Comparative analysis of Cd and Zn impacts on root distribution and morphology of *Lolium perenne* and *Trifolium repens*: implications for phytostabilization

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Comments on the figures :

For figure 1 and 2, I would split the profiles for Lolium and Trifolium for better readability

Abstract

Backgrounds and Aims Plant phytostabilization potential is a direct function of root systems. An experimental design was developed to investigate the impact of Cd and Zn on the root distribution and morphology of *Lolium perenne* and *Trifolium repens*.

Methods Seedlings were transplanted into columns filled with washed quartz and irrigated daily with Cd- or Zn-containing nutrient solutions during one month. Roots were then harvested at different depth and scanned to determine root length density (RLD) and diameter. Pot experiments were also performed to quantify metal, lignin and structural polysaccharides concentrations as well as cell viability.

Results Lolium perenne accumulated Cd and Zn in the roots while *T. repens* was unable to restrict heavy metal translocation. Cadmium and Zn reduced rooting depth and RLD but induced thick adventitious roots in *L.* perenne. This root swelling was related to Cd-induced lignification occurring in exodermis and parenchyma of central cylinder. Hemicellulose and lignin did not play a key role in root metal retention. Cadmium slightly reduced mean root cell viability while Zn increased this parameter in comparison to Cd.

Conclusions Even though plant species like *Lolium perenne* and *Trifolium repens* may seem suitable for a phytostabilization strategy based on their shoot metal tolerance, exposure to toxic heavy metals drastically impairs their root distribution

and could jeopardize the setting up of phytostabilization trials. The metal-induced alterations of root properties differ clearly according to the considered pollutant and plant species.

Keywords

Heavy metal contamination; root distribution; root diameter; lignin and structural polysaccharides; *Lolium perenne*; *Trifolium repens*.

1. Introduction

Urbanization, industrialization and the use of heavy-metal containing amendments in agriculture have resulted in soil contamination with heavy metals in many areas of the world. Among the various remediation technologies applicable to metal-polluted soils (Mulligan et al. 2001), phytostabilization is a low-cost strategy aiming at limiting pollutant dispersion out of the contaminated area (Kidd et al. 2009; Pilon-Smits 2005; Vangronsveld et al. 2009). This management strategy is suitable for sites where the land value is low in comparison to the cost of remediation (Robinson et al., 2009).

Various processes are involved in the control of heavy metal dispersion by means of phytostabilization. Plants may decrease the metal bioavailability in the polluted soil, which can be enhanced through the use of adapted soil amendments (Houben et al. 2012, Kumpiene et al. 2008; Lambrechts et al. 2011; Mench et al. 2003). Plant cover may also reduce contaminant leaching (Houben et al. 2012), as well as stabilize soils and control water and wind erosion (Reubens et al. 2007,

Lambrechts et al., submitted). Plants may help stabilize contaminants by adsorption or accumulation in roots (Vangronsveld et al. 2009), but the translocation to the shoots should remain limited to avoid contaminant transfer to the food chain (Kidd et al. 2009). The selection of adequate plant species is therefore a fundamental aspect for phytostabilization. The selected plants must also be perennial plant-species well-adapted to the local environmental conditions, with a rapid installation, high biomass production, and resistance to pollution (Pilon-Smits 2005). Root biomass and distribution within the soil profile is especially crucial for the long-term maintenance of the plant cover. Moreover, the entire root system architecture will contribute greatly to mechanical soil stabilization and erosion control, thanks to the tensile strength and friction or adhesion properties of single roots and to the morphological characteristics of the root system (Mattia et al. 2005; Reubens et al. 2007).

Several pilot studies and landscape applications of phytostabilization have been performed (e.g., Boisson et al. 2009; Dominguez et al. 2008), but more fundamental research is still needed to better understand the interactions between heavy metals, soil, plant roots and microorganisms in the rhizosphere (Vangronsveld et al. 2009). In particular, the establishment of a plant cover could be jeopardized because of heavy metal phytotoxicity. Chlorosis, necrosis and growth inhibition are common visible symptoms of toxicity (Foy et al. 1978; Kabata-Pendias 2001). Metal impacts on plants are generally ascribed to (1) blocking of the essential biological functional groups of enzymes and/or modification of the active conformation of biomolecules due to metal affinities for thioyl-, histidyl- and carboxyl-groups; (2) displacement of essential cations from specific binding sites, leading to inhibition of enzyme activity; (3) induced oxidative stress by increased reactive oxygen species (ROS) due to perturbation of the mitochondrial and photosynthetic electron transfer chain and

inhibition of the antioxidant defense system; (4) competition with essential nutrients during root uptake; and (5) mutagenic effect. This leads, for instance, to perturbation of the water and nutrient status, and impairment of photosynthesis and selective permeability of cell walls (Broadley et al. 2007; Clemens 2006; Kabata-Pendias 2001; Sharma and Dietz 2009; Verbruggen et al. 2009).

Most studies dealing with heavy metal toxicity and phytostabilization have focused on shoots, although studying roots is of crucial importance, as it is the gateway for heavy metals uptake and the main accumulation compartment for most plant species (Lux et al. 2011). Studying the effects of heavy metals on roots is fraught with difficulties, however. Indeed, roots grow in an opaque medium from which they cannot be extracted or observed without introducing artifacts (Lynch 1995). Moreover, it is almost impossible to avoid root contamination with polluted soil particles when working with real soils. To overcome these difficulties, many scientists have worked with hydroponic cultures or with agar medium (Zhu et al. 2011). These methods are useful for analyzing the impact of heavy metals on root morphology and physiological mechanisms. Heavy metals affect the root system by (1) growth inhibition (Fusconi et al. 2007; Larbi et al. 2002); (2) alteration of the nutrient status (Sandalio et al., 2001); (3) alteration of root anatomy and increased root diameter (Lux et al. 2011); (4) ultrastructural modifications (Sresty and Rao 1999); (5) modification of root architecture by induction of lateral roots (Ďurčeková et al. 2007); and (6) accelerated maturation and lignification (Ederli et al. 2004; Schützendübel et al. 2001). Cell walls, mainly composed of cellulose, hemicellulose and pectins may constitute the main metal accumulation compartment in roots (Deiana et al. 2000; Nishizono et al. 1987). Therefore, the metal-induced modification of their composition may have an impact on both root metal retention and root morphological properties

(Lux et al., 2011; Zhu et al., 2012). However, when working with hydroponic or agar medium conditions, nothing is known about the impact of heavy metals on tri-dimensional root architecture (Zhu et al. 2011). Yet, this knowledge is crucial to assess the phytostabilization potential of a plant species and a possible metal-induced modification of the root distribution, which would affect the soil phytostabilization potential.

