Bioindication of heavy metal contamination in vegetable gardens

David Martin¹, Pierre Vollenweider¹⁎, Alexandre Buttler² and Madeleine S. Günthardt-Goerg¹

¹ Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Zürcherstrasse 111, CH-8903 Birmensdorf, Switzerland. david.martin.mail@gmail.com, pierre.vollenweider@wsl.ch, madeleine.goerg@wsl.ch
² WSL and Ecole Polytechnique Fédérale de Lausanne – EPFL station 2, CH-1015 Lausanne, Switzerland. alexandre.buttler@epfl.ch
⁎ Corresponding author

Abstract
Private vegetable gardens are often contaminated with elevated levels of heavy metals (HM). Bioindication, using the visible symptoms developing in leaves, can provide simple, cost-effective and reliable detection of soil pollution. In this experiment five species of vegetables from different families were exposed to soil contaminated with HM dust given as copper/zinc/cadmium/lead = 640/3000/10/90 mg kg⁻¹ vs. uncontaminated control soil (copper/zinc/cadmium/lead = 28/97/0.1/37 mg kg⁻¹) to study HM uptake and stress reactions and validate markers of HM intoxication within each species. Each vegetable reacted specifically to the HM exposure. Characteristic leaf symptoms were found in Raphanus and Phaseolus associated with a reduced shoot:root biomass ratio, higher content of HM and cytochemical localisation of zinc matching reactions for stress, defence and detoxification. They were primarily attributed to the direct effects of zinc and the mixed indirect effects of all HM. Thus, two out of the five species showed a usable and promising bioindication potential to monitor HM pollution in vegetable gardens. In urbanized environments, bioindication using vegetables showing leaf symptoms can help to raise public awareness regarding food safety.

Keywords: heavy metal, soil, bioindication, plant response, tolerance, visible symptoms

1 Introduction

In Switzerland, private vegetable gardens are cultivated on approximately 50000 hectares of land divided into 900000 plots and according to the Swiss Federal Office for the Environment (FOEN), land use abuse (also with pesticides) and pollution with heavy metals (HM) are of worrying concern (HÖRLER 1992). Small cultivated surfaces and pollution variability make monitoring and risk assessment difficult. Bioindication techniques based on the recognition of visible symptoms in leaves provide simple, efficient and cost-effective methods for the identification of nutritional disorders (VOLLENWEIDER and GÜNTHARDT-GOERG 2005; KABATA-PENDIAS and PENDIAS 2001, especially Table 38, p. 87 and Table 40, p. 91; MARSCHNER 1995, especially Table 12.1, p. 462) and can help to raise public awareness regarding pollution issues (GARREC and VAN HALUWYN 2002). According to KABATA-PENDIAS and PENDIAS (2001), visible symptoms of HM toxicity vary within each species and even for individual plants. Those most commonly mentioned regarding shoots and leaves include stunting, chlorosis and discoloured dots or flecks (KABATA-PENDIAS and PENDIAS 2001; SANITÀ DI TOPPI and GABBRIELLI 1999). Symptom morphology and its distribution at leaf, twig and shoot level provide important diagnostic features (VOLLENWEIDER and
GÜNTHARDT-GOERG 2005; MARSCHNER 1995). Symptom validation based on the micro-localisation of metal contaminants and the characterization of plant physiological and structural reactions (VOLLENWEIDER et al. 2006) together with additional information on environmental conditions (MARSCHNER 1995) are helpful to increase the accuracy of visual diagnosis.

In this study, the reactions of vegetables to HM contamination were investigated experimentally. HM uptake, allocation, effects and symptoms in five species from different plant families were compared. Leaf symptoms and stress reactions were validated using microscopical analyses. Potential of leaf visible symptoms for the purpose of bioindication was evaluated by analysing the different plant responses.

