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Response of *Vicia faba* L. to metal toxicity on mine tailing substrate: Geochemical and morphological changes in leaf and root

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ABSTRACT

Vicia faba L. seeds were grown in a pot experiment on soil, mine tailings, and a mixture of both to mimic field situations in cultivated contaminated areas near mining sites. Metals in the substrates and their translocation in root, stem and leaf tissues were investigated, including morphological responses of plants growing on mine tailings. Metal concentration – and generally bioaccumulation – was in the order: roots > leaves > stems, except Pb and Cd. Translocation was most significant for Zn and Cd, but limited for Pb. Metal concentration in root and leaf was not proportional to that in the substrates, unexpectedly the minimum being observed in the mixed substrate whilst plant growth was retarded by 20% (38% on tailings). Calcium, pH, organic matter and phosphorus were the main influencing factors for metal translocation. The ultrastructure of *V. faba* L. cells changed a lot in the mine tailings group: root cell walls were thickened with electron dense Pb, Zn and C particles; in chloroplasts, the number of plastoglobuli increased, whereas the thylakoids were swollen and their number decreased in grana. Finally, needle-shaped crystalline concretions made of Ca and P, with Zn content, were formed in the apoplast of the plants. The stratagems of *V. faba* L. undergoing high concentrations of toxic metals in carbonate substrate, suggest root cell wall thickening to decrease uptake of toxic metals, a possible control of metal transport from roots to leaves by synthesizing phytochelators–toxic metal complexes, and finally a control of exceeded Ca and metal concentration in leaves by crystal P formation as ultimate response to stress defense. The geochemical factors influencing metal availability, guaranty a reduction of metal content in plant growing on mixed tailing/soil substrate as far as carbon te are not completely dissolved.

1. Introduction

Mine tailings, the solid waste from mineral resource exploitation, causes severe environmental problems. It is reported that there are more than 1000 abandoned mines in Korea (Kim et al., 2003), and about 100,000 to 500,000 mine sites are abandoned or inactive in the United States (Carrillo-Chavez et al., 2003). Unfortunately it appears that in some places, the dams built to retain the mine tailings have collapsed. Since 1970, there have been 35 reported major mine tailing dam failures around the world. In 2000 alone, there were five reported accidents in China, Romania, Sweden, and USA (Macklin et al., 2003). As a result, the lower regions of river and farmland are covered with a thick layer of mine tailings and severe environmental disasters take place.

In these contaminated areas, the bare surfaces of the mine tailings-covered soils will accelerate surface soil erosion by rain, underground water contamination due to leaching, and atmosphere pollution by wind, particularly if there is no vegetation cover (Conesa et al., 2007). Thus, the degree of contamination may increase and the range expands (Salvarredy-Aranguren et al., 2008). In spite of some remediation by mechanical sludge removal, most of the contaminated farmlands are often still covered with spills and some of them are cultivated at present (Liu et al., 2005). Because of high concentrations of toxic metals, poor soil physical structure, nutrient deficiency, and potentially high-pH value found in the mining waste, it is very difficult for plants to grow. Chronic lower level intakes of toxic elements have deleterious effects on man and other animals, since there is no efficient mechanism for their elimination and the detrimental impact becomes apparent often after several years of exposure (Bahemuka and Mubofu, 1999).

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Herbaceous leguminous plants can be used as pioneer species, which solve not only the problem of nitrogen deficiency in mine tailings because of their N_2-fixing ability, but also improve soil quality, and reduce weed occurrence (Yang et al., 2003). Our previous field trials found that some herbaceous legumes (e.g. soybean, Adzuki bean, mung bean and peanut) could successfully grow on lead/zinc mine tailings-covered soil (Liu et al., 2005). The most important result was that the toxic element concentrations in the seeds were the lowest among all those considered crops, which include cereal (rice, maize, and sorghum), vegetables (*Ipomoea*, *Capsicum*, taro and string bean), and legumes (soybean, Adzuki bean, mung bean, peanut and Faba bean), even if they grew on highly contaminated soil. The considered toxic element (Cr, Co, Ni, Cu, As, Zn, Cd, and Pb) concentrations in the seeds were lower than the corresponding threshold levels.

High concentrations of metals can cause severe damages to plants, hence inhibit their growth and reproduction. The leguminous *Vicia faba* L. might be of interest to grow on metal-contaminated sites with deficient nitrogen. But since it is considered as sensitive to metal uptake, it is thus of interest to check if it may develop some special mechanisms to grow on mine-tailings contaminated substrate and to inhibit toxic metal uptake and storage.

For this purpose, a pot experiment on soil and mine tailings was carried out to: (i) identify the metal translocation from substrate to plant tissues of *V. faba* L.; (ii) evaluate the plant growth responses; (iii) determine the ultrastructural cell changes under extreme experimental conditions; (iv) discuss the mechanisms of control of toxic metals uptake and storage in the root and leaf cells, and the relationship between metal contamination of substrate and chemical and morphological responses of plants as stratagems of survival.

2. Materials and methods

2.1. Pot experiment

2.1.1. Substrates for plant growth
Mine tailings were collected from a small abandoned lead–zinc mine in a valley from the Pyrénées Mountains, southern France (Brunel et al., 2003). The soil sample taken as a control, was collected at about 1 km from the abandoned mine. After air-drying, these soil and mine tailings samples were passed through a 2-mm sieve.

To simulate the condition of mine tailings-covered farmland and to comply with the major toxic metal concentrations such as, for example, found in Chenzhou mine tailings-contaminated area (China, Liu et al., 2005), mine tailings and soil were mixed in a proportion of 1 part mine tailings (290 g) to 4 parts soil (1160 g). Since the very sandy mine tailings texture may affect the physical and chemical properties of the mixed substrates, fine pure sand mixed with soil was also used as a control in the same proportion. Soil, mine tailings and pure sand were amended with NPK fertilizer at the rates of 200 mg N kg^{-1} substrate as Ca(NO$_3$)$_2$, and 100 mg P kg^{-1} substrate as KH$_2$PO$_4$. All nutrients were mixed adequately with these substrates, respectively. The treatment in a pot experiment included 4 groups of 1450 g sample: soil (S), soil + pure sand (S + S), soil + mine tailings (S + T) and mine tailings (T). All treatments were in triplicates. Substrates were irrigated with deionized water and allowed to equilibrate for 3 weeks prior to transplanting.

2.1.2. Greenhouse experiment
A greenhouse experiment was carried out for 8 weeks. *V. faba* L. seeds (Commercial mark: Qidou 2) were cleaned and surface-sterilized with NaClO, and then germinated. When the roots were

about 1 cm in length, seedlings were transplanted into the 12 pots (diameter 20.5 cm, triplicate treatments) with 12 seedlings per pot, watering with deionized water twice a week. Twelve days later, the heights of the seedlings were measured.

2.1.3. Samples collection and pre-treatment
At the end of the greenhouse experiment, roots and leaves were collected for ICP-MS analysis. To avoid extra contamination from metals, careful procedures of separation, drying and grinding were carried out. Particularly roots were cleaned according to the procedure by Wang et al. (2003). The substrates were sampled before transplanting. After air-dried at room temperature, and sieved with a nylon mesh (2 mm), the finest fraction (<2 mm) was ground in an agate mortar and stored in plastic bags for analysis.

2.2. Transmission electron microscopy

Ultrastructural studies were performed on root and leaf tissues from control plants ($n = 3$) and plants grown on mine tailings ($n = 3$) to determine plant responses under extreme conditions. For each plant, samples of the middle part of the third leaf from the stem tip and samples of root tips were chosen. They were cut in small pieces and fixed in a solution of glutaraldehyde (3%) for 4 h. After rinses, they were post-fixed in osmium tetroxide (1%) for 3 h, dehydrated in graded alcohol, and embedded in epon-araldite resin. Ultra-thin sections were collected on copper/palladium grids. Some of them were contrasted with uranyl acetate for 30 min. Ultra-thin sections were examined with a Hitachi H600 transmission electron microscope.

2.3. Energy dispersive X-ray microanalysis

Energy-dispersive X-ray microanalysis (EDXM) was carried out on unstained ultra-thin sections of root and leaf samples prepared as described above for transmission electron microscopy. The ultra-thin sections were collected on carbon-coated copper grids. EDXM were performed on a JEOL 2010 transmission electron microscope equipped with an EDAX 9100 microanalyzer at acceleration voltage 200 kV, take-off angle 25.02°, cooled with liquid nitrogen.

2.4. Metal concentration analysis

To measure total metal and major element concentrations in the plant tissues and substrate samples, 0.1 g leaf or substrate powder was completely dissolved by acid digestion method (bi-distilled HNO$_3$–HF and H$_2$O$_2$) using a well-calibrated procedure (Hernandez et al., 2003), which includes blanks and reference material. Metal concentrations were measured by ICP-MS (PerkinElmer ELAN 6000) and Ca and P by ICP-AES (see for quality control, Liu et al., 2005). The different soil parameters have been analyzed at the INRA Laboratory of Soil Science—Arras (France) following classical methods.

2.5. Data analysis

All statistical analyses were conducted with SPSS for Windows 6.0. To compare the effects on seedling height and metal concentrations in leaves between treatment groups, ANOVA was used.

