PRELIMINARY OBSERVATIONS ON THE METAL TOLERANCE AND RESILIENCE CAPACITY OF *HELICHRYSUM ITALICUM* (ROTH) G. DON GROWING ON MINE SOILS

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Abstract

Wild specimens of *Helichrysum italicum*, with their soil clod, were gathered from sites with different contamination levels by heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn) in the abandoned Niccioleta mine (Tuscany, Italy). We examined the effects of heavy metals (HM) on the morphology of *H. italicum* growing on mine soils, with the following objectives: to determine the fate of HM within the soil-plant system; to highlight morphological modifications at anatomical and cytological level; to ascertain the plant tolerance to heavy metals, and their resilience capacity. Plants appeared macroscopically not affected by toxic signals. Light microscopy and TEM observations of leaves show a clear difference in the cell organization of not-contaminated and contaminated samples. Trichomes of the polluted plants present a completely different morphology in comparison to non-polluted ones, with a stalk of 3-4 cells and a large secreting apical cell, a palisade parenchyma less organized, and a reduction of leaf thickness. A gradual restoration of cell organization suggests that somewhat resilience occurred in plants. The resilience capacity points to *H. italicum* as an useful species in remediation projects.

Keywords: *heavy metals, mine soils, plant morphology, Helichrysum italicum, ultrastructure*

Introduction

Heavy metal accumulation produces significant physiological and biochemical responses in vascular plants. Plants growing on abandoned mine sites are genetically tolerant to high metal concentrations. Therefore, they are of particular interest to ascertain possible damages to physiological functions caused by heavy metals, in view of future application in environmental restoration projects (Rascio and Navari-Izzo, 2011; Bini et al., 2013). Yet, in the last decades, several technologies have been proposed to cleanup contaminated land. One of the most promising technique is phytoremediation, a technique which utilizes plants to remove, eliminate, or decrease environmental contaminants (heavy metals, organics, radionuclides, explosives), attenuating the related risk (Bini, 2009). This

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technology is potentially little destructive, environmental friendly and cost-effective, and is particularly suitable at sites where contamination is rather low and diffused over large areas, its depth is limited to the rhizosphere or the root zone, and when there are no temporal limits to the intervention. It has been calculated, indeed, that with present accumulator plants, at least 3-5 years are needed to have appreciable results in clean up a moderately contaminated soil (McGrath, 1998).

In Italy there are numerous abandoned mine sites, where both metalliferous (e.g. *Silene paradoxa*) and non-metalliferous plants (e.g. *Taraxacum officinale*) grow (Bini, 2012).

The Tuscan district is one of the most known at worldwide level for the long mining history, dating back to the Palaeolithic(Lattanzi and Tanelli, 1985); all the mine sites (totally 34) in the district were closed in early 90s'.

Mining activity has left profound signs in the whole area, both from morphological (e.g. gully and rill erosion, landslides) and environmental point of view (waste disposal, acidification, metal contamination, vegetation decline), arising the problem of land management.

Up today, however, restoration programs are by far insufficient in comparison to the numerous sources of contamination. An agreement between local government (Regione Toscana) and the national estate ENI (responsible for the mine district) has been signed in 2009, reporting procedures, time and responsibility for clean-up, restoration and safety of numerous contaminated sites (mine dumps, flotation basins, roasting waste) in the Metalliferous Hills and the adjacent Scarlino plain.

In recent years, a survey of soils and plants growing on contaminated areas at several abandoned sulphide mines in Italy was carried out by working groups of the Universities of Florence, Siena, Cagliari, Bologna, Udine and Venice, in order to evaluate the ability of these plants to colonize mine waste and to accumulate metals, in the perspective of an ecological restoration of contaminated sites. We investigated the heavy metal concentration of the waste material, and the soils developed from, in order to determine the extent of heavy metal dispersion, and the uptake by plants, and deserved attention to wild plants growing at that sites, to find out new metal-tolerant species to utilize in soil remediation. The results of these investigations, with particular emphasis on the Tuscan areas, are available in current literature (Bini, 2012).

Although current literature on phytoremediation is rather abundant, the physiological mechanisms that control and regulate the above processes are not yet elucidated, also because many of these are site-specific(Rascio and Navari-Izzo, 2011). The specificity depends first of all upon the kind of contaminant, its composition and possible transformation (evolution), and, consequently, upon the vegetal species to be utilized for remediating a contaminated site. The choice of plants is a crucial aspect for the practical use of this technique, given the ability to accumulate metals in their tissues, being genetically tolerant to high metal concentrations. Further investigation, therefore, is needed in order to elucidate both the mechanisms involved and selection of plants, possibly applying genetic engineering to increase plant biomass.