In this paper, an experimental design was developed to assess together the impact of heavy metals on root morphology and root architecture at different depths, in order to assess the possible reduction in plant potential to stabilize soil because of metal pollution. The two selected plant species, *Lolium perenne* (perennial ryegrass) and *Trifolium repens* (white clover), exhibit drastically different root systems and were successfully tested during phytostabilization pot experiments (Arienzo et al. 2004; Lopareva-Pohu et al. 2011; Pichtel and Salt 1998; Santibáñez et al. 2008). The study focused on the impact of Cd and Zn, two common metal pollutants in industrial contaminated soils, on root distribution (biomass and length) and morphology (root diameter) with depth. This analysis was carried out for different heavy metal concentrations. The observed alterations of the root system were investigated by physiological measurements regarding lignin and structural polysaccharide contents and cell viability.

2. Materials and methods

2.1. Column experiment - root distribution with depth

The experiment was performed in a growth chamber under fully controlled environmental conditions (16 h photoperiod, 24°C day, 22°C night, relative moisture 80%, light intensity 100 µmol.m^{-2}.s^{-1}). The two selected plant species were *Lolium perenne* cv. Mondial and *Trifolium repens* cv. Alice. Two weeks after sowing in a pot filled with loamy soil, plant seedlings, selected for uniformity, were transferred into PVC columns (1 plant per column) of 55 cm height and 25 cm diameter filled with washed quartz and were allowed to grow for 2 weeks with a daily irrigation of Yoshida's nutrient solution. These columns were previously tested in order to develop an experimental device which allowed study root system with depth without disturbing root architecture setting up. The use of washed quartz enabled to easily recover roots from the substrate and avoid root contamination by particles. Moreover, washed quartz (particle size: 0.3-0.6 mm) exhibited a low compaction hazard in comparison to others substrates. The washed quartz was not added directly into the columns, but poured into a plastic bag inserted into the columns. The bottom of the plastic bag was perforated to allow free drainage of excess water. Moreover, the bottom of the columns was filled up with a 5-cm layer of coarse sand to facilitate drainage and put on a perforated dish. Columns were irrigated each day with 2 l of appropriate nutrient solution (see below). The irrigation volume was adapted in order to add enough nutrients for plant growth and to avoid heterogeneity of the water content with depth, which could strongly affect the root distribution. To avoid splash effects during irrigation, a thin layer of gravel was added at the top of the washed quartz. During preliminary tests, a gradual accumulation of nutrients and heavy metals was detected in the columns. Therefore, pots were flushed weekly with deionized water according to the recommendations of Zobel et al. (2007).

Five different treatments were performed for each plant species, with 3 replicates per treatment. The control treatment consisted of Yoshida's nutrient solution (Yoshida et al. 1976). All the columns received the Yoshida's solution for 2 weeks after seedlings transplantation, and then the treatments were applied for 4 weeks. For the other treatments, Cd or Zn was added to this nutrient solution to achieve the following concentrations: for *Lolium perenne*, Zn 500 µM, Zn 1000 µM, Cd 25 µM, Cd 50 µM; for *Trifolium repens*, Zn 50 µM, Zn 100 µM, Cd 5 µM, Cd 10 µM. These concentrations were selected based on previous hydroponic and pot tests, and showed the lower tolerance of *Trifolium repens* to heavy metals in comparison to *Lolium perenne* (Lambrechts et al. 2013).

After 4 weeks of plant growth, shoots were harvested, weighed and dried at 70°C in an oven for 3 days. Shoot Cd and Zn concentrations were obtained by dissolving 50 mg of shoot dry matter in an open crucible with HNO_3 (AnalaR NORMAPUR 65%). The mixture was gently heated on a hot plate until complete dryness. The residue was dissolved again with aqua regia (HCl AnalaR NORMAPUR 37% and HNO_3 65%) and filtered (Whatman 41). Finally, the solution was diluted to 10 ml with deionized water and analyzed with an AA Spectrometer (Thermo Scientific S Series). After shoot harvest, dishes at the bottom of the columns were replaced by a thick PVC circle. The substrate was then gradually pushed up into the column by means of a hydraulic jack, and 5-cm thick slices were cut with a blade-cutter. Roots were separated from the washed quartz and rinsed with deionized water, then dried with absorbent paper and fresh weighted. Roots were then stored into FAA solution (ethanol 70% - acetic acid 100% - formaldehyde 35%, 18:1:1 by volume) for further analyzes.

Root scanning was performed with a custom flat bed scanner (Medion 3600 DPI) whose scanning window can be filled with water to enable an easier positioning of the roots (Lobet and Draye 2013). Roots were spread out in the water and scanned in positive transparency mode and 8-bit grey scale at 600 dpi. Image analysis was performed with the open source software ImageJ (Abràmoff et al. 2004). A macro was written to routinely determine root length density and root diameter. Briefly, for every images the following steps were perfomed: (1) root object were retrieved using a thresholding algorithm (Kapur et al. 1985), (2) total length was computed based on the skeletonized image (Arganda-Carreras et al. 2010) and (3) root diameter histogram was obtained by creating and euclidian distance map of the root object and combining it with the skeleton (this manipulation yields a skeleton image in which every pixel is equal to the root diameter at its position).