3. Results

3.1. Substrate characteristics

Table 1 showed the physical and chemical characteristics of the substrates. The selected soil was fit for plant growing with a low

Treatment	pH (in H_2O)	$CaCO_3$ (g kg^{-1})	OM (g kg^{-1})	OC (g kg^{-1})	Total P (g kg^{-1})	Total N (g kg^{-1})	C/N	CEC (cmol$^+$ kg^{-1})	Ca^{2+} (cmol$^+$ kg^{-1})
S	5.1	1	63.6	36.98	0.45	3.40	11.56	3.5	1.27
T	8.6	287	10.9	6.36	0.10	0.38	35.33	0.2	1.96
S + T	5.2	72.5	50.4	29.33	0.36	2.65	15.50	2.7	1.44

acidy (pH 5.1), low-CaCO$_3$ content (1%), high-organic matter content and moderate contents of total P, N, and C/N ratio. The mine tailings were a sandy waste (83.6% sand) with low content of clay (3.1%), hence the water holding capacity was quite low. pH value of the mine tailings was rather high (8.6) and CaCO$_3$ content was very high, which suggested that ore was processed under alkaline conditions. The same situation was found in the mine tailings from Chenzhou Pb/Zn mine, China (Liu et al., 2005) and from Mount Uludag, Turkey (Gûleryûz et al., 2002). Otherwise, the other chemical characteristics of the mine tailings, such as low-organic matter content, total P and total N, were not conducive for plant growth. However, mixing of these materials (soil and tailings) represented field situations of potential contaminated cultivated areas in various mining regions of the world.

3.2. Metal concentrations in the substrates

Metal concentrations in the substrates were listed in Table 2. The mine tailings were characterized by very high concentrations of Pb, Cd, Cu and Zn, which exceeded the corresponding maximum permissible limits for agricultural soils (see Türkdogan et al., 2003; Hernandez et al., 2003) by 580, 429, 30 and 19 times, respectively. In the soil sample, the total concentrations of these four metals still exceeded the corresponding upper limit, as well as the world ranges for metals in non-polluted agricultural soil (Kabata-Pendias and Pendias, 1992). This soil from the Pyrénées Mountains was derived from mineral enriched bedrock (Brunel et al., 2003), but might also have been affected by the surrounding mining activities, mainly by atmospheric dust deposition. No significant differences were detected between S and S + S taking into account the dilution by the proportion of sand. The concentrations of Pb, Cd and Cu in the group of S + T were similar to those in the mine tailings-covered soils of Chenzhou lead/zinc mine (Table 2), previously reported by Liu et al. (2005). Only Zn was about three times higher in our present experimental substrate than that in Chenzhou soil. On the opposite, the concentrations of Cr, Co and Ni fell below world ranges reported for non-polluted soils (Kabata-Pendias and Pendias, 1992), so the effects of these three metals were not supposed to significantly affect plant growth.

3.3. Metal concentrations in plant tissues of V. faba L.

Pb, Cd, Cu and Zn were the most severe contaminants in the mine tailings, and Mn is an essential element for plant growth,

which was taken into consideration as an indicator for nutrient plant absorption. Our attention was thus focused on these five elements.

Among the five elements, Zn concentration in all plant tissues of *V. faba* L. was the highest, followed by Mn. Pb concentration was quite low (except on mine tailing plants) even though it was very high in the corresponding substrates (Fig. 1). The results showed that *V. faba* L. contained Zn and Mn, in a range of 76.6–944 mg kg^{-1} in the leaf tissue, and 251–5903 mg kg^{-1} in the root tissue. *V. faba* L. also absorbed and stored some Cu, Pb and Cd, with a range of 0.24–27.8 mg kg^{-1} in the leaf tissue, but much more in the root tissue (17.6–1577 mg kg^{-1}).

Except Cd and Mn, the metal content in roots was the highest in *V. faba* L. growing on mine tailings. In stems and leaves, only Pb had the highest concentrations in plants of the mine tailings group. We had expected that the concentrations of the considered metals in plant tissues would linearly increase with that in the corresponding substrates. But, when comparing the four treatments (Fig. 1), the results clearly showed that all these four toxic element concentrations and also the nutrient elements such as Mn, were almost the lowest in the S + T group. The S + S group did not show any differences in plant metal content compared to the S group, indicating no effect of substrate physical characteristics on metal absorption by plants.

The degree of metal absorption and translocation from substrate to vegetal tissues was estimated using the BAF (bioaccumulation factor, see Table 3). Generally, the BAF of Mn, Cu, Zn and Cd were in the order of root ≫ leaf > stem indicating that a major portion of the elements was distributed in roots, whereas only a small portion was transferred to leaf (8–44% for Mn, Cu and Zn) and to stem tissues (3–27% for Mn, Cu and Zn). The rest (about 38–85%) remained in roots. The exception was Pb (and Cd in plant growing on S + T), which was in the order of root ≫ stem > leaf. For Cd and Pb respectively, only about 1–7% of the total concentration was distributed in the leaves, 3–11% in the stems, whereas 86–96% was in the roots. Therefore, less proportion was transferred to the upper parts of the plants. Similar results were reported by Chen et al. (2003) for soybean plants, where Cd accumulated in roots between 2 and 20 times by comparison to stems and leaves.

Cd and Zn had the highest BAF values for the root, leaf and stem tissues of *V. faba* L. growing on soil (Table 3), indicating that these elements are the most mobile and reactive elements under acid conditions (as usually found in such soils, Hernandez et al., 2003), followed by Mn and Cu. Lead was the most stable element.

| Treatment	Cr	Mn	Co	Ni	Cu	Zn	Cd	Pb
In the present experiment study								
S + S[a]	95.1	249.2	7.4	108.1	83.7	195.6	1.1	72.1
S	118.9	311.5	9.2	135.1	104.6	244.5	1.3	90.1
S + T[a]	99.0	689.5	9.1	114.9	116.8	3105.1	9.7	1626.5
T	19.7	2201.8	8.6	33.8	165.5	14547.6	42.9	7771.8
In Chenzhou mine tailing contaminated soils[b]	28.8–63.5	–	5.7–13.6	10.0–31.6	110.1–148.9	529.6–1251	3.5–11.1	852–1444

(–) not measured.

[a] Calculated values based on the mixing ratios between soil and pure sand or mine tailings.

[b] Liu et al. (2005).
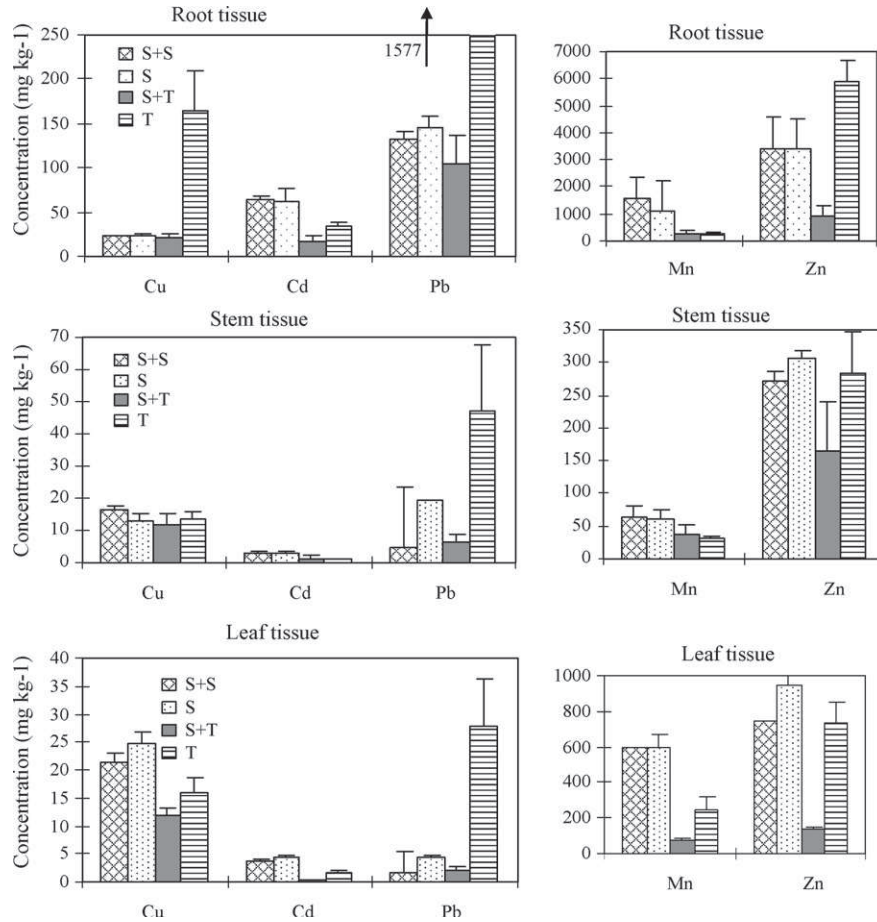
Fig. 1. Total metal concentrations in the root, stem, and leaf tissues of *Vicia Faba* L. (S + S: soil mixed with pure sand; S: soil; S + T: soil mixed with mine tailings; T: mine tailings) (mean ± S.D., $n = 3$) based on three replicates of the three independent series.

Table 3
BAF values of root, stem, and leaf tissues of *Vicia faba* L. (S + S, soil mixed with pure sand; S, soil; S + T, soil mixed with mine tailings; T, mine tailings).

