrations (<1$mgkg^{-1}$). This is likely due to the low solubility of the mineral Chromite (Cr_2O_3) occurring in serpentinite. Despite its low solubility, chromium in plants generally shows higher values than cobalt, but only occasionally high chromium concentrations are found, as in *Cerastium exile* and *Plantago holosteum* (Vergnano et al., 1982).

Copper is a minor component in serpentine soils, where it is released by parent material, and may form low-soluble compounds (native copper, oxide and carbonate). The actual range of Cu in mafic rocks is 30-160 $mgkg^{-1}$ (Oorts, 2013), whereas it is generally much lower in serpentine soils (1-30 $mgkg^{-1}$), with uniform distribution along the profile, depending on coarse texture. In addition to soil texture, several other soil parameters, in particular pH and organic matter, control its distribution and behaviour. Generally, in normal soils Cu is accumulated in the upper few centimetres, having a tendency to be adsorbed by organic compounds in nutrient biocycling (Lombini et al., 2004; Llugany et al., 2003). However, carbonates, clay minerals, and oxyhydroxides of Mn and Fe may transport copper also in deeper soil layers.

Copper is an essential element to plant, and the extraction with EDTA has been widely used as an indicator of Cu availability to plants. Levels >30mgkg^{-1} EDTA-extractable Cu could indicate that plant growth may be affected by Cu toxicity symptoms. Instead, concentrations <0.75 mgkg^{-1} could indicate deficiency conditions (Kabata-Pendias, 2011). Mean concentrations in normal plants are in the range 3-8 mgkg^{-1} (Kabata-Pendias and Mukherjee, 2007). However, Cu-tolerant plants and Cu-hyperaccumulators (e.g. *Aeolanthus biformifolius*) may contain up to 13000 mg Cu kg^{-1}, and have been largely utilized in phytomining (Kabata-Pendias and Mukherjee, 2007). Naturally elevated Ni contents are observed in soils derived from serpentine rocks, where Ni ranges from 770 to 7375 mgkg$^{–1}$ (Kabata-Pendias, 2011). Nickel in such soils is slightly mobile and occurs mainly in the residual fraction, in over 50% of its total contents. However, in surface soil horizons Ni occurs mainly in bound organic forms, a part of which are relatively easily soluble chelates. Bonding of Ni to organic ligands may be very strong, and can highly affect its mobility. Conversely, the remobilization of Ni from solid phases appears to be possible in the presence of fulvic and humic acids. The role of Ni on plant growth is not well known, neither if it is essential to plants or not. According to D’Amico et al. (2014), available Ni seems the most important soil character in determining vegetation distribution in serpentine soils of xeric inner-Alpine environment, while Ni excess most often precludes the presence of plant species.

Some native plants (e.g. *Alyssum bertolonii*) from serpentine sites are able to accumulate leaf concentrations of over 6000 mg Ni kg$^{–1}$.(Kabata-Pendias, 2011). Indeed, *Alyssum bertolonii* was firstly recognized by Baker (1981) as nickel hyperaccumulator.

Long-term experimental data indicate that available Ni is readily adsorbed, and it may be phytotoxic when EDTA-extractable concentration is higher than 20mgkg^{-1} (Archer and Hodgson, 1987). There is evidence that Ni is toxic at concentrations of 0.3 mg L^{-1}, while exchangeable Ni at concentrations much higher than 0.3 mg L^{-1} have been shown to produce no toxicity symptoms in bioassay plants (Proctor, 1971).

The serpentine syndrome

The serpentine vegetation differs from the conterminous non-serpentine areas, showing unusual macroscopic characters such as xenomorphic foliage, dwarfism, prostrate outcome, root shortening (i.e. serpentinomorphosis or “the serpentine syndrome”, as proposed by Jenny, 1980). Similarly, at microscopic level, cytomorphological characteristics of the roots and variations in biochemical parameters such as LPO and phenols have been recorded in serpentine vegetation (Gabbrielli et al., 1990). Light microscopy observations on serpentinophytes (*Thymus ophioliticus* and *Stachys serpentini*) showed depressed mitotic activity in the meristematic zone, and consequent reduced root growth (Maleci et al., 1997, 1999; Giuliani et al., 2008). Similar findings were observed by Lombini et al.