Among wild plants growing on mine sites in Tuscany, *Helichrysum italicum* is a typical Mediterranean species that is well known for its composition and for different pharmaceutical utilization (Bianchini et al., 2009):
- essential oil producer
- cosmetics
- anti-inflammatory, antibacterial, antioxidant.

Given the above characteristics, for the utilization and commercialization of derivatives from *H. italicum* the European Union requires that the absence of chemical impurities and heavy metals be certified (Bullitta et al., 2010).
The objectives of this work were:
- to determine the fate of HM within the soil-plant system at mine sites;
- to highlight morphological modifications at anatomical and cytological level;
- to ascertain the plant tolerance to heavy metals, and the resilience capacity.

Materials and methods

Site description

The mine district of Niccioleta-Boccheggiano was particularly active in the first half of last century, producing 80 million tons of pyrite (Lattanzi and Tanelli, 1985). It is extended for more than 3 Km (direction N-S) and 600 m (direction E-O), at elevation between 400 and 50 m a.s.l.
Ore minerals (iron oxides and sulphides) are located within the Mesozoic limestone ("calcare cavernoso" formation) and at the tectonic contact limestone/Palaezoic basement. The extracted ore was transported to local factories in the Scarlino plain for transformation in sulphuric acid, producing relevant amounts of pyrite ash, which were disposed randomly on the land, thus determining diffused contamination of agricultural land (1982). In the last decade of operational work, pyrite ash was used to prepare commercial pellets. The production cycle included the formation of more than 1.5 million tons of different waste materials (acid water, mud, mine soils, earthy fines, pyrite ash). Upon abandonment and closure (1992), safety works have been partly realised in the mining area; however, there are still sites where industrial waste is present and clean-up and restoration should be carried out.

Soil sampling and laboratory analyses

Four contaminated areas and a non-contaminated site (background control), were selected and sampled according to the procedures described by Margesin and Schinner (2005). Soil samples (Spolic Technosols) were collected in the proximity of waste material, as follows:
- Niccioleta 1 - soil proximal to waste material;
- Niccioleta 2 - soil on waste material;
- Niccioleta 3 - soil distal from waste material;
- Solmine - soil on pyrite ash, close to an industrial plant.

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In the period between spring–summer 2015, soil pits were opened and described following Italian national guidelines. All locations were sampled for topsoil (0–30 cm). Afterwards, soil samples were recovered to the laboratory for routine and geochemical analyses. For the analysis of pseudo-total metal content, 0.2 g of soil samples was subjected to a complete digestion in the microwave (model 1600-Ethos, Milestone) in closed containers made of Teflon. The breakdown was performed in 5 mL of aqua regia (37% HCl + 65% HNO_3, 1:3) and 1 mL of 48% HF, and then 1 mL of cold supersaturated H_3BO_3 was added. Full information on field sampling and laboratory methods is available in Wahsha et al. (2012a, b).

Plant sampling

At four of the previously selected sites, *H. italicum* specimens were sampled with their corresponding soil clod. Plants of the different sites, at gathering, presented normal appearance, and did not evidence particular diversities in shape and dimension of the leaves. The plants with their natural substrate were transferred at the Botanical Garden of the University of Florence, under the supervision of the authors. Plant samples were rinsed gently with tap and distilled water to remove the adhering soil, then were divided into leaves and roots, dried in ventilated oven at 80 °C for 2 days. Dried tissues were grinded in an agate mill (<100 μm). For each sample, 0.5 g of powder were digested in an acid mixture of 5 mL 65% HNO_3 and 3 mL 30% H_2O_2 in open vessels on the hot plate, followed by filtration with filter cellulose Whatman n. 42. Both soil and plant samples were analyzed for heavy metals (Cd, Co, Cr, Cu, Mn, Ni, Pb, Zn, Fe). The concentration of metals was determined by inductively coupled plasma–optical emission spectroscopy (ICP–OES, Perkin Elmer model 5300DV) according to the method reported by Margesin and Schinner (2005).