2 Material and methods

2.1 Treatments and growth conditions

Between three and twenty seeds, according to species, from Allium schoenoprasum L., Lactuca sativa L., Phaseolus vulgaris L., Raphanus sativus L. with two cultivars, and Spinacia oleracea L. (supplied by Samen Mauser, Winterthur) were planted in 0.9 l pots filled with an agricultural topsoil (pH. 6.4) from the Swiss midland region with or without mixed HM contamination (10 pots each per species and treatment). This soil showed no nutrient deficiency (HERMLE et al. 2006; see this reference for soil physical and chemical properties) and was not fertilised prior to or during the experimentation. The HM contamination was established experimentally mixing dust from the filters of a non ferrous smelter to the soil which resulted in copper (Cu)/zinc (Zn)/cadmium (Cd)/lead (Pb) = 640/3000/10/90 mgkg⁻¹ of which 40 % (Cu), 70 % (Zn), 85 % (Cd), and 10 % (Pb) was either mobile or easily mobilizable (NOWACK et al. 2006). This level of soil contamination and HM bio-availability clearly exceeded Swiss VBBo limits¹ but remained partly below levels found at different polluted sites throughout Europe (DICKINSON 2000; HORVATH and GRUIZ 1996; ERNST 1972). It thus reproduced in a realistic way soil pollution levels existing at “brown field” sites. The control treatment (CO) without HM contamination contained: Cu / Zn / Cd / Pb = 28 / 97 / 0.1 / 37 mgkg⁻¹. Plants were cultivated from September to December 2004 in a greenhouse with the following growth conditions: night/day: 11/13 hours, 0/200 W/m², 16/18°C, 85 %/60 % relative humidity. Pots were arranged in rows by species to simulate conditions in a normal vegetable garden and to monitor individual as well as overall plant development. The water supply from below was standardised between plants, species and treatments by placing all pots on the same 1 cm thick and water-saturated felt mat. When competition within a given species was signalled by close contact between shoots, the plants were systematically thinned to reduce the plant density per pot in a similar way throughout both treatments.

2.2 Methods

Visible leaf symptoms were monitored as soon as the first leaves had fully developed (3rd to 4th week of treatment). Leaf sampling for microscopical analysis occurred shortly before total plant harvest (12th week). Leaf disks of one cm diameter were excised and either im-

¹ Verordnung über Belastungen des Bodens (VBBo, 1998) : Cu/Zn/Cd/Pb = 150/300/2/200 mg/kg.
Immediately analysed under the microscope or fixed by infiltration in 2.5 % glutaraldehyde (buffered at pH 7.0 with 0.067 M Sørensen phosphate buffer) under evacuation before storing at 4°C. Upon harvest, plants were separated into shoots and roots. After carefully removing soil particles manually, roots were washed in demineralised water and a root aliquot was fixed and stored in view of microscopical analysis, using the same procedure as for the leaves. Root and shoot parts were weighed after drying for at least 12 hours at 65°C and the mean single plant mass per pot was determined. For elemental analyses, roots and shoots from 3 pots each per species and treatment were pooled (3 pots = 1 sample), milled (ultra centrifuge mill coated with wolfram carbide), digested with a high pressure microwave system (UltraClav by Milstone: 240°C, 120 bar) in a mixture of HNO3 (65 %) and HF (40 %) and analysed in duplicates (spread < 10 %) by ICP-AES (Optima 3000 by Perkin Elmer). All tests were carried out according to ISO (International Standard Organisation) 17025. Zinc was micro-localised cytochemically according to VOLLENWEIDER et al. (2005); changes in the cell and tissue structure and the physiological responses to HM stress were characterised as in VOLLENWEIDER et al. (2003 and 2006). The statistical significance was tested using ANOVA (SAS Institute Inc., Cary NC).

3 Results

3.1 Visible symptoms and biomass reduction

Each of the five tested species reacted in a species-specific way to the HM contamination. Two species (Raphanus, and Phaseolus) showed characteristic HM leaf symptoms in the form of early occurring depressed and bleached leaf blade necrosis on older leaves (Fig. 1B vs. A) or gradients of vein browning culminating at the vein basis and increasing with leaf age in trifoliate leaves (Fig. 2B vs. A), respectively. Symptom localisation matched the micro-localisation of Zn (see 3.3). The other species showed either unspecific (chlorosis in Allium) or no symptoms (Lactuca and Spinacia) (not shown). The two Raphanus cultivars showed differences in symptom intensity only. The HM exposure significantly reduced the growth in all species and the biomass of the HM-treated plants upon harvesting reached only 12.6 % in the worst (Spinacia) and 56.1 % in the best case (Allium) of that accumulated in the meantime by control plants (Fig. 3). Spinacia thus suffered the largest biomass reduction followed by Raphanus, Lactuca, Phaseolus and Allium. Shoots were more affected than roots in Allium, Raphanus and Phaseolus as shown by reduction in shoot:root biomass ratio amounting to 45 %, 25 % and 23 % respectively whereas the root biomass was more severely reduced than that of shoot in Spinacia and Lactuca (38 % and 9 % increase in shoot:root biomass ratio of HM vs. CO treatment).
Fig. 1. Caption see page 174.
Fig. 3. Root and shoot biomass of control and HM-treated plants from 5 different vegetable species (*Phaseolus*: shoots including pods; *Raphanus* roots including the still small radish) after 12 weeks exposure to mixed HM contamination ( Means + SE, N = 9). Species are ordered according to decreasing biomass reduction in the HM treatment. All means are significantly different in the HM vs. the control (CO) treatment for both roots and shoots as well as for shoot vs. root within each species and treatment (*P* < 0.0001).