(2004) in the serpentinophyte *Silene armeria* and by Rascio et al. (2008) in rice plants (*Oryza sativa*) exposed to high metal concentrations. The metal-exposure inhibited root growth and also interfered with correct root morphogenesis, causing disordered division and abnormal enlargement of epidermal and cortical cell layers in the apical region. Morphological observations of rice leaves using light microscopy revealed that the metal treatment, in addition to hampering elongation, led to a decrease in leaf blade thickness, a feature reported also by Maleci et al. (2014) for *Taraxacum officinale* grown on metal-enriched soils.

The different tolerance mechanisms responsible for plant adaption to high concentrations of heavy metals in the soil can be summarized in the capacity of the plant either to limit metal uptake and translocation or to accumulate the metal in non toxic forms (Baker, 1981; Gabbrielli et al., 1990). An excluder plant, for example, takes up from the soil only small amounts of a toxic metal, and localizes it preferentially in roots, thereby excluding that element from the metabolic processes (Rascio and Navari-Izzo, 2011). The majority of plants, including many serpentine species, tend to limit metal absorption to roots so that leaf concentration is generally low (Vergnano Gambi et al., 1982). Only a few species, which have reached a high degree of adaption and are endemic of serpentine soils, are able to translocate metals from roots to their aerial parts, as occurs for Ni in *Silene italica* and in *Alyssum bertolonii* (Gabbrielli et al., 1990) and are referred to as hyperaccumulator plants (Baker, 1981; Rascio and Navari-Izzo, 2011). Hyperaccumulator plants include species that accumulate >10000 $mgkg^{-1}$ (Mn or Zn), >1000 $mgkg^{-1}$ (Cu, Co, Cr, Ni, Pb) or >50 $mgkg^{-1}$ (Cd) in their shoots (Wenzel et al, 2003).

Serpentinophytes, therefore, have attracted great interest for the study of resistant mechanisms to PTEs penetration, and could constitute a proxy for non-accumulator plants in the development of phytotechnologies for remediation of contaminated sites (Hill et al., 2014). Indeed, the ideal proxy is spatially defined in the environment (i.e. endemic of serpentine soils), and represents an open-space laboratory to model the distribution of metals at contaminated sites and to assess plant tolerance to metal contamination. The mechanisms of resistance of a serpentinophyte to the excess of PTEs are still poorly investigated (Dinelli et al., 1997; Lombini et al., 1998 Lombini et al., 2004). It was general opinion that metal tolerance in metallophytes (e.g. *Thlaspi caerulescens*) is metal-specific (Ernst et al., 1992). Conversely, there have been previous reports suggesting that co-tolerance is likely to involve low level multi-metal tolerance (e.g. Symeonidis *et al.*, 1985). The possibility that resistance to stress factors occurring on serpentine ecotypes, such as high amounts of Ni and Co or the unfavourable Mg/Ca ratio (Lombini et al., 2004; D'Amico et al, 2014), may provide enhanced protection against metal toxicity. Several previous investigations (Wahsha et al., 2012; Franco et al., 2009, and references therein) have shown that excess metal causes DNA damage, peroxidation reactions and early death of epidermal cells. This early cell-death response (apoptosis) was interpreted as a detoxification mechanism helping to protect deeper cell layers of the meristematic zone, essential for root growth (Lombini et al., 2004).

The hypothesis raised so far (Vergnano Gambi, 1975), that the cell wall could constitute a barrier against the penetration of PTEs inside the plant tissues, is supported by metabolic modifications that make possible the plant tolerance to very high heavy metal concentrations, and to low levels of essential nutrients such as P, K and Ca (Rascio and Navari-Izzo, 2011).