	Root tissue				Stem tissue				Leaf tissue			
	S + S	S	S + T	T	S + S	S	S + T	T	S + S	S	S + T	T
Mn	6.35	3.60	0.33	0.13	0.26	0.19	0.05	0.01	2.38	1.88	0.10	0.11
Cu	0.26	0.22	0.17	0.88	0.18	0.12	0.10	0.07	0.24	0.23	0.10	0.08
Zn	17.43	14.23	0.25	0.39	1.39	1.28	0.04	0.02	3.83	3.96	0.04	0.05
Cd	57.77	44.97	1.46	0.75	2.45	2.13	0.11	0.03	3.30	3.25	0.02	0.04
Pb	1.75	1.77	0.06	0.22	0.06	0.23	0.00	0.01	0.02	0.05	0.00	0.00

BAF = $[M]_{plant}/[M]_{substrate}$, with $[M]_{plant}$: the metal concentration in the plant tissue and $[M]_{substrate}$: the metal concentration in the substrate.

3.4. Effects of metals on V. faba L. seedling growth

The influence of the substrate on seedling growth was estimated using the restraining index calculation (Table 4). The results indi-

Table 4
Mean height[a] and restraining index (RI)[b] of *V. faba* L. seedling in response to mine tailings treatments (mean value ± S.D., $n = 36$).

	S	S + S	S + T	T
Mean height ± S.D. (cm)	8.69 ± 2.24	8.07 ± 2.35	7.07 ± 1.90	5.52 ± 2.18
RI$_{height}$ (%)	0	8.78	19.99**	37.59**
Biomass (g dry matter)	8.27 ± 0.93	8.67 ± 1.93	6.35 ± 1.44	6.26 ± 2.94
RI$_{biomass}$ (%)	0	−4.65	30.32**	32.13**

[a] The mean height of the seedling from the soil surface to the seedling tips.

[b] Restraining index (RI) = (mean value of control group − mean value of treated group)/mean value of treated group × 100% (soil group was indicated as control group).

** Difference is significant at 0.01 level.

cated that in the S + T and T groups, *V. faba* L. seedling growth was significantly ($p < 0.01$) restrained by 20% and 38%, respectively, as compared to the control group (S).

The restraining equation is $y = 12.4x − 14.4$, $R^2 = 0.974$ ($n = 36$); where y corresponds to restraining index and x corresponds to treatments.

The growth performance was retarded significantly in the S + T group despite the lowest metal concentrations found in the plant tissues. The dry biomass of *V. faba* L. was decreased significantly ($p < 0.01$) in the S + T or T groups with a restraining index of 30.3 or 32.1, respectively.

3.5. Ultrastructure changes in root tissues of V. faba L. grown on mine tailings

The root cambium or cortex cells of control plants did not exhibit any ultrastructural alterations. Cambium cells were undifferentiated with a central nucleus, a large cytoplasm rich in rough

endoplasmic reticulum, free ribosomes and mitochondria (Fig. 2). Small vacuoles containing electron dense materials were scattered throughout the cytoplasm. Root cortex cells were bounded by a thin cell wall (103–294 nm) (Fig. 3a). The periplasmic spaces were narrow. However, few small electron dense particles were observed on the surface of cell walls and in intercellular spaces (Fig. 3a, arrows).

Roots of *V. faba* L. grown on mine tailings were macroscopically altered. They were dark in color whereas roots of control plants were light. Transmission electron microscopic examination showed ultrastructural changes in root tissues. The cell walls were thickened (588–705 nm) and consisted of an inner weakly dense layer and an outer dense layer (Fig. 3b$_1$). Numerous electron dense particles of various sizes were observed in intercellular spaces, where they were often adsorbed on the surface of cell walls (Fig. 3b$_1$ and 3b$_2$, arrows). Some of them were localized inside the outer layer of cell walls (Fig. 3b$_2$, small arrows). In highly damaged root cells, the electron dense particles sometimes in clusters, were distributed at the cell periphery (Fig. 3c, arrows) and on the surface of nuclear envelope (Fig. 3c, arrow heads). X-ray probe microanalysis showed
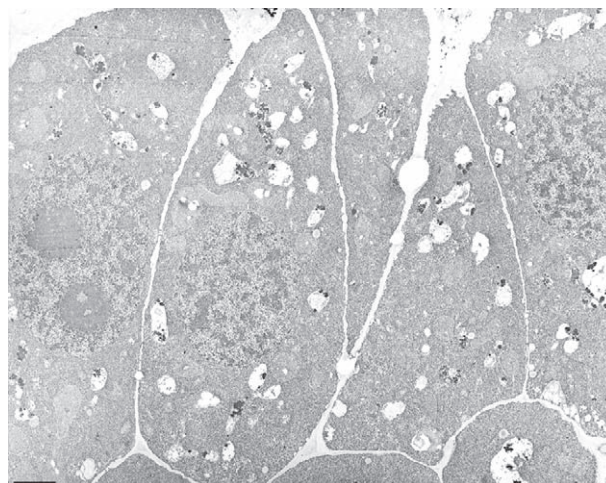
Fig. 2. Electron micrograph showing root cambium cells of *Vicia faba* L. grown in control soils. No structural changes were observed. N: nucleus; V: vacuole. Bar = 3 μm.
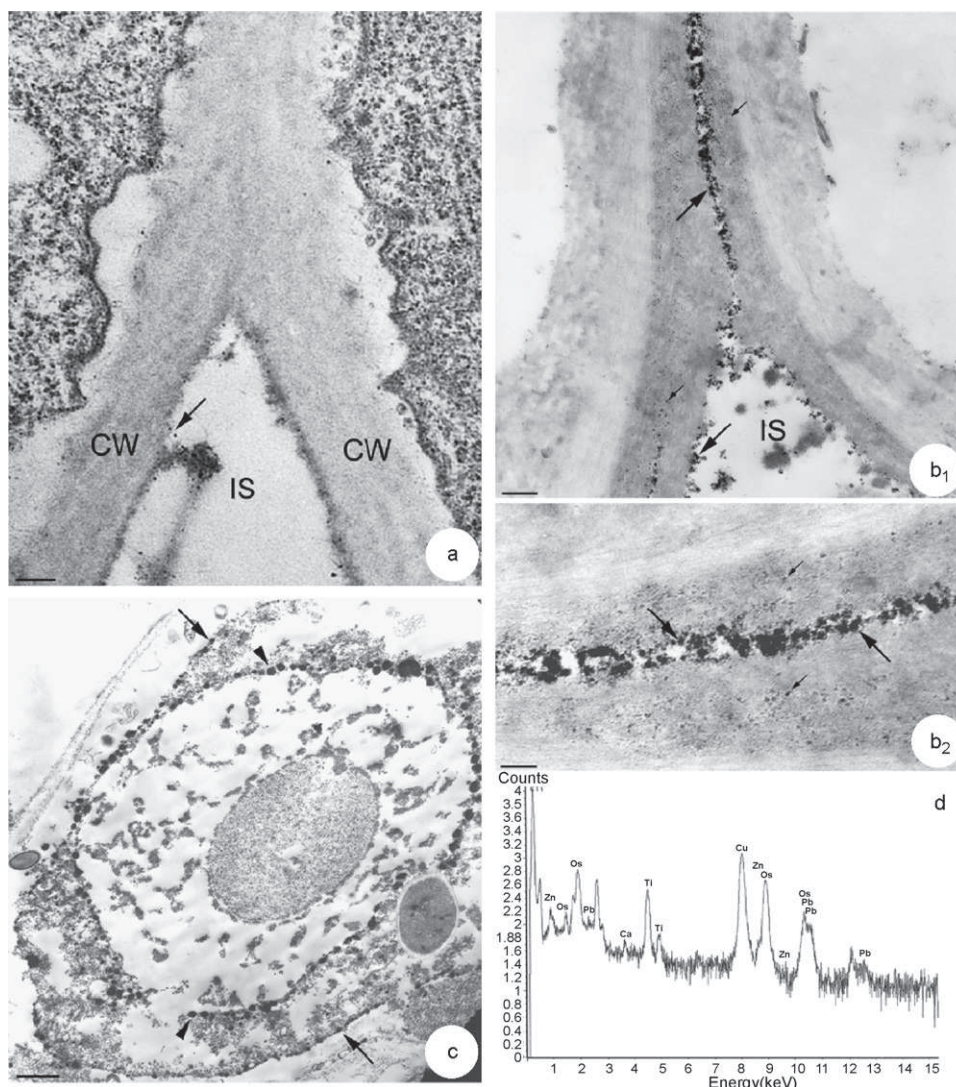
Fig. 3. Evidence for the accumulation of metal particles in root tissues of plants grown on mine tailings (b$_1$, b$_2$, c and d) compared to control plants (a). (a) Electron micrograph showing the presence of small dense particles on the surface of cell wall (CW) and in intercellular spaces (IS) in root tissue of control plants. Bar = 0.2 μm. (b$_1$ and b$_2$) Distribution of metal particles in root cells of *V. faba* L. grown on mine tailings. (b$_1$) Dense particles mainly localized along the surface of cell walls and in intercellular spaces. Bar = 0.2 μm. (b$_2$) High magnification showing clusters of dense particles on the surface of cell walls (arrows) and small dense particles inside the cell walls (small arrows). Bar = 0.1 μm. (c) Electron micrograph of a damaged root cell displaying dense particles on the surface of its plasma membrane (arrow) and nuclear envelope (head arrow). Bar = 1 μm. (d) Spectrum of X-ray microanalysis from dense particles localized on the surface of cell wall of root cells from *V. faba* L. grown on mine tailings. Pb, Zn, Ca and C peaks were observed. Cu peak may be disturbed by the section support. Os and Ti are probably due to sample process.
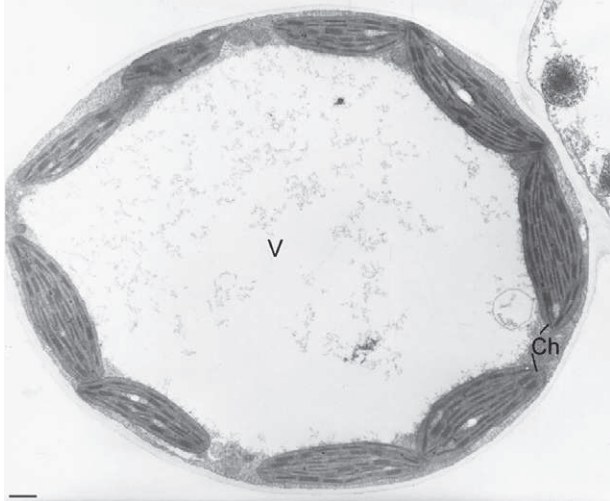
Fig. 4. Electron micrograph showing the general appearance of a leaf cell from control plant. Note the large central vacuole (V) surrounded by lens-shaped chloroplasts (Ch). Bar = 1 μm.