2.2. Pot experiments – physiological measurements

A pot experiment was performed in order to assess heavy metal concentrations in shoots and roots. Pots were smaller than columns (13 cm diameter, 16 cm height). All the experimental conditions were the same as for the columns, but the irrigation volumes were adapted in order to preserve the same solution/pot volumetric ratio as for the column experiment. All the treatments were replicated 5 times. After 4 weeks of growth, the number of tillers was counted for *Lolium perenne*, as well as the numbers of leaves and stolons for *Trifolium repens*. Shoots were harvested and fresh weighed. Roots were separated from quartz, rinsed with deionized water, dried with absorbent paper and fresh weighted. The number of nodules in *Trifolium repens* was

counted for each root system. Shoots and roots were oven dried at 70°C for 3 days, dry-weighed, and the Cd and Zn concentrations were measured as previously described. Translocation factors were calculated as the ratio between the metal concentration in the shoots and concentration in roots.

A second pot experiment was performed next to deepen the analysis of metal impact on roots. All the experimental conditions were identical as for the first pot experiment, with 10 replications per treatment. After 4 weeks of treatment, roots of half of the pots were harvested and put together per treatment, oven dried then crushed with mortar and liquid nitrogen to get fine powder. Concentrations of lignin and structural polysaccharides (cellulose and hemicellulose) were assessed according to Van Soest et al. (1991). Briefly, the crushed plant sample was exposed successively to a neutral detergent solution during 1h at 100°C to obtain by filtration the NDF fraction (neutral detergent fibers), then to an acid detergent solution during 1h at 100°C to get the ADF fraction (acid detergent fibers), and then to sulfuric acid 72% during 3h to obtain the ADL fraction (acid detergent lignin). The ADL fraction was incinerated at 550°C during 3h, and the mass loss allowed us to calculate the percentage of lignin. The difference between ADF and ADL gave the percentage of cellulose, and the difference between NDF and ADF the percentage of hemicelluloses.

For *Lolium perenne*, some roots of the five remaining root systems were randomly harvested for direct measurement of cell viability. The remaining parts of the root systems of *Lolium perenne* and *Trifolium repens* were fixed into FAA solution for further histochemical analyses. Specific measurements of cell viability were

250 performed on freshly harvested *Lolium perenne* apex roots, through the reduction of
251 2,3,5-triphenyltetrazolium chloride (TTC) into formazan (Lutts et al. 2004), with eight
252 replications per treatment. Approximately 50 mg of fresh tissue were excised, quickly
253 rinsed in deionized water containing 0.05% Tween 20 and incubated in darkness in
254 tubes containing 5 ml of 0.5% TTC in 50 mM K_2HPO_4 pH 7.0 for 15 h. Samples were
255 then filtered (Whatman n°4), rinsed with deionized water and incubated in 3 ml
256 ethanol 94% at 80°C during 5 min under gentle agitation (80 rpm). After
257 centrifugation at 5000 g during 1 min, extracted formazan was quantified
258 spectrophotometrically at 487 nm (Shimadzu UV Spectrophotometer UV-1800). The
259 viability index is defined as the absorbance measured per gram of fresh tissue.
260
261 For the root systems stored into FAA solution, cross-sections were performed on
262 random roots at 1 cm below the crown. Selected roots were dehydrated in a graded
263 ethanol series, embedded in paraffin and sectioned at 5 µm with a rotary microtome.
264 Serial cross-sections were stained with safranine-fast green and observed with a light
265 microscope Leica DM500 equipped with a camera Leica ICC50. Analysis of root
266 diameter and lignification percentage was assessed thanks to image analysis
267 software ImageJ. For the lignification percentage, a macro was developed to convert
268 the color image into a binary one based on a fixed threshold, which only selects
269 lignin-colored pixel, and to compare the binary area to the total root area.
270
271 2.3. Statistical analysis
272
273 Data of plant growth and development related parameters, metal concentrations,
274 lignin and structural polysaccharides relative contents, parameters related to cross-

sections and cell viability were statistically analysed through ANOVA 1, considering five metal treatments for each plant species (Student-Newman-Keuls test; SAS System for Windows, version 9.2). For relative contents of lignin, hemicelluloses and cellulose, an arcsinus transformation was applied to obtain normal distribution of the data. Statistical significance was assessed at the 5 % level, with a Welch correction when there was no equality of the variance (Levene's test).

3. Results

3.1. Column experiment – modification of root distribution

The addition of heavy metals into the nutrient solution led to a decrease in the shoot biomass of Cd 50µM and Zn 1000µM in comparison to control plants for *Lolium perenne* (Table 1). Regarding *Trifolium repens*, heavy metal addition did not impact the shoot dry weights in comparison to control plants. The concentrations of Cd and Zn (Table 1) increased in shoots of *Lolium perenne* and *Trifolium repens* as a function of the concentration in the nutrient solution. The differences of shoot concentrations between the low pollutant dose and the highest one were statistically significant for Cd, but not for Zn.

Fig. 1 presents the Root Weight Density (RWD), Root Length Density (RLD) and root diameter depth profiles for *Lolium perenne*. The mean RWDs estimated for the whole column were the following, in descending order: C 0.63^{A} $g.dm^{-3}$, Cd25 0.38^{B} $g.dm^{-3}$, Zn500 0.20^{BC} $g.dm^{-3}$, Cd50 0.09^{C} $g.dm^{-3}$, Zn1000 0.06^{C} $g.dm^{-3}$. Hence, Cd and Zn significantly decreased the root biomass production by *Lolium perenne*. The

analysis of the RWD profile (Fig. 1A) showed that the decrease in root fresh weights was dependent on root depth. Roots were detected until 40 cm depth for control plants, but the maximum root depths decreased for the metal treatments: Cd25 35 cm, Cd50 30 cm, Zn500 25 cm, Zn1000 20 cm. These observations suggest that heavy metals drastically impaired root elongation. Nevertheless, RWDs were also severely affected in the top layer. At this level, the different treatments are ranked as follows: C 0.36^{A} $g.dm^{-3}$, Cd25 0.17^{B}, Zn500 0.16^{B}, Cd50 0.07^{C}, Zn1000 0.05^{C}. Although Cd25 and Zn500 exhibited similar values in the top layer, the RWD between the two treatments differed in the deeper layers: RWD for Zn500 decreased strongly from the first to the second layer, whereas RWD for Cd25 only slightly decreased and remained similar to control values as off 10 cm depth.