Fig. 1, p. 172. Visible symptoms (B vs. A), micro-localisation of Zn in the leaf (C-I, N, O) and stress reactions (G-M) in *Raphanus* after a 12 week exposure to mixed HM. B vs. A visible symptoms included leaf chlorosis and necrotic flecks (arrows). D vs. C micro-localisation of Zn in cortical parenchyma (coP), xylem (xyl) and phloem (phlo) and (F vs. E) throughout the leaf blade tissues next to a necrotic fleck. H vs. G cells inside necrosis (nec) completely collapsed, oxidized and showed large accumulation of Zn (detail in I). O vs. N inside cells, Zn often accumulated in chloroplasts (chl). L-M vs. J-K conducting fine roots showed few stress and defence reactions. L vs. J cell walls were not thickened by HM exposure (orange tones indicate pectins). M vs. K the most prominent change was found in pericycle (per) which was partly collapsed. Abbreviations: Uep: upper epidermis; meso: mesophyll; Lep: lower epidermis; ve: vein; end: endodermis; cc: central cylinder; cor: cortex. Bars: 50 μm (C-H, J, L); 20 μm (I, K, M); 10 μm (N, O).

Fig. 2, p. 173. Leaf visible symptoms (B vs. A), micro-localisation of Zn in the leaf (E, F) and stress reactions (D, H, K, L vs. C, G, I, J) in *Phaseolus* after a 12 weeks exposure to mixed HM. B vs. A visible symptoms included leaf chlorosis and gradients of browning (brown spots) along veins in older trifoliate leaves (arrows). D vs. C browning (♦) consecutive to oxidation of cell wall and cell content at the junction between the leaf vein and leaf blade. E, F micro-localisation of Zn in HM treated samples. Most signals were found in secretory hairs at the leaf surface (F; Zn accumulation is indicated by the pink staining of cell walls). H vs. G thickening of cell walls with lignin-like material in different vein tissues; the red autofluorescence of chlorophyll is reduced. K, L vs. I absorbing fine roots from the HM treatment showing sizeable stress and defence reactions. Cell walls in exodermis (exo) and cortex (cor) were thickened with pectins (orange tones) and polyphenolic material (green-bluish tones) and cells tended to collapse; nuclei were frequently condensed (cn). L vs. J traumatic zones with thickening of cell walls appeared in central cylinder (cc) and probably resulted from divisions in the pericycle (per) layer. Abbreviations: adcol and abcol: ad- and abaxial collenchyma; scler: sclerenchyma; xyl: xylem; phlo: phloem; end: endodermis. Bars: 50 μm (C-E, G, H), 20 μm (I, K), 10 μm (F, J, L).
3.2 HM uptake and allocation

As shown in Fig. 4A-D, HM absorption and translocation varied considerably between species (no analyses available for Spinacia because of small biomass). The HM treatment significantly increased the Zn, Cd and Cu concentration in both root and shoot parts of all tested species (vs. the control) with the exception of Cd (no significant increase) in the shoots of Allium and Cu in the roots of Raphanus and Allium. In the controls, Cd was either below or near the detection limit of 0.6 mg kg\(^{-1}\) dry mass. Lead remained below the detection limit of 3 mg kg\(^{-1}\) dry mass in Raphanus and in the shoots of all other species. Raphanus was exceptional in allocating more Zn, Cd and Cu to the shoot than to the root and, regarding Zn, even in the control treatment. At species level, the HM treatment increased the Zn concentration in the shoots of Raphanus > Phaseolus = Allium > Lactuca (control similarly Raphanus = Phaseolus > Allium = Lactuca), but in the roots the sequence was dissimilar for Raphanus: Phaseolus > Allium = Lactuca > Raphanus (Fig. 4A). The increase in the concentration of copper followed a rather similar pattern to that observed in the case of Zn (Fig. 4C). Lead concentration was also highest in Phaseolus > Lactuca > Allium > Phaseolus, but highest in its roots (Phaseolus > Allium > Lactuca > Raphanus) (Fig. 4D). Lead concentration was also highest in Phaseolus > Lactuca > Allium > Raphanus (Fig. 4D). As indicated in Fig. 4 and according to Kabata-Pendas and Pendas (2001, Tab 36, p. 83), leaf toxicity was clearly exceeded (or close to the toxicity limit) for (1) Zn in roots of all species and shoots of Raphanus, Phaseolus, Allium (and Lactuca), (2) Cu in roots of Phaseolus, Allium and Lactuca and shoots of Raphanus, (Allium and Phaseolus), (3) Cd (in roots of Phaseolus, Allium and Lactuca and shoots of Raphanus and Lactuca) and (4) lead (in roots of Phaseolus). No Zn or Cu deficiency was noticed in plants from the control treatment.