that the particles localized either on the cell walls or inside the cells were mainly made up of Pb, Zn and C (Fig. 3d). The signal of Cu and Ti might be mainly associated to the support grid as well as Os to the chemical treatment.

3.6. Ultrastructure changes in leaf tissues of V. faba L. grown on mine tailings

The ultrastructural organization of mesophyllic cells of control plants did not display any abnormalities (Fig. 4). A large vacuole occupied the intracellular space. It was surrounded by a thin cytoplasm containing mitochondria and regularly arranged chloroplasts. These latter were lens-shaped with a typical arrangement of grana and stroma thylakoids and contained few small plastoglobuli (Fig. 5a). The number of thylakoids in grana ranged from 4 to 12.

With regard to leaves of V. faba L. grown on mine tailings, a chlorosis occurred after 25 days of culture as compared to leaves of control plants. Transmission electron microscopic analysis showed damage symptoms in leaf tissues. First, a gradual disorganization of chloroplasts was observed (Figs. 5b–d). They were more rounded than chloroplasts in leaves of control plants (Fig. 4b). They contained a considerably reduced system of grana and stroma thylakoids. Most thylakoids were swollen and their number in grana ranged from 2 to 4 (Fig. 5c). An increase in the number and size of plastoglobuli as well as the number of starch granules was also observed (Fig. 5b and c). In some cells, chloroplasts were totally disorganized with a disruption of their envelope and a high swelling of thylakoids (Fig. 5d). The second main alteration observed in leaf tissues of V. faba L. grown on mine tailings was the presence of crystalline concretions. These structures appeared often adsorbed on the surface of cell walls at the level of intercellular spaces (Figs. 5b and 6a, arrows). They were needle-shaped and measured 68.5 nm ± 14.9 length and 6 nm ± 1.4 diameter. They were often in clusters and surrounded by an amorphous material (Fig. 6b and d, arrows). In some cells, they were observed inside the cell walls and in periplasmic spaces (Fig. 6c, arrows and d). In this case, the plasma membrane in the vicinity of crystals was disrupted. Some rare needle-like structures were also found in the cytoplasms underlying plasma membranes (Fig. 6b, arrow head). An accumulation of crystalline structures could be observed in some cells (Fig. 7a). Their X-ray emission spectrum showed that they were mainly made up of P, Ca, C and O (Fig. 7b), suggesting they were calcium phosphate salts. A slight signal of Zn was also detected. No signals of other

metals (such as Pb and Cd) were observed, but may be due to analytical detection limits. The electron diffraction diagram carried out on these crystalline structures displayed the characteristic rings of apatite structures (Fig. 7c). This suggested that crystals in leaf tissues of V. faba L. exposed to very high concentrations of metals and growing on alkaline substrate, consisted of calcium phosphate in an apatite-like structure.

4. Discussion

This study was performed by growing seedlings of V. faba L. on mine tailings and soil plus soil mixed with mine tailings, preferably to soil artificially added with metals or on hydroponic solutions, in order to mimic closely field situations occurring in metal-contaminated places. The data clearly showed both chemical and morphological plant responses depending on the kind of metal and on substrate conditions.

### 4.1. Chemical response of V. faba L. to metal contamination

From the chemical data obtained in this experiment, it was shown that V. faba L. reacted to the increase of metal concentration in the substrate. However, this response was not linear and depended on metal, substrate characteristics, and plant tissue.

4.1.1. Behaviour of metal in plant in relation to metal content in the substrate

Up to a certain critical limit of metal concentration, some mechanisms of plant uptake regulation take place regarding metal contamination. This was attested by the concentrations and the BAF, which generally decreased from roots to leaves, and with increasing level of substrate contamination. Nevertheless, metal concentration in plant depends not only on substrate metal concentration level but also on metal type and on the characteristics of the substrate regulating metal availability. Zn and Mn were significantly stored in plant roots and leaves, whereas Cu, Pb and Cd were particularly concentrated in roots. Metal accumulation is in the order of root > leaf > stem, except for Pb and Cd (root > stem > leaf): this indicated the plant ability to regulate metal transfer. V. faba L. greatly bioaccumulates Zn and Cd whatever the shoots and roots for plant growing on soil most probably in relation to its nutrient role for Zn and its high mobility for Cd under acid conditions. But with increasing substrate metal content, the relative metal accumulation is decreased, which indicated a limit to metal translocation. Pb was found to be the most concentrated in the shoots and roots of plants growing on mine tailings compared to other substrates. It could be deduced that in response to high concentrations of toxic metals in the mine tailings, V. faba L. root cells synthesized certain phytochelators (PCs)–metal complexes, probably to be PC–Pb complexes. These PC–metal complexes might influence toxic metal transport to the stems and leaves. PC–metal complex was also reported in other plants such as tobacco (Vogeli-Lange and Wagner, 1990) and wheat (Stolt et al., 2003). These results suggest accumulation of Cd and Zn, and Pb exclusion as tolerance strategies of V. faba L. Similar data have been reported by Vogel-Mikuš et al. (2005) in *Thlaspi praecox* Wulf.

4.1.2. Influence of physico-chemical characteristics of the substrate on plant response to metal contamination

Changes in physico-chemical characteristics of substrates might affect metal mobility. A pH decrease in the substrate causes an increasing metal solubility, which would result in an increased flux from bound forms to soil solution and thus, to increasing uptake into plant (Weis and Weis, 2004). In our study, a negative correlation between BAF of metals and pH of the substrates was observed
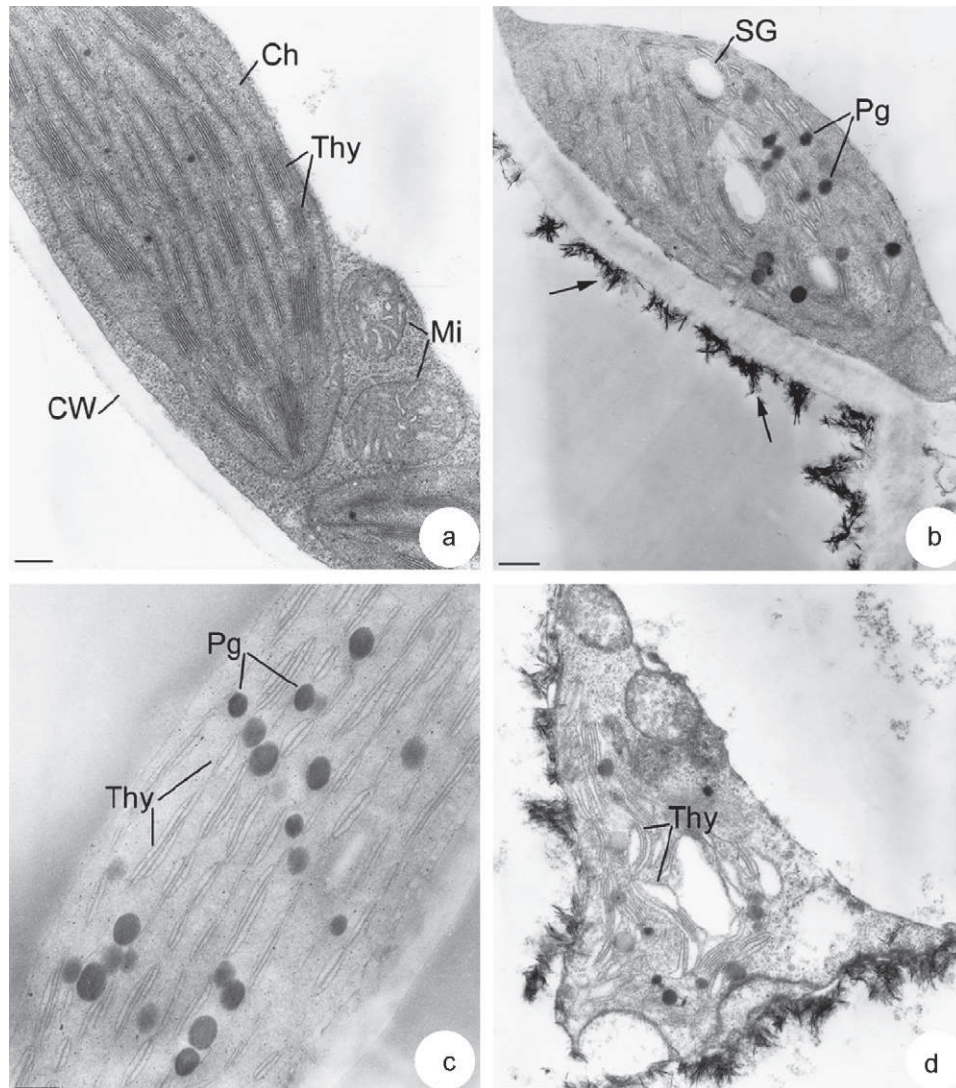
Fig. 5. Gradual ultrastructural damages of chloroplasts in leaf cells of *V. faba* L. grown on mine tailings. (a) Chloroplast (Ch) in mesophyllic cell from control plant. Observe the numerous thylakoids (Thy) and few small plastoglobuli scattered throughout the stroma. CW: cell wall; Mi: mitochondria. Bar = 0.2 μm. (b and c) Chloroplasts in mesophyllic cells from *V. faba* L. grown on mine tailings. *Note*: (i) the numerous big plastoglobuli (Pg), (ii) the swollen thylakoids (Thy) and their reduced number. The arrows indicate electron dense needle-like structures on the surface of cell wall. SG: starch granule. Bars = 0.4 μm (b) and 0.2 μm (c). (d) Final stage of chloroplast damages in mesophyllic cells of *V. faba* L. grown on mine tailings. Observe the absence of boundary membrane, the high dilation of thylakoids (Thy) and the presence of needle-like structures on the surface of plasma membrane. Bar = 0.4 μm.