Mean RLD values for the whole column were the following, in descending order: C 8.21^{A} $m.dm^{-3}$, Cd25 4.05^{B}, Zn500 2.3^{BC}, Cd50 1.77^{C}, Zn1000 1.13^{C}. These values, as well as Fig. 1B, show that RLD followed the same trend as RWD, except for Cd25. Indeed, for this treatment, the RLD were lower than those of control plants until 20 cm depth, instead of 5 cm for the RWD. The analysis of root diameter (Fig. 1C) showed that the presence of heavy metals induced an increase in the mean root diameter in the top 5-cm layer in comparison to the control plants, which exhibited relatively constant mean root diameters until 35 cm depth. This increase was significant for Cd25, Cd50 and Zn500, but not for Zn1000. However, a higher mean root diameter was only significant for the first 5-cm soil layer in the case of Cd50 and Zn500 treatments, and the root diameters decreased strongly in the deeper layers in comparison to control. This was not the case for Cd25: the mean root diameters were higher than those of control plants until 35 cm depth.

Fig. 2 presents the RWD, RLD and root diameter depth profiles for *Trifolium repens*. Mean RWD values for the whole column were, in descending order: C 0.37^{A} g.dm^{-3}, Zn100 0.26^{AB}, Zn50 0.18^{B}, Cd5 0.18^{B}, Cd10 0.18^{B}. Analysis of Fig. 2A revealed that the trends observed for the whole column could also be detected in the top soil layer, but not deeper. Rooting depth was not affected by the presence of Cd and Zn in the nutrient solutions. The mean RLDs for the whole root systems were the following, in descending order: C 3.23^{A} m.dm^{-3}, Zn100 2.40^{A}, Zn50 2.40^{A}, Cd5 2.29^{A}, Cd10 2.26^{A}. Therefore, Cd and Zn did not significantly affect the total root lengths. As for RWD, differences between treatments were mainly observed in the top substrate layer for Cd but not for Zn (Fig. 2B). Indeed, for this depth, there was a significant difference between Cd treatments and the control. Some peaks of mean root diameter (Fig. 2C) could be detected for the control and Cd treatments between 30 and 40cm depth, but this was not the case for the two Zn treatments. Roots of Zn100 exhibited a higher mean diameter in the first 15 cm, but below this depth the values decreased faster than the other treatments.

3.2. Pot experiment – growth and metal concentrations

For the two considered plant species, shoot biomass was higher than root biomass whatever the considered treatment (Fig. 3A & 3B). This biomass production generally decreased when heavy metals were introduced into the device in comparison with control plants. As far as *Lolium perenne* is concerned (Fig. 3A), root biomass production was generally more affected than shoot ones by the addition of Cd and Zn. Cadmium 50 µM induced a decrease in the shoot and root dry weights. The addition of Zn into the nutrient solution did not significantly affect shoot and root

biomasses. For *Trifolium repens* (Fig. 3B), Cd 10 µM significantly decreased both the shoot and the root dry weights in comparison to the control. On the contrary, Zn did not affect plant biomass, as for *Lolium perenne*. Heavy metals also affected plant development, especially for the Cd treatments in *Trifolium repens* (Fig. 3D & 3E). Indeed, addition of Cd significantly decreased both the number of leaves and stolons in comparison to controls. Zn 50 µM had no impact on plant development, and Zn 100 µM decreased the number of stolons. Cd and Zn treatments had no impact on the number of tillers for *Lolium perenne* (Fig. 3C). Heavy metals affected also the occurrence of root nodules for *Trifolium repens* (Fig. 3F), especially for Cd 10 µM.

The increase in Cd concentration in contaminated nutrient solution led to significantly higher concentrations in both shoot and root tissues of the two plant species (Table 2). A similar trend was observed for Zn, but the increase between Zn 500µM and Zn 1000µM was not significant for *Lolium perenne* roots. Calculation of translocation factors indicated that zinc treatments led to lower values of Zn translocation in comparison to control for *Lolium perenne*, but not for *Trifolium repens*. Moreover, translocation factors were lower for *Lolium perenne* than for *Trifolium repens*.

3.3. Pot experiment – lignin and structural polysaccharides

The analysis of the relative content of lignin, hemicellulose and cellulose were performed for the whole root systems of the two plant species (Fig. 4). Structural polysaccharides and lignin content differed strongly between the controls of the two plant species, especially for cellulose and hemicelluloses. Indeed, their relative

amounts were clearly higher in *Lolium perenne* in comparison to *Trifolium repens*. Zinc excess decreased cellulose, hemicelluloses and lignin content in *Lolium perenne* roots. These modifications were proportional to the exogenous Zn concentration. Cadmium also decreased the root cellulose and hemicellulose content, but lignin remained unaffected. Zn50 and Cd5 treatments did not lead to detectable changes in the lignin and structural polysaccharides contents in *Trifolium repens* in comparison to controls. However, all fractions markedly increased in response to Zn100 and Cd10.

3.4. Pot experiment – cross-sections and cell viability

This complementary experiment focused mainly on *Lolium perenne* roots, which exhibited clearer response to heavy metals than *Trifolium repens*. Fig. 5 shows the results of the analysis of cross-sections performed on randomly selected roots 1 cm below the crown. A significant increase in the root diameter for the metal treatments in comparison to control was observed for *Lolium perenne* (Fig. 5A), confirming the increase in mean root diameter previously recorded in the top layer of the column experiment for the contaminated treatments (Fig. 1C). A visual comparison of the root systems for the different treatments (Fig. 5) allowed complementing the previous observations. The plant crown of Zn500, Zn1000 and Cd50 showed a proliferation of short and thick adventitious roots, which was not observed for Cd25 and control plants. The assessment of root lignification for these roots thanks to safranine staining and image analysis with ImageJ revealed two different trends for Cd and Zn (Fig. 5B). Contamination with Cd led to a local increase in root lignification compared to control plants, whereas Zn decreased the values of this parameter. This Cd-

induced increase in lignification was observed mainly for the exodermis as well as for the parenchyma cells in the central cylinder.