An explanation of serpentinophyte tolerance to normally toxic elements could be a metabolic adaption of plants against the possibility that a metal could achieve the most active metabolic sites; a second possibility is that the whole enzymatic complex could have undergone alteration in such a way that it functions normally even in the presence of high amounts of toxic metals (Gonnelli et al., 2001). For example, high Ni concentrations in *Silene italica* inhibit root growth and induce an increase in peroxidase activity (i.e. Ni-induced stress), whereas do not have effects on *Alyssum bertolonii* (Gabbrielli et al., 1990; Barzanti et al., 2011). The preferential Ni distribution in shoot epidermis and sclerenchyma of *A. bertolonii* (Vergnano Gambi, 1975) suggests the possibility of formation of localized complexes which impede the translocation of metal toxic doses to cytoplasm and cell organelles. This hypothesis is supported by light microscopy observations that show depressed mitotic activity in the meristematic zone of *S. italica*, which is the principal cause of reduced root growth. Conversely, *A. bertolonii* does not show signs of Ni-induced stress. As a matter of fact, the metal content of serpentine plants affects significantly the plant metabolism, and therefore the major or minor adaption to the adverse environmental conditions (soil depth, drought, nutrient deficiency, microelement levels). Indeed, the fact that metal accumulation occurs not only at root level, but also in leaves, flowers and sometimes even in seeds, could be an indication of metal essentiality for the plant metabolism (Rascio et al., 2008).

Results and Discussion of previous and current studies

Heavy metals in soils and plants from selected serpentine sites

A summary of basic statistics on heavy metals in soils and plants from different serpentine outcrops in Italy is reported in Tables 1 and 2, respectively.

The levels of trace elements (Ni, Cr, Co, Cu, Zn,) in serpentine soils investigated by several authors (Vergnano Gambi et al., 1982; Angelone et al., 1993; Robinson et al., 1997 ; Dinelli et al., 1997; Lombini and Dinelli, 1998; Galardi et al., 2007; Bonifacio et al., 2010) are similar to those reported in the literature for worldwide soils developed from the same parent material. All the authors report high concentrations especially of Cr and Ni, and minor amounts of Co, Cu, Zn; metals are most abundant in subsoil than in topsoil.

Consistently, Cecconi et al. (1990), in serpentine soils outcropping close to Impruneta (Florence), *locus classicus* for serpentine plants, found the following results (range toposoil-subsoil, mgkg^{-1}): Co 121 -181; Cr 913-2097; Cu 104-114; Ni 913- 3149; Zn 64-60.

Table 1 *Descriptive statistics of total metal concentrations in serpentine soils (mgkg^{-1}dw)*

metal	Ni	Cr	Co	Cu	Zn
mean	2342	3502	99	28	91
min	299	115	63	9	56
max	2685	4470	252	39	126

Source: Vergnano et al., 1982; Angelone et al., 1993; Robinson et al., 1997; Dinelli et al., 1997; Lombini and Dinelli, 1998; Galardi et al., 2007; Bonifacio et al., 2010.

In a paper on the biogeochemistry of serpentine soils from Tuscany, Angelone et al. (1991) reported metal concentrations somewhat less than above. Metal extractability by EDTA proved very low for all metals. Indeed, EDTA-extractable elemental concentration was much less than total concentrations (Co 17 mgkg^{-1}, Cr 2mgkg^{-1}, Cu 6 mgkg^{-1}, Mn 540 mgkg^{-1}, Ni 130 mgkg^{-1}, Zn 5 mgkg^{-1}), as expected for metals strongly bound in crystal lattice of minerals (e.g. Chromite Cr_2O_3). Most EDTA-extractable element concentrations (Mn, Co, Cr, Ni, Zn), decrease with depth, confirming close relationship with the organic matter. Significant correlation between total and extractable Ni and Cu have been found, while total and extractable Co results significantly correlated to extractable Zn (r = 0.71).

The sequence of metal extractability (Co>Mn>Cu>Ni>Zn>Cr) suggests that the total content of major and trace elements in soil does not provide information about the extent of potential availability of elements to plants. However, Co is one of the most easily extractable elements, in spite of its low total content in soils and its low concentration in the extractable fraction. Extractable Cr concentrations close to critical ones have been found in upper horizons of serpentine soils. The same behaviour has been observed for extractable Ni, which achieves concentration levels that can induce toxicity symptoms to plants.