(Tables 1 and 3), with the highest BAF for Zn and Cd observed for low-pH soil conditions.

Moreover, it was observed that metal concentration in plants was not linearly correlated to that in the substrate, and unexpectedly the lowest metal concentrations were in the group growing on mixed soil + tailing. Indeed, we measured the CEC in the mixed substrate (S + T) after plant growing. It showed a significant increase $(10.57 \pm 0.12\,\mathrm{cmol^+\,kg^{-1}})$ compared to the initial value (Table 1), and particularly for exchangeable Ca, compared with the value expected by simple mixing calculation $(4.24\,\mathrm{cmol^+\,kg^{-1}})$. This high value might be explained by an increase in mine tailing carbonate dissolution under more acid conditions (brought by the soil) and particularly in the surrounding roots (Chojnacka et al., 2005). This process is probably enhanced by mine tailing sulphide dissolution, which is known to increase acidity (Salvarredy-Aranguren et al., 2008). This led to increased calcium availability as exchangeable form. Moreover, the slight increase of substrate pH (compared to soil alone) resulting by mixing soil and mine tailings has also probably increased metal binding to soil organic matter, which might

strongly reduce metal availability. The increasing exchangeable Ca particularly obvious for the mixture S + T substrate might be competitive for metals (even if Ca content is not increased in those plants, Fig. 8). The lowest metal concentration of metals found in the tissue of plants growing on soil + tailings might be explained by the regulating role of calcium as indicated by the translocation factor between metal contents in leaf over root (Fig. 9).

Together with calcium, phosphorus may play a limiting role for metal translocation and contamination to plant. Ca content in the plant tissue was generally higher in the mine tailings group (except for leaves in the S + T group), whereas phosphorus was the less. The ratio Ca/P (Fig. 8) indicated that leaves of plants growing on mine tailings were very much enriched in Ca compared to phosphorus. In the soil group, the significant translocation of Ca probably inhibits part of the transfer of Zn and mainly that of Cd and Pb to leaves. When exchangeable Ca increases and P is low (soil + tailing group), then Ca, P and Cd translocation is restrained whereas that of Zn and Pb is increased, compared to what happens in the soil group. This might explain why in spite of the lowest metal concentration,
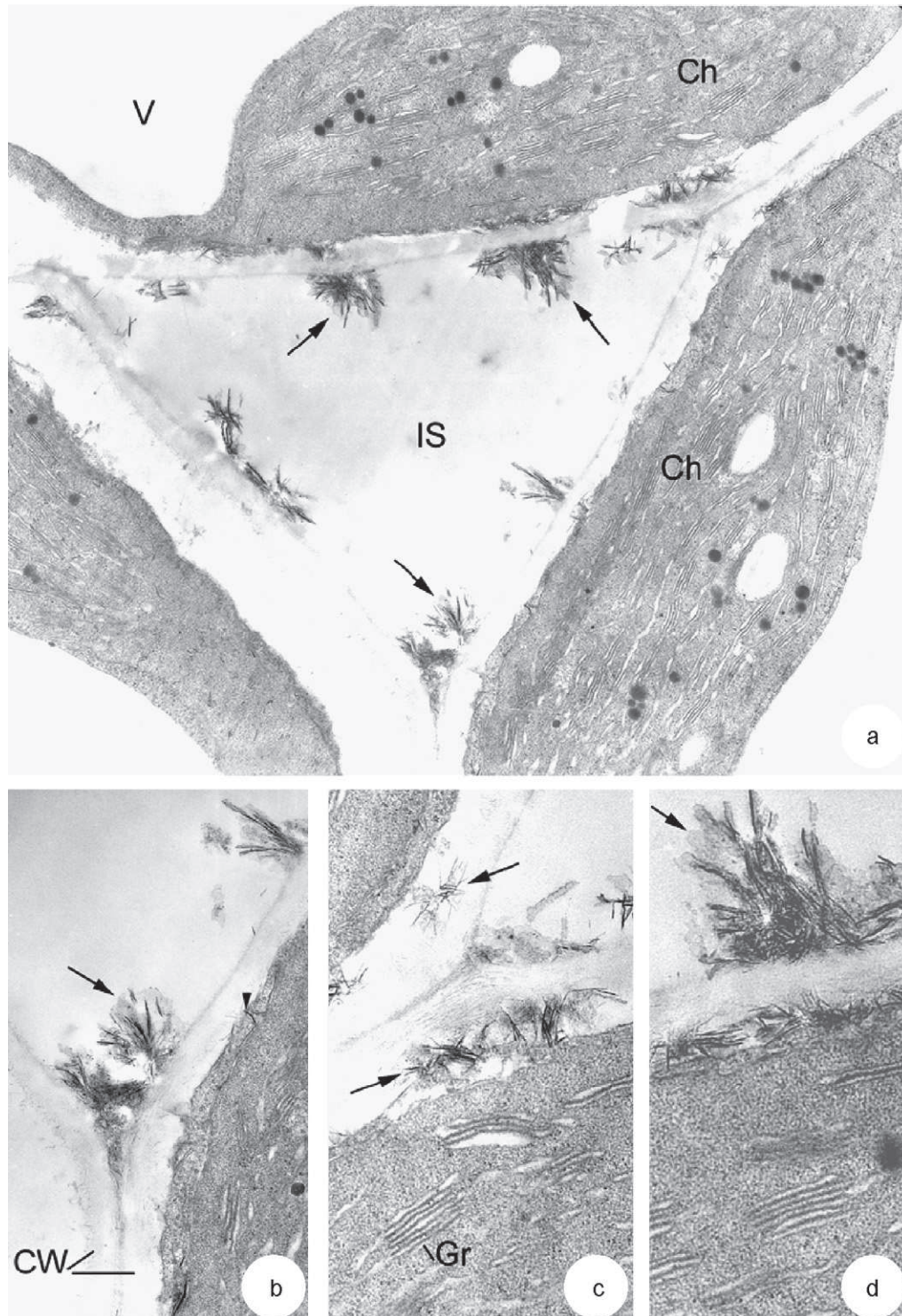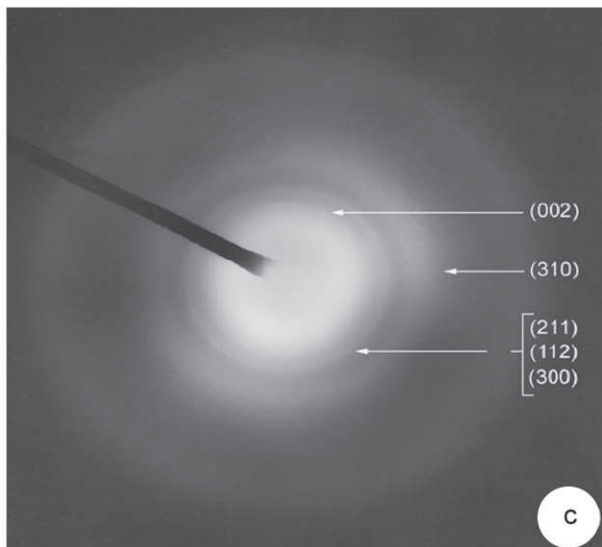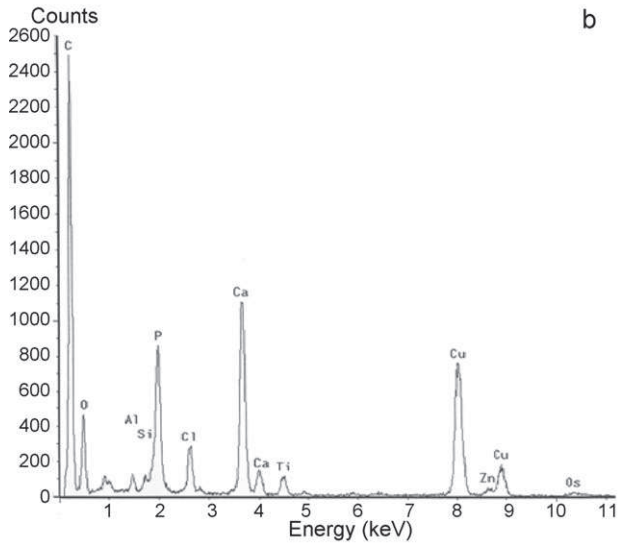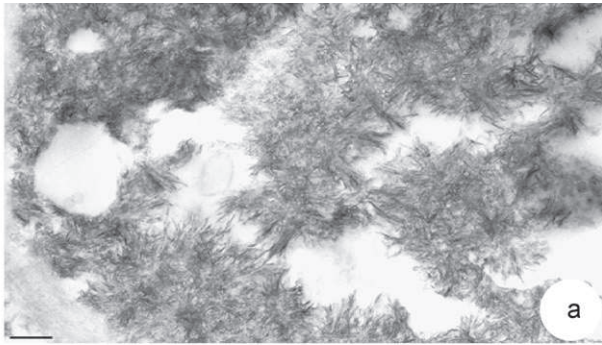
Fig. 6. Crystalline structures in leaf tissues of *V. faba* L. grown on mine tailings. (a) Aggregates of crystalline structures (arrows) on the surface of cell walls at the level of intercellular space (IS). Ch: chloroplast. Bar = μm. (b–d) Distribution of crystalline structures on the surface of cell walls (CW) (b and d), inside the cell wall, in periplasmic spaces (c and d, arrows) and in cytoplasms underlying plasma membrane (b, arrow head). Note the amorphous material surrounding needle-like structures (b and d, arrows). Gr: grana. Bars = μm.