The Cd and Zn induced short adventitious roots were compared to apex roots of control plants in terms of cell viability for *Lolium perenne* (Fig. 6). Zn1000-contaminated root cells led to higher cell viability than the control and Cd-treated roots.

4. Discussion

4.1. Contrasted plant species response to heavy metal exposure

Lolium perenne and *Trifolium repens* exhibited drastically different responses to Cd and Zn (Table 2). Despite lower Cd and Zn concentrations in contaminated nutrient solutions for *Trifolium repens* in comparison to *Lolium perenne* (5 and 10 times less for Cd and Zn, respectively), the accumulation of those two elements in the shoots were within the same range for the two species. Given the higher metal concentrations in root tissues of *Lolium perenne* (3 times more than for *Trifolium repens*), it appears that perennial ryegrass is able to accumulate Cd and Zn mainly in the roots and limit their translocation to the shoots. Therefore, shoot growth and development were not significantly affected by metal contamination in this species (Fig. 3). On the contrary, *Trifolium repens* translocated more metals to the shoots, which impaired shoot development. Yang et al. (1995) observed the same patterns for white clover and perennial ryegrass with Cd hydroponic experiments. Moreover, for the same endogenous metal concentration as used here for *Lolium perenne*,

shoot growth and development of white clover were more affected than ryegrass by metal contamination, especially for Cd. This may be explained by more efficient mechanisms allowing *Lolium perenne* to cope with metal accumulation in shoots tissues. Macnicol and Beckett (1985) collected data from many hydroponic and soil experiments to determine critical tissue concentrations of heavy metals for different plant species, beyond which toxic effects are detected. They indicated critical Cd shoot concentrations of 10 $mg.kg^{-1}$ for white clover and 30-35 $mg.kg^{-1}$ for ryegrass, and for Zn 250-300 $mg.kg^{-1}$ for clover and 370-560 $mg.kg^{-1}$ for ryegrass. These values confirm the lower level of metal tolerance of *Trifolium repens* comparatively to *Lolium perenne*. The lower biomass production of white clover could also be explained by the Cd-induced inhibition of symbiotic nitrogen fixation (Broos et al. 2004), as suggested in the present study with a reduction of the occurrence of root nodules. Bidar et al. (2007, 2009) reported a higher sensitivity to metal-induced oxidative stress in *Lolium perenne* than in *Trifolium repens*. This suggests that for a given plant species, plant behavior could vary depending on the considered cultivar (Grant et al. 2008) or that oxidative stress is not the major cause of growth inhibition, at least in *Lolium perenne*.

4.2. Impact of heavy metals on the root system distribution and morphology

Pot experiment suggested that *Lolium perenne* was suitable for phytostabilization purpose due to its low metal translocation from roots to shoots and its shoot tolerance to heavy metals (Table 2; Fig. 3). Indeed, ryegrass shoots were only significantly affected by exposure to Cd 50µM in comparison to control and could provide an important plant cover to limit pollutant dispersion by water and wind

450 erosion. However, Cd and Zn decreased both the rooting depth and the RLD for this
451 plant species (Fig. 1). Therefore, soil exploration by roots may progressively be
452 limited while increasing heavy metal concentrations, which in turn could lead to
453 nutrient deficiencies and thus affect the durability of the plant cover. Moreover, this
454 induced decrease in the total root length, especially in the top layer of soils, could
455 reduce plant anchorage and the roots' potential to stabilize soils (Reubens et al.
456 2007). Concerning *Trifolium repens,* Cd treatments led to a significant decrease of
457 biomass and RLD in the top soil layers (Fig. 2). Therefore, as for ryegrass, metal
458 exposure also restricts the potential of white clover for phytostabilization. Some metal
459 hyperaccumulator plant species, such as *Thlaspi caerulescens* or *Sedum alfredii,* are
460 able to maintain or increase their root distribution under metal contaminated
461 conditions (Li et al. 2009; Schwartz et al. 1999). However, these plant species always
462 display a low biomass and a shallow root system and are therefore not
463 recommended for phytostabilization (Mench et al. 2010). These observations
464 suggested the requirement, during the development of a phytostabilization strategy,
465 to combine plant installation with the use of adapted amendments to overcome this
466 metal-induced plant growth inhibition (Kumpiene et al. 2008; Lambrechts et al. 2011;
467 Mench et al. 2010).
468
469 The reduction of root depth and RLD in *Lolium perenne* (Fig. 1) was linked to a
470 Cd- and Zn-induced proliferation of short and thick adventitious roots (Fig. 5) which
471 contributed greatly to the observed increase of mean root diameter in the top soil
472 layer and to the drastic decrease of root depth. The reduction of root growth may
473 result from cell death, inhibition of cell division, a decrease in the rate of cell
474 elongation or different processes occurring simultaneously (Delpérée and Lutts 2008;