3.3 Structural injuries

Microscopical investigation focused on Raphanus and Phaseolus since they showed the most characteristic leaf symptoms of HM contamination and their shoots contained elevated levels of HM. In Raphanus, a strong cytochemical Zn signal was found in (1) the leaf vein (adaxial cortical parenchyma, xylem and phloem; Fig. 1D vs. C), (2) the leaf blade (upper and lower epidermis and mesophyll; Fig. 1F vs. E) and (3) leaf hairs (not shown) of HM-treated samples, whereas control plants showed no signal (Fig. 1C, E, G, N). Zinc was especially abundant in collapsed and necrotic leaf blade sections (Fig. 1H, I). In the mesophyll, Zn was frequently found in chloroplasts (Fig. 1O vs. N). Beside necrosis, only a few other stress reactions were observed in leaves (not shown) and fine roots (Fig. 1L, M vs. J, K), notably a partial collapse of pericycle cells together with an apparent reduction in cell division (Fig. 1M vs. K).

In Phaseolus, Zn was micro-localised in HM-treated samples only – mainly where the leaf blade branched onto the main vein (Fig. 2E). Zinc often accumulated in secretory hairs on the leaf surface (Fig. 2F). Oxidation of cell walls and cell content (Fig. 2D vs. C) also occurred at the junction of the leaf blade and vein, where Zn was micro-localised (Fig. 2E), and was responsible for the formation of brown dots on the upper leaf surface (arrows in Fig. 2B). Thickening of cell walls with lignin-like material (Fig. 2H vs. G) was detected in oxidised tissues. Moderate injuries were found in the leaf blade and included the degradation of chlorophyll (Fig. 2H vs. G) and the accumulation of starch (not shown). The different structural changes gave indications on the underlying physiological processes triggered by the HM exposure: vein browning and cell wall thickening revealed oxidative
Fig. 4. Concentrations of Zn, Cd, Cu and Pb (mg kg⁻¹ dry mass) in roots and shoots of 4 different vegetable species (*Phaseolus* shoots without pods; *Raphanus* roots including the still small radish), after 12 weeks exposure to mixed HM contaminated or control (CO) soil (Means + SE, N = 3). Lower and upper leaf deficiency and toxicity limits according to KABATA-PENDIAS and PENDIAS (2001, Table 36, p. 83) are indicated with dashed and solid line respectively. Significance of the HM vs. CO treatment
within the same species and organ is indicated by * \((P < 0.05)\), ** \((P < 0.01)\) and *** \((P < 0.001)\). Significant difference of the means for shoots vs. roots in the same treatment and species is indicated similarly by +. Significant difference to another species within the same treatment and organ is indicated as R (Raphanus), L (Lactuca), P (Phaseolus) and A (Allium) for significance at \(P < 0.05\).
stress and changes to the chloroplast structure showed signs of accelerated senescence (VOLLENWEIDER et al. 2006). In fine roots, structural injuries were observed in deeper tissue layers in the absorbing (Fig. 2K, L vs. I, J) rather than in the conducting zone (not shown). In exodermis and cortex, they included partial cell collapse, cell wall thickening with pectins and polyphenols and degeneration of cell content. In the central cylinder, traumatic zones developed in the endodermis and pericycle of the absorbing zone only (Fig. 2L vs. J) whereas no structural change was detected in conducting root segments.