plant growth was reduced for S + T group, whereas non significant other macroscopic morphological disorders were observed during the time of the experiment.

Finally, in the mine tailings group, when both Ca and metal contents were very high, translocation occurred almost in the same way as in the soil group, even if it was proportionally lower in the different tissues. But the concentrations were so high that critical limits were exceeded, and consequently some biological processes were taking place to defend against chemical stress as discussed below.

According to Kim et al. (2002), a protective effect of Ca^{2+} on Pb^{2+} toxicity occurs for rice. It may involve multiple mechanisms including competition at the entry level, and that Pb^{2+} and Cd^{2+} may compete with divalent cations for transport into roots of

Fig. 7. Apatitic structure of crystals in leaf tissues of *V. faba* L. grown on mine tailings. (a) Clusters of crystalline structures inside a cell leaf. Bar = 0.3 μm. (b) Spectrum of X-ray microanalysis from crystals shown on micrograph (a). Note the great peaks of Ca and P. Zn was also detected. Cu peak may be disturbed by the section support. Os and Ti are probably due to sample process. (c) Electron diffraction diagram obtained from crystalline structures shown on micrograph a. The main diffraction rings, which are characteristic of apatitic calcium phosphate are indicated by Miller indices.

rice plants. This process could explain part of Pb storage in roots in these carbonate conditions. But when the critical limit was reached (mine tailing conditions), bioaccumulation of Pb became significant. An increase of Ca concentration under Cd stress would be a possible mechanism for reducing toxic effects of Cd, and a
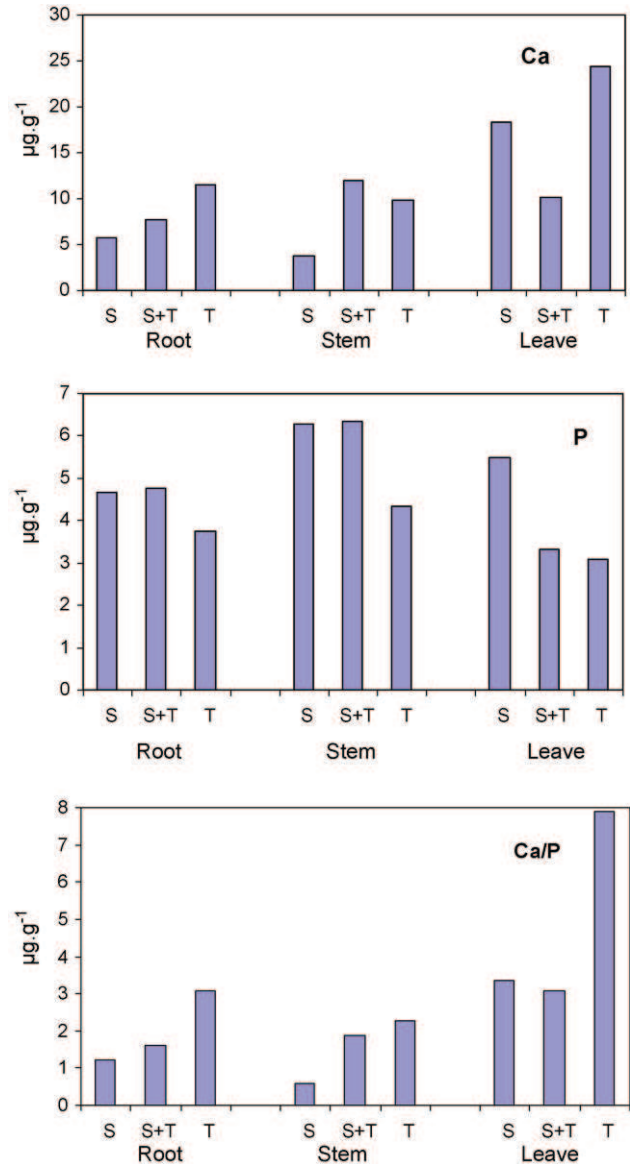
Fig. 8. Ca and P concentrations, and Ca/P ratio in the roots, stems and leaves of *V. faba* L. growing on soil (S), soil mixed with mine tailings (S + T), mine tailings (T).

decrease of Ca concentration under Cd toxicity may be a symptom of a damaged intercellular defence system. Osteras and Greger (2003) have also found interaction between heavy metals and calcium, which explained the observed concentrations of metals in bark and wood. As a whole, in such a carbonate environment, even
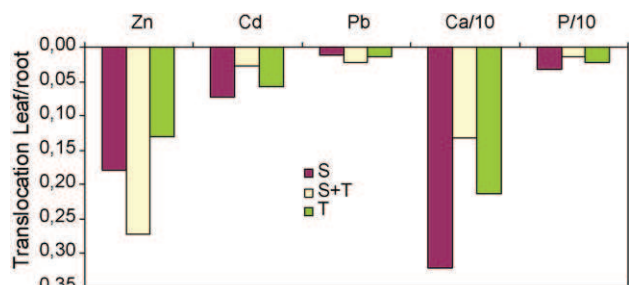
Fig. 9. Translocation of Zn, Cd, Pb, Ca and P from root to leaf (concentration ratio) of *V. faba* L. growing on soil (S), soil mixed with mine tailings (S + T), mine tailings (T). Notice that Ca and P ratio is divided by 10.

if metal concentration was rather high, the metal translocation was probably limited by the competition with calcium, the metal binding to organic matter, and the ability of *V. faba* L. to accumulate metals in roots using some regulating mechanisms of toxicity.

4.2. Damages on V. faba L. as a response to metal contamination: ultrastructural changes and growth reduction

In soils where control plants were grown, total metal concentrations of Pb, Cd, Cu and Zn exceed maximum permitted values for cultivated soils. Indeed in *V. faba* L. growing on soils, the occurrence of dense particles enriched in metals on the surface of cell walls (but not inside the cells of roots or leaves) of the same type of those growing on mine tailings, and the absence of any alteration of cell ultrastructure, suggest that some stratagems of defence already took place regarding such a soil metal content. This characteristic permitted *V. faba* L. to avoid being damaged within a certain threshold value. This value was exceeded in the mine tailings group, where the regulating potential was surpassed, resulting in all these metal concentrations increased strongly in all plant tissues. Results showed that high concentrations of metals in the mine tailings reduced *V. faba* L. growth by 38%. Among the concerned metals, Bolan et al. (2003) have shown that increasing addition of Cd in soils enhanced Cd concentration in plants, resulting in decreased plant growth (i.e. phytotoxicity) and cell ultrastructural changes in both roots and leaves.

In the roots, one of the ultrastructural modifications induced by high-metal content in substrate concerns the increase of cell wall thickness. This is one of the mechanisms that plant could develop to limit metal absorption in roots. Indeed, thickening of cell wall was observed in shoots of *V. faba* L. exposed to Cd or Cu (Liu et al., 2004b) and in marine macroalgus exposed to Cu (Andrade et al., 2004). This phenomenon seems to be associated to increased activity of peroxidase (Liu et al., 2004a, b). This enzyme is able to catalyze lignin synthesis (Arduini et al., 1995) and is induced in higher plants exposed to toxic metals (Prasad, 1996). Roots of *V. faba* L. also showed high amounts of electron-dense particles of metals on the surface and within the cell walls. In the more strongly damaged cells, these particles were found within the cells, where they accumulate at the nuclear and plasma membranes. EDX analyses have shown that these particles were Pb- and Zn-enriched. These observations attest that metals have probably entered within the root cells. Similar Pb deposits have also been shown along plasma membranes of *Sesbania* root cells by Sahi and Sharma (2005).