475 Sresty and Rao 1999). In our work, Zn treatments induced an increase in cell viability
476 in comparison to the control (Fig. 6). Moreover, cell viability was not affected in
477 response to Cd treatments, as observed also by Delpérée and Lutts (2008) with
478 *Solanum lycopersicum* after contamination with Cd 250µM during 14 days. Fusconi
479 et al. (2007) observed with *Pisum sativum* a decrease of cell viability only for Cd
480 250µM, but not for the lower Cd concentrations. They attributed the reduction of plant
481 growth to a decrease of apex length in relation to the inhibition of mitotic activity.
482
483 However, some authors suggested that root growth inhibition could be rather
484 attributed to metal-induced inhibition of cell elongation occurring through several
485 mechanisms. Our work demonstrates that such inhibition was linked with root
486 swelling (Fig. 5). Cytoskeleton, and especially microtubules, constitutes a major
487 target of heavy metals. Přibyl et al. (2005) showed that heavy metals could induce a
488 disintegration of microtubules followed by a steep increase in cell width and a
489 decrease in growth rate for green alga *Spirogyra decimina* exposed to Cd. Heavy
490 metals could also damage cell membrane, as shown by Bidar et al. (2008) for *Lolium*
491 *perenne* and *Trifolium repens*. This loss of membrane integrity would result in turgor
492 loss and inhibition of cell expansion (Poschenrieder and Barceló 2004). However,
493 heavy metals at lower concentrations could also act on cell elongation by decreasing
494 cell wall extensibility without damaging cell membrane. Exposure of *Pinus sylvestris*
495 roots to Cd concentrations exceeding cell detoxification capacity led to accumulation
496 of H_2O_2 because of an imbalance of the redox systems (Schützendübel et al. 2001).
497 H_2O_2 is a signaling intermediate which could increase the mechanical strength and
498 lower the extensibility of plant cell walls, following an abnormal lignification
499 (Ďurčeková et al. 2007; Ederli et al. 2004; Schützendübel et al. 2001). This

500 premature xylogenesis shortened the root elongation zone and therefore reduced
501 root growth (Ďurčeková et al. 2007, Lunáčková et al. 2003).
502
503 Cd-induced lignification was detected in the present study based on the cross-
504 sections of ryegrass roots (Fig. 5). We demonstrate that lignification, linked to root
505 swelling, was localized in *Lolium perenne* in the exodermis and in the parenchyma
506 cells in the central cylinder. Lux et al. (2011) showed that root tissues which
507 increased their size after metal exposure varied according to plant species and Cd
508 concentration. However, the lignin contents were not significantly different between
509 control and Cd-treated whole root systems (Fig. 4). This suggests that the short and
510 thick adventitious roots represented only a small part of the whole root biomass,
511 which included also longer and finer roots formed before the exposure to metal
512 toxicity, for which only their apices were exposed to Cd-induced lignification.
513
514 Several cell wall compounds may adsorb heavy metals (Chen et al. 2013) and
515 Cd-induced lignification is accordingly sometimes reported as a plant adaptation
516 mechanism to tolerate heavy metals (Ederli et al. 2004, Maksimović et al. 2007)..
517 Metals can sorb to carboxylic (hemicelluloses, pectin and lignin), phenolic (lignin,
518 waxes, fat), hydroxylic (structural polysaccharides, lignin, pectin) and carbonyl
519 groups (lignin) (Pejic et al. 2009). In contrast, cellulose could not efficiently bind metal
520 ions efficiently due to its unbranched structure (Zhu et al. 2012). Although
521 hemicelluloses, pectins and lignin may bind heavy metal compounds, their sorbing
522 efficiency depends strongly of the considered plant species and metal types. Indeed,
523 Hu et al. (2010) obtained the highest metal binding potential for hemicelluloses from
524 rice bran, as well as Nawirska (2005) for pectin and hemicelluloses in pomace fibers.

However, Chen et al. (2013) showed that hemicelluloses negatively impact Cd adsorption in willow roots, while pectins enhanced Cd adsorption. In the present study, hemicellulose and lignin relative contents increased in *Trifolium repens* after exposure to heavy metals (Fig. 4), although metal translocation factors remained unaffected (Table 2). An opposite trend was recorded for *Lolium perenne*: metal translocation to the shoots decreased while relative contents of hemicelluloses and lignin also decreased after Cd and Zn contamination. This suggests that hemicelluloses and lignin did not play a key role in metal root accumulation in the tested species. However, pectins, which were not quantified in the present work, may contribute to heavy metal retention in roots, as well as other mechanisms also taken into account in metal homeostasis, such as chelation, vacuole sequestration, *etc.* (Clemens 2006; Verbruggen et al. 2009).

Zn 1000 µM induced interesting and drastically different results than Cd treatments for ryegrass roots. Indeed, root lignification decreased in comparison to other treatments (Fig. 5), and such a trend was also observed for the lignin content at the whole root system level (Fig. 4). Therefore, root swelling could not be linked to root lignification in this case. The higher cell viability detected for Zn 1000µM indicated also that other mechanisms act on root swelling (Fig. 6). However, to our knowledge, scientific data are missing about the Zn-induced root swelling mechanisms. Cell viability measurements showed that root elongation inhibition and swelling were not linked to cell death. Sresty and Rao (1999) proposed that Zn affected mainly cell elongation instead of cell division. The mechanism may be a disturbance of cytoskeleton, leading to an increase in cell width (Přibyl et al. 2005).

549 However, the data in the present study were not sufficient to support such
550 hypothesis.
551
552 4.3. Comparison of the experimental devices
553
554 When designing a pot experiment, experimental factors such as pot size, rooting
555 medium, nutrient content, *etc.* should be cautiously selected (Poorter et al. 2012).
556 Therefore the pots size was adapted in the present study based on the scientific
557 objectives: column size allowed not to limit root system setting up and was suitable
558 for root distribution study. However, such experimental devices are costly in terms of
559 space occupation, maintenance and harvest times, thus limiting the feasible number
560 of treatments and replicates. Therefore, smaller pots containing the same rooting
561 medium and metal concentration are often used to investigate metal-induced
562 modifications of root properties. Although the same ratio volume pot / volume
563 irrigation was considered for pots and columns for daily irrigation, biomass production
564 was higher in pots than in columns, especially for *Trifolium repens* (Table 1; Fig. 3).
565 During preliminary experiments, an experimental duration of 8 weeks instead of 4
566 weeks was tested, among other things, and higher biomass production was observed
567 for plants into columns in comparison to pots (results not shown), because of a
568 limitation of root development after this duration. The difference in biomass
569 production after 4 weeks of treatment may be linked to different water content
570 gradient between the two containers, which is a typical and often-unrecognized
571 artifact with pot experiments (Passioura 2006). Because of the rapid drainage of
572 nutrient solution irrigated each morning, water content was low at the top of the
573 column device, and increased gradually with depth. This water content gradient, as