Microscope observations

Microscope observations were carried out in autumn, 2015. Small pieces of leaves (taken in the middle part of the leaf length), were withdrawn from plants of each contaminated site and from the control. Fresh material was pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at a pH 6.8, post-fixed in 2% OsO4 in the same buffer, then dehydrated and embedded in Spurr’s epoxy resin. Semithin sections of embedded material were stained with Toluidine Blu, and observed with a Leitz Light Microscope for a general overview of the leaf morphology.
Ultrathin sections were stained with uranyl acetate and subsequently with lead citrate to observe leaf ultrastructure. Observations were performed with a Philips EM 300 transmission electron microscope.

Results and discussion

Heavy metals in soils

Heavy metal concentration in mine soils is reported in Table 1. The highest HM levels, as expected, were recorded in soils from waste at Niccioleta 2, and the lowest level was found at the site Solmine, in the proximity of industrial plants producing TiO_2 with chromium impurities. This fact explains the higher Cr concentration recorded at this site in comparison to Niccioleta; however, Cr levels are largely below the national regulations. Cadmium concentration, instead, is very high at every site.

At Niccioleta, a dispersion aloe is clearly apparent from waste material to distal soil, where most HM (Cr, Cu, Cd, Zn, Mn) concentrations dramatically drop; conversely, Pb, Ni, Fe, Co concentrations are higher at distal soils in comparison to proximal soils. This could be due to waste material composition and disposal.

Table 1. *HM in soils (0-30 cm)*

Site	Cr	Pb	Cu	Cd	Zn	Ni	Fe	Mn	Co
					mg kg^{-1}				
Niccioleta1 proximal	27.0 ± 1.3	16.7 ±0.7	169 ±7	20.7 ±1.8	660 ±14	38.1 ±2	27110 ±32	8350 ±11	16.8 ±2.3
Niccioleta2 waste	29.2 ±1.6	2936 ±10	394 ±2	26.0 ±1.5	667 ±10	44.0 ±2.7	41810 ±22	16270 ±24	16.4 ±1
Niccioleta3 distal	18.3 ±2.1	60.5 ±2.0	26.0 ±4.0	8.0 ±0.8	76.4 ±6.4	76.9 ±9.6	32130 ±31	6010 ±33	51.2 ±6
Solmine pyrite ash	36.5 ±1.1	91.2 ±6.0	37.8 ±1.1	13.4 ±1.7	427 ±13	59.8 ±3.5	28770 ±18	2810 ±39	52.1 ±8

Heavy metals in plants

The elemental concentrations in leaves and roots of *H. italicum* collected at the same sites than soils are reported in Table 2. The highest metal concentration for most HM, as expected, was found in plants collected at site Niccioleta 2 (waste soil); moreover, not-essential elements (Pb. Cd) and critical elements (Cu, Ni, Fe, Cr) are preferentially accumulated in roots in comparison to leaves, while essential elements (Zn, Mn, Co) are preferentially accumulated in leaves. This suggests an exclusion strategy of the plant with regard to potentially phytotoxic elements. Furthermore, as in the case of soils, plants growing at distal sites present a net drop of metal concentration in both leaves and roots. It is noteworthy to point out, however, that any signal of toxicity was apparent at the time of sample collection: this may be explained with tolerance mechanisms activated by the plant in the presence of disturbance factors such as high HM concentrations.

Table 2. *HM in plants*

Site		Concentration (mgKg^{-1})								
		Cr	**Pb**	**Cu**	**Cd**	**Zn**	**Ni**	**Fe**	**Mn**	**Co**
Niccioleta 2	leaves	<0.1	38.72	49.3	0.4	0.74	<0.1	1052	860.5	3.08
Helichrysum	roots	14.3	208.62	62.3	0.66	0.52	0.8	3047	638.5	2.9
Niccioleta 3	leaves	n.d.	2.87	0.08	n.d.	0.31	n.d.	0.79	1.473	n.d.
Helichrysum	roots	n.d.	2.76	0.11	n.d.	0.35	n.d.	21.8	1.177	n.d.
Solmine	leaves	13.3	8.42	18.6	3.37	0.93	<0.1	511	73.7	4.89
Helichrysum	roots	48.4	441.9	30.7	3.59	7.19	5.17	2592	134.5	5.98

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Translocation factor

In order to assess the fate of HM in the soil-plant system, we have calculated the translocation factor as the ratio between metal content in leaves and roots. The main results are summarized hereafter:

- the less mobile elements are Cu (average TF=0.79), Fe (average TF=0.34), Pb (average 0.17); Ni and Cr are nearly immobile; these metals tend to be blocked in the roots, suggesting some exclusion mechanism by plants;
- conversely, Zn and Mn (average TF=1,4; 1,35, respectively) appear to be relatively well translocated from roots to shoots, as micronutrients;
- Cd (TF=0.60); Co (TF=1.04) present translocation factor closet to 1.00, suggesting a limited mobility of these metals.