Data on major and trace element concentrations in plants (both typical and preferential serpentinophytes) from selected serpentine outcrops are reported in Table 2. A noteworthy variability in element concentrations in the same species and among the various species of plants examined was observed. Such a variability can be generally ascribed to differences among species, the type of vegetal organs, stage of growth, and edaphic conditions, as indicated so far by Vergnano Gambi et al. (1982). Nickel is perhaps the most investigated heavy metal in plants, because of its role in controlling the establishment of plant communities (D'Amico et al., 2014), and the commercial importance. The majority of the examined plants show a leaf concentration below 0.85 mg Ni kg^{-1}, with the exception of *Armeria* and *Plantago*. In *Minuartia laricifolia* and *Thymus ophioliticus* Ni is mainly accumulated in woody stems, which is consistent with findings from other Italian serpentine areas, where Ni accounts for 2500 mgkg^{-1} in soils (Vergnano Gambi et al., 1982). Conversely, there are no marked differences in Ni accumulation among the typical serpentinophytes and the preferential ones both at leaf and root level, with the exception of *Alyssum* species (Galardi et al., 2007). Nickel is the one element always reaching the highest values in plant tissues, none of the species examined concentrating chromium or cobalt to the same extent. Generally chromium, despite its low solubility, shows higher values than cobalt, but only occasionally (e.g. in *Cerastium exile* and *Plantago holosteum*) high chromium concentrations are found (Vergnano Gambi et al., 1982). It is likely that higher Ni uptake favours also the uptake of cobalt. Among preferential serpentinophytes, no species is able to accumulate, particularly at leaf level, nickel, cobalt or chromium.

The trace element abundances in normal plants are generally below the levels considered excessive or toxic (Kabata-Pendias, 2011). However, some exceptions have been recorded for non-serpentinophytes growing on serpentine soils from Tuscany (Bini et al., 2004): high Mn values have been found in *Erica arborea* (420 mgkg^{-1}); *Spartium junceum* contained high Cr concentrations (5.5 mgkg^{-1}), while Ni levels > 10 mgkg^{-1} have been detected in shoots of *Juniperus communis.* Nickel was also present in relatively high concentrations in plants from serpentine soils in xeric conditions (Angelone et al., 1993): in *Pinus pinaster* needles (15.6 mgkg^{-1}), and in *Cistus monspeliensis* branches (28.9 mgkg^{-1}).

Table 2. *Metal concentration in serpentine plants (mgkg^{-1}dw)*

	Zn	Cu	Ni	Cr	Co
Alyssum bertolonii shoots	59	8	2118	14	14
Alyssum bertolonii roots	44	12	1866	168	24
Silene armeria shoots	62	8	27	11	4
Silene armeria roots	71	24	97	48	15
Minuartia laricifolia subsp. ophiolitica shoots	48	9	91	55	10
Minuartia laricifolia subsp. ophiolitica roots	60	9	118	39	9
Biscutella gr. *laevigata* shoots	35	5	21	8	3
Biscutella gr. *laevigata* roots	69	8	92	36	4
Thymus striatus subsp. *ophioliticus* leaves	-	-	35	7.6	<0.5
Thymus striatus subsp.*ophioliticus* roots	-	-	100	32	<0.5
Stachys recta subsp. *serpentini* roots	-	-	23	3.5	<0.5
Cistus salvifolius stems	-	-	52	12	<0.5
Pinus pinaster old needles	-	-	15	1.06	<0.5

(source: Vergnano, 1975, 1982; Gabbrielli et al., 1990; Angelone et al., 1991, 1993; Robinson et al., 1997; Lombini and Dinelli, 1998; Maleci et al., 1997)

Morphological observations on serpentinophytes

Macro- and micromorphological and phytochemical characters of some serpentine plants from different serpentinite outcrops in Tuscany were examined by Gabbrielli et al. (1990), Maleci et al. (1997, 1999) and Giuliani et al. (2008) in comparison to non-serpentine plants. The serpentinophyte *Silene italica* showed thinner apical root areas than non-serpentine plants, with depressed mitotic activity, and a corresponding increase in peroxidase activity (Gabbrielli et al., 1990). Conversely, *Alyssum bertolonii*, a well known Ni hyperaccumulator, did not exhibit signs of nickel-induced stress (Gabbrielli et al., 1990). In *Thymus striatus* subsp.