574 well as nutrient content gradient, were detected during preliminary tests. The addition
575 of coarse material at the bottom of the columns to facilitate drainage and the weekly
576 flushes with deionized water should partly have alleviated this artifact. However, the
577 residual water content gradient may have affected the root distribution with depth of
578 *Trifolium repens*, as observed with small peaks for RWD, RLD and root diameter
579 (Figure 4) at the bottom of the device. On the contrary, for the pot experiment, root
580 medium was more saturated with water despite the presence of coarse particles at
581 the bottom of the containers. Therefore, the higher duration of contact between the
582 roots and the nutrient solutions for the pot experiment may lead to an increase in
583 nutrient and metal uptake. Indeed, Cd and Zn mineralomasses were higher in shoots
584 grown in pots. It may be assumed that the metal exposure of root systems led to the
585 same impacts for the two experimental devices, but that these effects may be
586 exacerbated with the pot experiment, despite the same concentrations in the
587 contaminated nutrient solutions and the same irrigation ratios.
588
589 **Conclusions**
590
591 Even though plant species like *Lolium perenne* and *Trifolium repens* may
592 seem suitable for a phytostabilization strategy based on their shoot metal tolerance,
593 exposure to toxic heavy metals drastically impairs their root distribution within the
594 substrate profile, leading to a reduction of their soil stabilization potential.
595 Assessment of plant potential for phytostabilization purpose should therefore be
596 based on both shoot and root behaviors against toxic metal exposure. This metal-
597 induced reduction of root distribution was linked for *Lolium perenne* with a
598 proliferation of thick and short adventitious roots. However, the mechanisms leading

to such a root swelling differed between Cd and Zn. The increase in root diameter was linked to Cd-induced root lignification, localized in exodermis and parenchyma of central cylinder. Exposure to Zn increased the root diameter without increasing root lignification, and higher cell viability was detected. Moreover, the composition of root cell walls was differently affected by metal exposure according to the considered plant species, with an increase in lignin, hemicelluloses and cellulose contents for *Trifolium repens*. These cell wall modifications did not affect the root metal retention. This study demonstrates that impacts of heavy metal toxicity on root properties differ according to the considered pollutant and plant species.

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References

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics international 11: 36-42

Ignacio Arganda-Carreras, Rodrigo Fernandez-Gonzalez, Arrate Munoz-Barrutia, Carlos Ortiz-De-Solorzano, "3D reconstruction of histological sections: Application to mammary gland tissue", Microscopy Research and Technique, Volume 73, Issue 11, pages 1019–1029, October 2010.

Arienzo M, Adamo P, Cozzolino V (2004) The potential of *Lolium perenne* for revegetation of contaminated soil from a metallurgical site. Sci Total Environ 319: 13-25

Bidar G, Garcon G, Pruvot C, Dewaele D, Cazier F, Douay F, Shirali P (2007) Behavior of *Trifolium repens* and *Lolium perenne* growing in a heavy metal

629 contaminated field: Plant metal concentration and phytotoxicity. Environ Pollut 147:
630 546-553

631 Bidar G, Verdin A, Garcon G, Pruvot C, Laruelle F, Grandmougin-Ferjani A, Douay F,
632 Shirali P (2008) Changes in fatty acid composition and content of two plants (*Lolium*
633 *perenne* and *Trifolium repens*) grown during 6 and 18 months in a metal (Pb, Cd, Zn)
634 contaminated field. Water Air Soil Poll 192: 281-291

635 Bidar G, Pruvot C, Garçon G, Verdin A, Shirali P, Douay F (2009) Seasonal and
636 annual variations of metal uptake, bioaccumulation, and toxicity in *Trifolium repens*
637 and *Lolium perenne* growing in a heavy metal-contaminated field. Environ Sci Pollut
638 R 16: 42-53

639 Boisson J, Bouchardon JL, Carrey A, Charissou AM, Colpaert J, Faure O, Guérin V,
640 Joulian C, Pottecher G, Remon E, Vangronsveld J, (2009) Evaluation des
641 performances de la phytostabilisation sur un grand site. 2^{e} Rencontres Nationales de
642 la Recherche sur les Sites et Sols Pollués, ADEME, Paris.

643 Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. New
644 Phytol 173: 677-702

645 Broos K, Uyttebroek M, Mertens J, Smolders E (2004) A survey of symbiotic nitrogen
646 fixation by white clover grown on metal contaminated soils. Soil Biol Biochem 36:
647 633-640

648 Chen G, Liu Y, Wang R, Zhang J, Owens G (2013) Cadmium adsorption by willow
649 root: the role of cell walls and their subfractions. Environ Sci Pollut R: 1-8.

650 Clemens S (2006) Toxic metal accumulation, responses to exposure and
651 mechanisms of tolerance in plants. Biochimie 88: 1707-1719

652 Deiana S, Manunza B, Palma A, Premoli A, Gessa C (2001). Interactions and
653 mobilization of metal ions at the root-soil interface. In: Gobran GR, Wenzel WW,
654 Lomobi E (ed) Trace elements in the rhizosphere. Academic press, pp 127-148

655 Delpérée C, Lutts S (2008) Growth inhibition occurs independently of cell mortality in
656 tomato (*Solanum lycopersicum*) exposed to high cadmium concentrations. J Integrat
657 Plant Biol 50: 300-310

658 Domínguez MT, Maranón T, Murillo JM, Schulin R, Robinson BH (2008) Trace
659 element accumulation in woody plants of the Guadiamar Valley, SW Spain: a large-
660 scale phytomanagement case study. Environ Pollut 152: 50-59

661 Ďurčeková K, Huttová J, Mistrik I, Ollé M, Tamás L (2007) Cadmium induces
662 premature xylogenesis in barley roots. Plant Soil 290: 61-68

663 Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responses induced by high
664 concentration of cadmium in *Phragmites australis* roots. Physiol Plantarum 121: 66-
665 74