Plant morphology

In this study, any evident variation of the macromorphology was recorded in *H. italicum* by HM excess; indeed, all the plants examined (control and contaminated samples), present similar morphology and dimensions. Consistently, any apparent toxicity symptoms were visible in numerous plants of the same area. However, we observed a little difference in leaves colour (a less intense green colour in specimens of contaminated sites in comparison to control). These statements are consistent with findings by Kupper et al. (1998), who noted that transition metals such as Cd, Cu, Ni, Pb, Zn may substitute for Mg in the chlorophyll molecule, thus reducing the photosynthetic function, which results in colour change (i.e. chloroplasts number decrease, and consequently the chlorophyll production). It is likely that analogous process would occur in our studied plants.

Although the leaf morphology of *H. italicum* does not present particular differences among the specimens of various sites, at microscopic level morphological and histological changes were observed.

Light microscopy observations show a clear difference in the cell organization of not-contaminated (Fig. 1) and contaminated samples. In particular, the secreting trichomes, which are responsible for the characteristic flavour of the plant, present a different morphology in the polluted specimens with respect to the not-polluted ones. Indeed, the latter present the typical trichomes of the Asteraceae family, with two lines of cells bearing the secretion accumulated on the apical cuticular space (Fig. 2). Trichomes of the polluted plants, instead, present a completely different morphology, with a stalk of 3-4 cells and a large secreting apical cell (i.e. they are capitate hairs; Fig. 4).

Samples from contaminated sites, moreover, present a palisade parenchyma less organized (Fig. 3), and a reduction of leaf thickness proportional to HM concentration, as reported by Bini et al. (2012) and Maleci et al. (2014) in *Taraxacum officinale* specimens. The actual thickness, measured by microscopy (and which is not appreciable with the visual observation), decreases with increasing HM content. The different leaf thickness is consistent with the reduced, or even lacking, photosynthetic parenchyma, normally structured as palisade on the adaxial leaf surface, and spongy parenchyma on the abaxial surface, as observed in control plants. The poor structural organisations, and the reduced foliar thickness of the contaminated plants, are clearly related to soil contamination.

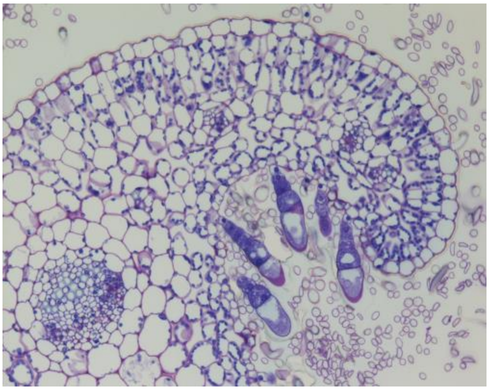

Figure 1: *Control plant.*
Transverse section of the leaf: chlorophyll parenchyma with trichomes bearing secretion (Toluidine blue). Light microscopy, 20x

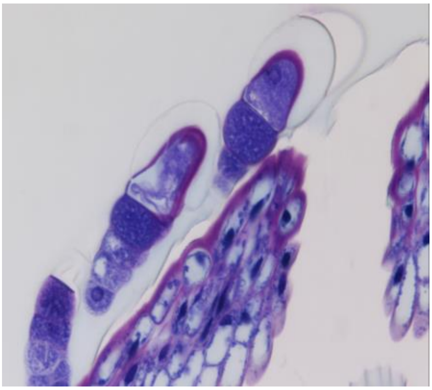

Figure 2. *Control plant.*
Secreting trichomes (particular). Light microscopy, 100x