The high concentrations of metals in mine tailings lead to important ultrastructural modifications in *V. faba* L. leaves as compared to control plants. One of them was the deep alteration of the chloroplasts, which in the final stage led to their lyses. This alteration was manifested in choroplasts by the swelling and the decreasing number of grana thylakoids. Similar chloroplast alterations were observed in cabbage (Molas, 2002), pine and birch (Kukkola et al., 1997, 2000) exposed to high-Ni concentrations, or on maize exposed to Cd and Zn (Jiang et al., 2007). Moreover, the number and size of plastoglobuli in the thylakoids stroma of *V. faba* L. leaves increased for plants grown on mine tailings. It is well known that the number of plastoglobuli increases with stress conditions due to nickel or copper exposure (Hernandez et al., 1995; Kukkola et al., 1997, 2000; Ouzounidou et al., 1997; Molas, 2002; Andrade et al., 2004).

Concretions were the second major ultrastructural modifications observed in the leaf mesophyll of *V. faba* L. grown on mine tailings. They were mainly localized in intercellular spaces and on the surface of the cell wall. These concretions could not be detected in the root cells or in the leaf cells of control plants. They were found as clusters of needle-like crystals surrounded by amorphous material. EDX and electronic diffraction analysis showed that they

Table 5
Element ratios in the hair-like crystals of leaf cells of *V. faba* L. due to metals.

Elements	Counts	Weight (%)	Atom (%)
P–K	8594	39.24	45.68
Ca–K	14,754	59.80	53.79
Zn–K	162	0.96	0.53
Total		100.00	100.00

were Ca–P crystals close to apatite composition (Table 5), which have already been described generally in pathological cases of animal tissues (Hering et al., 1987; Hollande et al., 1990; Cantet et al., 2001). To our knowledge, calcium phosphate crystals have not yet been described in plants. However, some mineral deposition containing phosphate could be detected in plants exposed to metals. Sarret et al. (2001) have observed Zn phosphate in dense granules in the leaves of *Phaseolus vulgaris* treated by Zn and Pb. Zn phosphate deposition has already been described in root or leaf cells of *Thlapsi caerulescens* (Vazquez et al., 1994; Zhao et al., 1998). Moreover, Cd phosphate was observed in the vacuoles of *Chlamydomonas acidophila* (Nishikawa et al., 2003).

In *V. faba* L. the formation of calcium phosphate crystals needs high Ca and P concentrations to allow their precipitation and thus, the question arises on the origin of these elements. In vegetal tissues, calcium is mainly concentrated in cell walls (Demarty et al., 1984) where it plays a role in the structure by forming bridges between the different polysaccharide molecules (Jarvis, 1984). It is thus possible that high concentrations of Zn, Pb and Cd, may substitute for Ca^{2+}. Thus, the Ca^{2+} ions exchanged in roots and stems could be transported in leaves by the apoplastic system. At this step, the concentration would be high enough to allow precipitation and crystallization in the apoplast. The displacement of Ca^{2+} ions from cells to apoplast might also explain the loss of choroplaste structure since Ca^{2+} is known to play a significant role in the maintenance of the thylakoids.

Concerning phosphorus ions, different hypothesis can be proposed. In the vegetal cells, the phytic acid is one of the ways of storage (Ravindran et al., 1994). This acid is usually stored in the cisternae of the endoplasmic reticulum. As soon as plants are exposed to stress such as metals, part of the phytic acid is transported in the vacuoles where it is linked to cations such as metals (Vats and Banerjee, 2004) and the other part is secreted in the intercellular spaces. For *V. faba* L., it can be proposed that the phytic acid, which is produced in the intercellular spaces might be associated to Ca^{2+} ions and thus, induce the formation of phosphate calcium minerals. Another origin of phosphate ions would be the polyphosphate hydrolysis. Indeed, it has been shown that the degradation of polyphosphates to orthophosphates is more important for plant tolerance to metals than the absolute amount of polyphosphates (Van Veen et al., 1994). In *V. faba* L., it can be proposed that the formed orthophosphates could precipitate with high-calcium concentrations and thus control metals. Indeed, Zn was detected in the calcium phosphate crystals. Other metals such as Cd and Pb might be present, but were below EDXM analytic detection limits. The higher Ca/P ratio in leaf cell (8.0) compared to that in crystals (1.3) indicated in the latter the relative increase of P relative to Ca, in relation to mineral precipitation. The role of phosphate has also been evoked to be a significant controlling factor of metal transfer from soil to plant particularly for Cd, Cu, Pb or Zn. Chojnacka et al. (2005) indicated that the higher phosphorus is in soil, the lower the metal is in plants, and more recently the addition of phosphorus to soil decreased the bioavailability of Cd and Zn and chloroplast damage in maize. Indeed, phosphate deposits in the vacuoles were found to complex Cd and Zn (Jiang et al., 2007).

However, the understanding of mechanisms of crystal formation needs further investigations. Particularly, investigations would be

necessary on ultrastructure of leaves and roots of plants growing on the mixed soil and mine tailings substrate, where plants showed alteration of growth but no macroscopic changes and surprisingly low calcium and metal contents in tissue.

5. Conclusion

Experiments were carried out to simulate a field response of *V. faba* L. growing on soil contaminated by metals from carbonate mining wastes. The results showed that *V. faba* L. was able to translocate and accumulate metals in different ways and to develop different stratagems depending on the critical concentration limits and the physico-chemical characteristics of the substrate.

Metal concentrations in plants were significant in plants growing both in enriched soils and waste, particularly for Zn and Mn. But Pb was the most concentrated in all tissue plants growing on the most contaminated substrate. Root was the favourite compartment of metal storage and of bioaccumulation. Bioaccumulation was favoured for Zn and Cd in the context of substrate decreasing pH, whereas calcium and probably organic matter binding regulated metal transfer. This might explain why the plant response to increasing metal concentration in substrate was not linear, contrary to the reduction in plant growth, as observed in the mixed soil–waste substrate where metals were the less concentrated in plant tissues. Phosphorus might also be a limiting factor for plants growing on highly contaminated substrates. Indeed, Ca, P and pH were supposed to be important factors for metal transfer to plant.

Moreover, on mine tailing substrate, the very high metal and calcium content led to significant ultrastructural changes in plant cells compared to soil. On soils, where translocation was favoured, dense particles of metals were stored in the cell walls. This suggests a mechanism of plant defence without cell ultrastructure alteration, within a certain critical limit of metal concentration in substrate. On mine tailings, where metal concentration limits were exceeded, plant growth was reduced by 38% and the cell walls were thickened in roots to limit metal absorption, whereas Pb- and Zn-enriched particles have entered the root damaged cells. In response to high concentrations of toxic metal, phytochelators Pb-complexes might be synthesized in root cells and control metal transport to stems and leaves. Moreover ultrastructure of leaf cells of plants growing on mine tailings was altered particularly for chloroplast and plastogobuli in relation to metal stress. Finally, concretions as Ca–P needle-like crystals close to apatite found in intercellular spaces and at the surface of the cell wall, were identified as another important regulatory mechanism. Zn was detected in those mineral phases, whereas Pb could not be detected probably in relation to analytical detection limit. These crystals are supposed to be the ultimate phase of plant response to such highly exceeded metal and Ca content, before dying.

The formation of such crystals would need more investigations as well as the behaviour of ultrastructure cell response in plants growing on the mixed soil–tailing substrate to conclude accurately on their origin and mechanism of formation.

Indeed, it is important to keep in mind that mixing soil + tailings in those proportions in cultivated mining areas led to low-metal content in *V. faba* L. despite a significant growth reduction. As long as substrate pH remains high, metal bioavailability will be low, but as soon as carbonates are consumed (as enhanced by sulphide oxidation) the mobility of metals and thus their toxicity would be increased for plants.

References

Andrade, L.R., Farina, M., Amado Filho, G.M., 2004. Effects of copper on *Entermorpha flexuosa* (Chlorophyta) in vitro. Ecotoxicology and Environmental Safety 58, 117–125.

Arduini, I., Godbold, D.L., Onnis, A., 1995. Influence of copper on root growth and morphology of *Pinus pinea* L. and *Pinus pinaster* Ait. seedlings. Tree Physiology 15, 411–415.

Bahemuka, T.E., Mubofu, E.B., 1999. Heavy metals in edible green vegetables grown along the sites of the Sinza and Msimbazi rivers in Dar es Salaam, Tanzania. Food Chemistry 66, 63–66.

Bolan, N.S., Adriano, D.C., Duraisamy, P., Mani, A., Arulmozhiselvan, K., 2003. Immobilization and phytoavailability of cadmium in variable charge soils. I. Effect of phosphate addition. Plant and Soil 250 (1), 83–94.

Brunel, C., Munoz, M., Probst, A., 2003. Remobilisation of Zn and Pb in a mountain stream contaminated by mining wastes during a moderate flood event (Ariège, France). J. Phys. IV 107, 233–236.

Cantet, S., Fanjul, M., Brémont, F., Midy, V., Hollande, E., 2001. Cytological characterization of apatitic calcium phosphate structures in bronchial epithelial tissue cultured from a child with cystic fibrosis (ΔF508). Virchows Archives 439, 683–690.