DOI: 10.6092/issn.2281-4485/4547

ophioliticus Lacaita stems were shorter than in *T. striatus* subsp. *striatus*, with closer internodes, creeping, highly lignified, and are intensely coloured by anthocyanins (Maleci et al., 1997; 1999); leaves were narrow and acute, flowers deeply coloured pink; bracts and calyces too were reddish and smaller (about 20%). The size difference was highly evident in leaves, *T. striatus* subsp. *ophioliticus* showing a width decrease of up to 40% in comparison to *T. striatus* subsp. *striatus.*

While macromorphological features of serpentinophytes have been widely investigated, micromorphological features did not receive great attention, notwithstanding their ability to tolerate high metal concentrations. Micromorphological characters of serpentine plants, as reported by Maleci et al. (1997; 1999) and Giuliani et al. (2008), exhibited a well developed wood, with a lignified stem pith. The whole plant presented long non-glandular trichomes, and two types of glandular trichomes, peltate and small clubbed trichomes. Data on trichome distribution showed that it is quite similar in all the plants examined (Maleci et al., 1999).

Chemical analyses of heavy metals showed Fe, Pb and Zn levels in serpentine-adapted plants lower than in non-serpentine plants; Mn, Cu and Cr, on the contrary, resulted slightly more abundant, while Ni was the only element at concentration up to ten-folds higher in serpentinophytes (3.8 $\mathrm{mgkg^{-1}}$) than in non-serpentine plants 0.36 $\mathrm{mgkg^{-1}}$), which is consistent with data from literature (Angelone et al., 1993). Also volatile compounds showed a different composition, with sesquiterpenes largely prevailing (up to 21% in *T. striatus* subsp. *ophioliticus* leaves). Differences between the two subspecies are mainly due to the edaphic conditions that induce the development of a particular (endemic) ecotype (Maleci et al., 1999).

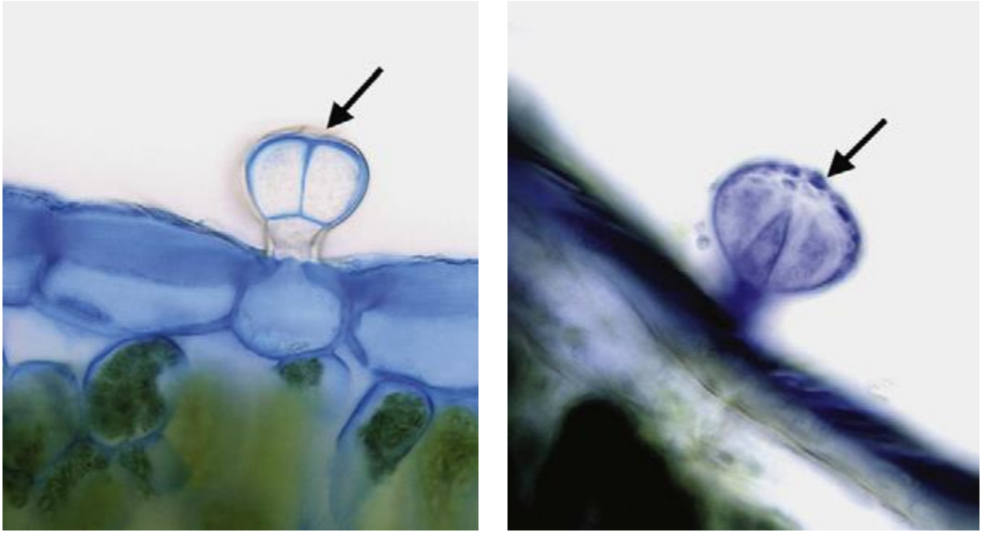
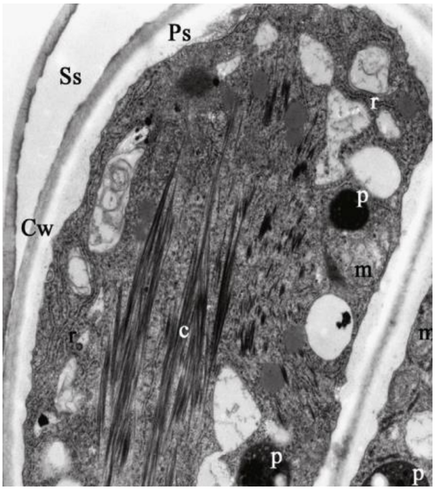