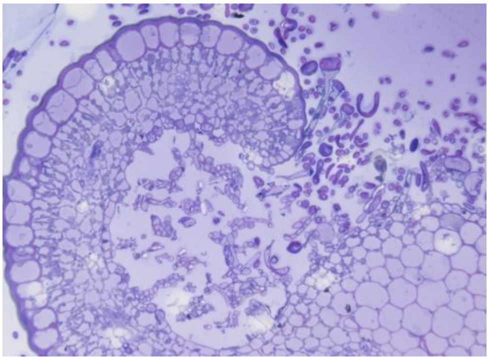

Figure 3. *Contaminated plant.*
Transverse section of leaf; less leaf thickness, big epidermal cells with thick cuticle. Light microscopy, 20x

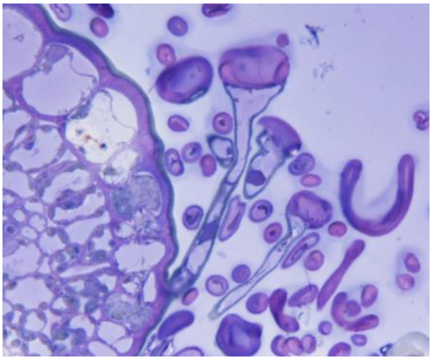

Figure 4. *Contaminated plant.*
Deformed glandular trichomes, with only one cell line (particular). Light microscopy, 100x

Micromorphological studies by T.E.M. on plant cells subjected to HM stress are nearly lacking. In a recent paper, Zhao et al. (2011) report the effects on the morphology and ultrastructure of plants subjected to lead-induced stress: reduction in size of different parts, thinner cell walls, swollen and deformed chloroplasts are recorded at ultrastructural level. Consistently, Maleci et al. (2014) report significant differences in leaf ultrastructure of *Taraxacum officinale* growing on mine sites. In our study, no deformation was observed in chloroplasts; however, in contaminated samples chloroplasts of the epidermal cells show difficult functioning (Fig. 7) and are smaller and numerically less than in the control, where

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chloroplasts are lacking in the epidermal cells (impermeable cutine, Fig. 5), while those of the internal cells bear abundant starch. Also glandular trichomes appear different in safe plants (Fig. 6) with respect to contaminated plants (Fig. 8).

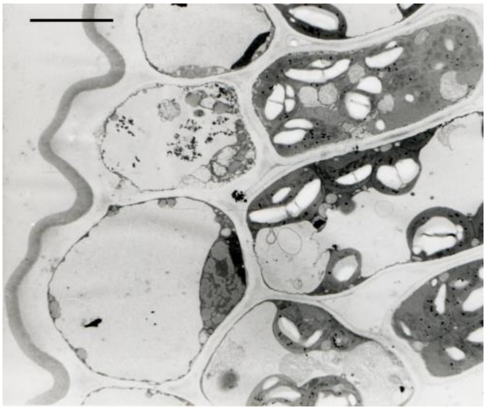

Figure 5. *T.E.M. - Control plant. Adaxial page of leaf. Epidermal cells lacking chloroplasts (impermeable cutine); internal cells with chloroplasts (grey) bearing abundant starch (white). Bar = 1 μm*

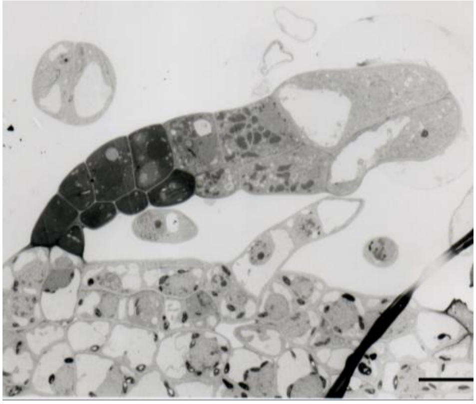

Figure 6. *T.E.M. - Control plant. A safe glandular trichome bearing two lines of basal cells and large apical secreting cells. Bar = 1 μm.*

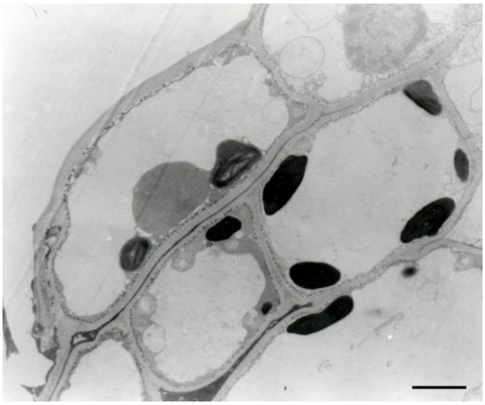

Figure 7. *T.E.M. – Contaminated plant. Epidermal cells with chloroplasts showing difficult functioning (black), likely due to heavy metals. Bar = 1 μm*