Carrillo-Chavez, A., Morton-Bermea, O., Gonzalez-Partida, E., Rivas-Solorzano, H., Oesler, G., Garcia-Meza, V., Hernandez, E., Morales, P., Cienfuegos, E., 2003. Environmental geochemistry of the Guanajuato Mining District, Mexico. Ore Geology Reviews 23, 277–297.

Chen, Y.X., He, Y.F., Yang, Y., Yu, Y.L., Zheng, S.J., Tian, G.M., Luo, Y.M., Wong, M.H., 2003. Effect of cadmium on nodulation and N_2-fixation of soybean in contaminated soils. Chemosphere 50 (6), 781–787.

Chojnacka, K., Chojnacki, A., Gorecka, H., Gorecki, H., 2005. Bioavailability of heavy metals from polluted soils to plants. Science of the Total Environment 337, 175–182.

Conesa, H.M., García, G., Faz, A., Arnaldos, R., 2007. Dynamics of metal tolerant plant communities development in mine tailings from the Cartagena-La Unión Mining District (SE Spain) and their interest for further revegetation purposes. Chemosphere 68 (6), 1180–1185.

Demarty, M., Morvan, C., Thellier, M., 1984. Calcium and the cell wall. Plant, Cell and Environment 7, 441–448.

Gûleryûz, G., Arslan, H., Kirmizi, S., Gûçer, S., 2002. Investigation of influence of tungsten wastes on the elemental composition of some alpine and subalpine plants on Mount Uludag, Bursa, Turkey. Environmental Pollution 120, 707–716.

Hering, F., Briellmann, T., Luond, G., Guggenheim, H., Seiler, H., Rutishauser, G., 1987. Stone formation in human kidney. Urological Research 15, 67–73.

Hernandez, L., Probst, A., Probst, J.-L., Ulrich, E., 2003. Heavy metal distribution in some French forest soils: evidence for atmospheric contamination. The Science of Total Environment 312, 195–219.

Hernandez, J.A., Olmos, E., Corpas, F.J., Sevilla, F., del Rio, L.A., 1995. Salt-induced oxidative stress in chloroplasts of pea plants. Plant Science 105, 151–167.

Hollande, E., Levrat di-Donato, J.H., Fanjul, M., Palévody, C., Puech, J., Ratovo, G., 1990. Calcium phosphate deposits in domes of human pancreatic adenocarcinoma cell Capan-1 cultures. Biology of the Cell 69, 191–203.

Jarvis, M.C., 1984. Structure and properties of pectin gels in plant cell walls. Plant, Cell and Environment 7, 153–164.

Jiang, H.M., Yang, J.C., Zhang, J.F., 2007. Effect of external phosphorus on the cell ultrastructure and the chlorophyll content of maize under cadmium and zinc stress. Environmental Pollution 147, 750–756.

Kabata-Pendias, A., Pendias, H., 1992. Trace Elements in Soils and Plants, 2nd ed. CRC Press, London, p. 413.

Kim, M.J., Ahn, K.H., Jung, Y., Lee, S., Lim, B.R., 2003. Arsenic, cadmium, chromium, copper, lead, and zinc contamination in mine tailings and nearby streams of three abandoned mines from Korea. Bulletin of Environmental Contamination and Toxicology 70, 942–947.

Kim, Y.Y., Yang, Y.Y., Lee, Y., 2002. Pb and Cd uptake in rice roots. Physiologia Plantarum 116 (3), 368–372.

Kukkola, E., Huttunen, S., Back, J., 1997. Scots pine needle injuries at subarctic industrial sites. Trees 11, 378–387.

Kukkola, E., Rautio, P., Huttunen, S., 2000. Stress indications in copper- and nickel-exposed Scots pine seedlings. Environmental and Experimental Botany 43, 197–210.

Liu, H.Y., Liao, B.H., Zhou, P.H., Yu, P.Z., 2004a. Toxicity of linear alkylbenzene sulfonate and alkylethoxylate to aquatic plants. Bulletin of Environmental Contamination and Toxicity 72 (4), 866–872.

Liu, H.Y., Liao, B.H., Lu, S.Q., 2004b. Toxicity of surfactant, acid rain and Cd^{2+} combined pollution to the nucleus of *Vicia faba* root tip cells. Chinese Journal of Applied Ecology 15 (3), 493–496.

Liu, H.Y., Probst, A., Liao, B.H., 2005. Heavy metal and As contamination in soils and crops affected by the Chenzhou lead/zinc mine spill (Hunan, China). The Science of the Total Environment 339, 153–166.

Macklin, M.G., Brewer, P.A., Balteanu, D., Coulthard, T.J., Driga, B., Howard, A.J., Zaharia, S., 2003. The long term fate and environmental significance of contaminant metals released by the January and March 2000 mining tilings dam failures in the Maramures County, upper Tisa Basin, Romania. Applied Geochemistry 18, 241–257.

Molas, J., 2002. Changes of chloroplast ultrastructure and total chlorophyll concentration in cabbage leaves caused by excess of organic Ni(II) complex. Environmental and Experimental Botany 47, 115–126.

Nishikawa, K., Yamakoshi, Y., Uemura, I., Tominaga, N., 2003. Ultrastructural changes in *Chlamydomonas acidophila* (Chlorophyta) induced by heavy metals and polyphosphate metabolism. FEMS Microbiology Ecology 44, 253–259.

Osteras, A.H., Greger, M., 2003. Accumulation of, and interactions between, calcium and heavy metals in wood and bark of *Picea abies*. Journal of Plant Nutrition and Soil Science-Zeitschrift fur Pflanzenernahrung und Bodenkunde 166 (2), 246–253.

Ouzounidou, G., Moustakas, M., Eleftheriou, E.P., 1997. Physiological and ultrastructural effects of cadmium on wheat (*Triticum aestivum* L.) leaves. Archives of Environmental Contamination and Toxicology 32, 154–160.

Prasad, T.K., 1996. Mechanism of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: changes in antioxidant system, oxidation of proteins and lipids, and protease actibities. Plant Journal 10 (1), 1017–1026.

Ravindran, V., Ravindran, G., Sivagolan, S., 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. Food Chemistry 2, 1333–2136.

Sahi, S.V., Sharma, N.C., 2005. Phytoremediation of lead. In: Shtangeeva, I. (Ed.), Trace and Ultratrace Elements in Plants and Soils. Series Advances in Ecological Researches. Witpress, Southampton, Boston, pp. 209–222.

Salvarredy-Aranguren, M.M., Probst, A., Roulet, M., Isaure, M.P., 2008. Contamination of surface waters by mining wastes in the Milluni Valley (Cordillera Real, Bolivia): mineralogical and hydrological influences. Applied Geochemistry 23 (5), 1299–1324.

Sarret, G., Vangronsveld, J., Manceau, A., Musso, M., Haen, J., Menthonnex, J.-J., Hazemann, J.-L., 2001. Accumulation form of Zn and Pb in *Phaseolus vulgaris* in the presence and absence of EDTA. Environmental Science and Technology 35, 2854–2859.

Stolt, J.P., Sneller, F.E.C., Bryngelsson, T., Lundborg, T., Schat, H., 2003. Phytochelatin and cadmium accumulation in wheat. Environmental and Experimental Botany 49, 21–28.

Türkdogan, M.K., Kilicel, F., Kara, K., Tuncer, I., Uygan, I., 2003. Heavy metals in soil, vegetables and fruits in the endemic upper gastrointestinal cancer region of Turkey. Environmental Toxicology and Pharmacology 13 (3), 175–179.

Van Veen, H.W., Abee, T., Kortstee, G.J.J., Periera, H., 1994. Generation of proton motive force by the excretion of metal-phosphate in the polyphosphate-accumulating *Acinetobacter johnsonii* strain 210A. Journal of Biological Chemistry 269, 29509–29514.

Vats, P., Banerjee, U.C., 2004. Production studies and catalytic properties of phythases (*myo*-inositolhexakisphosphate phosphohydrolases): an overview. Enzyme and Microbial Technology 35 (1), 3–14.

Vazquez, M.D., Poschenrieder, C., Barcelo, J., 1994. Compartmentation of zinc in roots and leaves of the zinc hyperaccumulator *Thlaspi caerulescens*. Botanica Acta 107, 243–250.

Vogel-Mikuš, K., Drobne, D., Regvar, M., 2005. Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonisation of pennycres *Thlapi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. Environmental Pollution 133, 233–242.

Vogeli-Lange, R., Wagner, G.J., 1990. Subcellular localization of cadmium and cadmium-binding peptides in Tobacco leaves: implication of a transport function for cadmium-binding peptides. Plant Physiology 92, 1086–1093.

Wang, W.S., Shan, X.Q., Wen, B., Zhang, S.Z., 2003. Relationship between the extractable metals from soils and metals taken up by maize roots and shoots. Chemosphere 53, 523–530.

Weis, J.S., Weis, P., 2004. Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. Chemosphere 30 (5), 685–700.

Yang, B., Shu, W.S., Ye, Z.H., Lan, C.Y., Wong, M.H., 2003. Growth and metal accumulation in vetiver and two *Sesbania* species on lead/zinc mine tailings. Chemosphere 52, 1593–1600.

Zhao, F.J., Shen, Z.G., McGrath, S.P., 1998. Solubility of zinc and interactions between zinc and phosphorus in the hyperaccumulator *Thlaspi caerulescens*. Plant, Cell and Environment 21, 108–114.