4 Discussion

This study systematically documents the important differences existing in HM uptake, allocation and stress reactions between several species from different plant families, in conformity with the already published evidences (KABATA-PENDIAS and PENDIAS 2001; PAGE et al. 1981). Visible symptoms were representative of those occurring with HM stress (KABATA-PENDIAS and PENDIAS 2001). The necrosis and the underlying changes in the cell structure of *Raphanus* suggested hypersensitive-like reactions (HR-like; VOLLENWEIDER et al. 2003) as the cause. Such stress effects result from cascade chains of oxidative stress reactions and can be elicited by different HM (DIETZ et al. 1999). Gradients of browning along veins increasing with leaf age in *Phaeolus* leaves were typical for an abiotic soil-borne stress factor (VOLLENWEIDER and GÜNTHARDT-GOERG 2005; MARSchNER 1995) and thus provided a clear bioindication of HM stress. The three species with characteristic (*Raphanus*, *Phaseolus*) or unspecific (*Allium*) visible symptoms were also those showing a reduced shoot:root biomass ratio and a Zn (*Raphanus* also Cu) concentration in the shoots above the upper limit of leaf toxicity. It strongly suggests that these metals directly impaired leaf physiology. The HM content in each plant organ upon harvest considerably varied between species but the relative concentration in root and shoot of each HM (with large root to shoot translocation for Zn and Cd, moderate for Cu and undetectable for Pb) showed consistent patterns with previous descriptions (SIEDLECKA 1995). In synthesis, the characteristic bioindications of HM effects as indicated by the visible leaf symptoms in *Phaseolus* and *Raphanus* can be in priority attributed to the effects of Zn and also Cu in shoots, whereas combined effects of several HM in the rhizosphere could contribute to unspecific symptoms such as stunting, chlorosis and biomass reduction in above-ground plant parts (VOLLENWEIDER et al. 2006).

Zinc micro-localisation matched that of the associated stress reactions. The strongest signal was detected in *Raphanus* where Zn accumulation in chloroplasts could significantly increase oxidative stress. Zn is an abiotic elicit of free radicals and reactive oxygen species (FR & ROS; DIETZ et al. 1999) while the photosynthetic electron transport system in chloroplast is the major source of ROS in plants (YAMASAKI et al. 1997). The overflow of the continuously running detoxification system inside chloroplasts and the whole cell (HIPPELI and ELSTNER 1996) caused by an additional source of FR and ROS can significantly contribute in initiating HR-like (RAO et al. 2000; SANDERMANN 1996) whose occurrence here is strongly suggested by the structural changes detected inside leaf necrosis. The lack of defence reactions in roots and leaves probably indicates that HM accumulated with little control, which resulted in serious injuries. Copper (and in a lesser way Cd) could also contribute in increasing stress in leaves if their micro-localisation matched that of Zn (not checked). In *Phaseolus* the role of Zn was also prominent in comparison to other HM, particularly since the Cd and Cu level in shoots did not reach or equalled the lower toxicity limit, respectively. In contrast to *Raphanus*, active detoxification and defence reactions were found in roots and
leaves of *Phaseolus*: Zn was sequestrated in and excreted through secretory hairs as observed for tobacco (*Choi et al.* 2001); cell wall thickening with lignin-like material, together with oxidation traces, suggested the involvement of different antioxidative enzymes in the apoplast and symplast, including peroxydases, and detoxification of ROS like H$_2$O$_2$ (*Gratao et al.* 2005; *Romero-Puertas et al.* 2004); sizable structural changes were also found inside roots together with the highest HM content measured in any plant part during this experiment. Structural changes in HM-treated *Phaseolus* thus suggest that 1) physiological and structural reactions inside roots contributed in limiting exportation of Zn to the shoot and 2) stress in leaves was alleviated by Zn excretion through secretory hairs and detoxification of oxidative stress. These reactions could further mitigate the stress caused by Cd and Cu: at least the translocation of these HM thus appears reduced judging from the large differences between the shoot and root concentrations of both metals.

In conclusion, this study shows that different species of vegetables subjected to similar growth conditions and HM exposure react differently as a consequence of their species-specific metal uptake, allocation and tolerance. This variability of plant response to HM exposure is expected to be larger in outdoor vegetable gardens as a consequence of the heterogeneities in soil contamination and growth conditions. The selection of a few bio-indicators to control site and vegetation contamination by HM appears therefore more promising than monitoring all species growing in a vegetable garden. Common vegetable crops can develop specific symptoms in their leaves even if exposed to realistic amounts of HM. *Phaseolus* and *Raphanus* were found to be especially well suited for revealing Zn and bioindicate a mixed HM contamination, as often occurs in polluted soils. To ascertain bioindication specificity, the appropriate species should be able to translocate high amounts of HM into their leaves and be sensitive to HM stress. Implementing vegetable bioindicators showing leaf symptoms of HM intoxication can help increase awareness to soil contamination issues and strengthen the link between food safety and the public.

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**5 References**


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