Figure 1. *Glandular trichomes stained with Alcian blu (left) and Nadi reagent (right) evidence (arrowed) the hydrophilic (polysaccarides) and lipophilic (essential oils) secretion, respectively.*

In *Stachys recta* subsp. *serpentini*, as reported by Giuliani et al. (2008), were observed scarce non-glandular trichomes, and glandular trichomes, in which a drop of secretion, composed of both hydrophile and lipophile substances, is present. The histochemical stains evidenced the production of both essential oil and polysaccharides and proteins (Fig.1). Polyphenols, evidenced by the $FeCl_3$ stain (not shown), are present too.

TEM observations on the ultrastructure of secreting trichomes present on leaves and inflorescences (Fig.2) evidenced osmiophilic plastids, rough and smooth endoplasmatic reticulum, all organelles responsible for both the lipophile and hydrophile secretion. Although it was not possible to ascertain the presence of metals, it is likely that the secretion efflux brings together heavy metals, detoxifying the cell, as suggested by Rascio et al. (2008) and Franco et al. (2009).



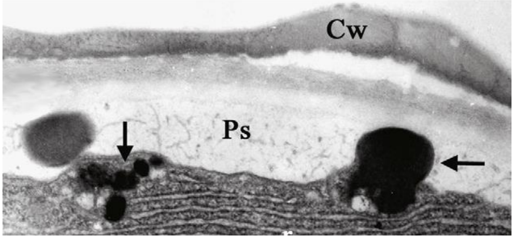


Figure 2
Left: ultrastructure of a secreting cell.
Right: particular showing drops of secretion (arrowed) extruded from the secreting cell. (Cw) Cell wall, (Ss) Subcuticular space, (Ps) Periplasmatic space, (p) plastids, (c) proteic crystals, (m) mitochondria, (r) rough endoplasmic reticulum.

Conclusions and perspectives

Data from literature and findings reported in this paper showed that plants growing on naturally metal-enriched (serpentine) soils are adapted to particular ecological conditions, i.e. they are (genetically) metal-tolerant. Resistance to high concentrations of heavy metals is a pre-requisite for plants that can be useful in re-vegetation techniques of soils at metal-contaminated sites. Plants that spontaneously occur at those sites can provide genes for the development of plants with a high restoration capacity, and therefore may be referred to as *proxies* of normal plants for remediation of metal-contaminated sites.

The selection of plants tolerant to target metals (particularly Co, Cu and Ni in this work) and efficient to translocate metals from roots to the aboveground organs (i.e.hyperaccumulators) is indeed a key factor in phytotechnology for soil restoration, especially by metal phytoextraction.

Conversely, metal-adapted non hyperaccumulating plants can be useful in exploring the possibility for phytostabilization, rather than too specialized hyperaccumulators.

Moreover, these plants can be utilized in landscape ecology projects and in metal recovery (phytomining), and receive social consent.

In conclusion, the information that soil scientists and ecologists may achieve combining soil and vegetation features of metal-enriched sites include:

(1) presence of vegetation communities known to be associated with a given mineralisation (e.g. metallophytes, serpentinophytes);

(2) presence of a lithological unit known to be favourable for site-specific vegetation (e.g. serpentinite); and/or

(3) presence of environmental features which may indicate a zone favourable to the connection between soils and vegetation (e.g. climate, elevation, aspect).

Many questions, however, need further attention. Fundamental topics for future research are:

- better knowledge of processes involved, by full scale investigations, laboratory and field trials;
- application of modern techniques (e.g. genetic engineering) to produce plants with high biomass production;
- find out new accumulator shrubby and arboreal plants.

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