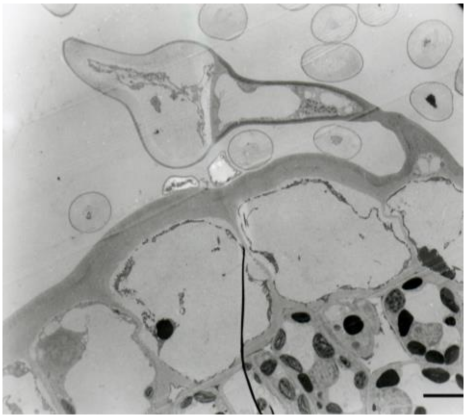

Figure 8. *T.E.M. - Contaminated plant. Damaged trichome on the adaxial page of leaf; osmiophile chloroplasts (black) in parechyma. Bar = 1 μm*

Signals of increasing contamination are the reduced starch production in chloroplasts (Fig. 9), and the occurrence of black points along the cell walls and the median lamella of contaminated plants (Fig. 10). Black points are likely HM concentrated along the cell walls as a detoxification process.
A gradual restoration of cell organization, with unusual production of starch, and an increasing reorganization of chlorophyll parenchyma suggest that somewhat

resilience is occurring in contaminated plants. The presence of stress-tolerant mycorrhizal fungi could contribute to reduce metal toxicity. The resilience capacity suggests that *Helichrysum italicum* could be a useful species in remediation projects.

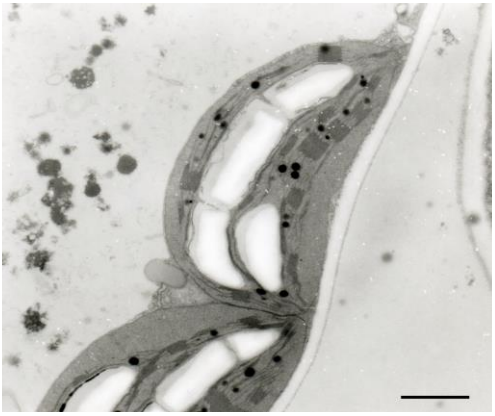

Figure 9. *T.E.M. Safe chloroplast with abundant starch (white) and thylakoids. Dark points are likely HM. Bar = 1 µm*

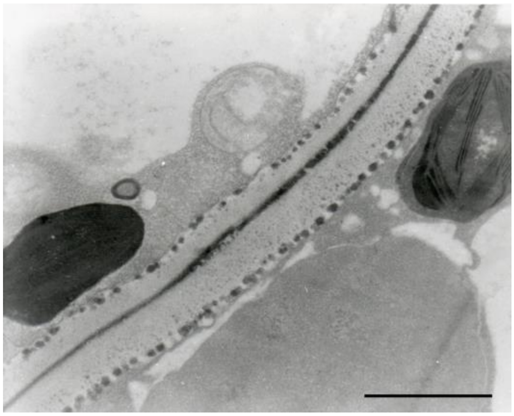

Figure 10. *T.E.M. Two contaminated cells: median lamella and walls bear heavy metals (black): detoxification? Bar = 1 µm*

Conclusions

Soil analysis shows that HMs are released from mine tailing; a fraction of HMs is taken up by *H. italicum* and transferred from soil to roots (e.g. Pb, Fe, Cd) and shoots (e.g. Zn, Mn, Co), as it is common in indicator plants.
H. italicum from mine soils proved tolerant plant, genetically adapted to survival.
The study shows that there is a relationship between high metal content in *H. italicum* plants and their modified morphology. HMs do not determine an evident modification of the macromorphology. Conversely, significant microstructure alteration is evident: the leaf thickness decreases, and the absence of a regularly structured cellular organisation is likely related to soil contamination.
A gradual restoration of leaf organisation suggests that somewhat resilience occurred in plants.
H. italicum can be used for phytoremediation projects (and particularly for phytostabilization) in environmental restoration of metal-contaminated areas.
The presence of heavy metals has a beneficial consequence, because the land affected by anthropogenic pollution may be restored by natural way at low cost.
Particular attention should be deserved in utilizing the aerial parts for drug extraction and phytotherapic preparations.

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