666 Foy CD, Chaney RL, White MC (1978) The physiology of metal toxicity in plants.
667 Annu Rev Plant Physiol 29: 511-566

668 Fusconi A, Gallo C, Camusso W (2007) Effects of cadmium on root apical meristems
669 of *Pisum sativum* L.: Cell viability, cell proliferation and microtubule pattern as
670 suitable markers for assessment of stress pollution. Mutat Res - Gen Tox and En
671 632: 9-19

672 Grant CA, Clarke JM, Duguid S, Chaney RL (2008) Selection and breeding of plant
673 cultivars to minimize cadmium accumulation. Sc Total Environ 390: 301-310

674 Houben D, Pircar J, Sonnet P (2012) Heavy metal immobilization by cost-effective
675 amendments in a contaminated soil: effects on metal leaching and phytoavailability. J
676 Geochem Explor 123: 87-94

677 Hu G, Huang S, Chen H, Wang F (2010) Binding of four heavy metals to
678 hemicelluloses from rice bran. Food Res Int 43: 203-206

679 Kabata-Pendias A (2001). Trace Elements in Soils and Plants, third ed. CRC Press,
680 Boca Raton

681

682 Kapur, JN; Sahoo, PK & Wong, ACK (1985), "A New Method for Gray-Level Picture
683 Thresholding Using the Entropy of the Histogram", Graphical Models and Image
684 Processing **29(3)**: 273-285
685

686 Kidd P, Barceló J, Bernal MP, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R,
687 Monterroso C. (2009) Trace element behaviour at the root–soil interface: implications
688 in phytoremediation. Environ Exp Bot 67: 243-259

689 Kumpiene J, Lagerkvist A, Maurice C (2008) Stabilization of As, Cr, Cu, Pb and Zn in
690 soil using amendments–a review. Waste Manage 28: 215-225

691 Lambrechts T, Gustot Q, Couder E, Houben D, Iserentant A, Lutts S (2011)
692 Comparison of EDTA-enhanced phytoextraction and phytostabilisation strategies
693 with *Lolium perenne* on a heavy metal contaminated soil. Chemosphere 85: 1290-
694 1298

695 Lambrechts T, Lequeue G, Lobet G, Lutts S (2013). Impact of cadmium and zinc on
696 root system of *Lolium perenne* and *Trifolium repens*. Comm Agr App Biol Sci 78: 19-
697 24.

698 Larbi A, Morales F, Abadía A, Gogorcena Y, Lucena JJ, Abadía J (2002) Effects of
699 Cd and Pb in sugar beet plants grown in nutrient solution: induced Fe deficiency and
700 growth inhibition. Funct Plant Biol 29: 1453-1464

701 Li T, Yang X, Lu L, Islam E, He Z (2009) Effects of zinc and cadmium interactions on
702 root morphology and metal translocation in a hyperaccumulating species under
703 hydroponic conditions. J Hazard Mat 169: 734-741

704 Lobet G, Draye X (2013) Novel scanning procedure enabling the vectorization of
705 entire rhizotron-grown root systems. Plant Methods 9: doi:10.1186/1746-4811-9-1

706 Lopareva-Pohu A, Verdin A, Garçon G, Lounès-Hadj Sahraoui A, Pourrut B, Debiane
707 D, Waterlot C, Laruelle F, Bidar G, Douay F, Shirali P (2011) Influence of fly ash
708 aided phytostabilisation of Pb, Cd and Zn highly contaminated soils on *Lolium*
709 *perenne* and *Trifolium repens* metal transfer and physiological stress. Environ Pollut
710 159: 1721-1729

711 Lunáčková L, Šottníková A, Masarovičová E, Lux A, Streško V (2003) Comparison of
712 cadmium effect on willow and poplar in response to different cultivation conditions.
713 Biol Plantarum 47: 403-411

714 Lutts S, Almansouri M, Kinet JM (2004) Salinity and water stress have contrasting
715 effects on the relationship between growth and cell viability during and after stress
716 exposure in durum wheat callus. Plant Sci 167: 9-18

717 Lux A, Martinka M, Vaculík M, White PJ (2011) Root responses to cadmium in the
718 rhizosphere: a review. J Exp Bot 62: 21-37

719 Lynch J (1995) Root architecture and plant productivity. Plant Physiol 109: 7

720 Macnicol RD, Beckett PH (1985) Critical tissue concentrations of potentially toxic
721 elements. Plant Soil 85: 107-129

722 Maksimović I, Kastori R, Krstić L, Luković J (2007) Steady presence of cadmium and
723 nickel affects root anatomy, accumulation and distribution of essential ions in maize
724 seedlings. Biol Plantarum 51: 589-592

725 Mattia C, Bischetti GB, Gentile F (2005) Biotechnical characteristics of root systems
726 of typical Mediterranean species. Plant Soil 278: 23-32

727 Mench M, Bussiere S, Boisson J, Castaing E, Vangronsveld J, Ruttens A, De Koe T,
728 Bleeker P, Assunção A, .Manceau A (2003) Progress in remediation and
729 revegetation of the barren Jales gold mine spoil after in situ treatments. Plant Soil
730 249: 187-202

731 Mench M, Lepp N, Bert V, Schwitzguébel JP, Gawronski SW, Schröder P,
732 Vangronsveld J (2010) Successes and limitations of phytotechnologies at field scale:
733 outcomes, assessment and outlook from COST Action 859. J Soil Sediment 10:
734 1039-1070

735 Mulligan CN, Yong RN, Gibbs BF (2001) Remediation technologies for metal-
736 contaminated soils and groundwater: an evaluation. Eng Geol 60: 193-207

737 Nawirska A (2005) Binding of heavy metals to pomace fibers. Food Chem 90: 395-
738 400

739 Nishizono H, Ichikawa H, Suziki S, Ishii F (1987) The role of the root cell wall in the
740 heavy metal tolerance of *Athyrium yokoscense*. Plant Soil 101: 15-20

741 Passioura JB (2006) Viewpoint: The perils of pot experiments. Funct Plant Biol 33:
742 1075-1079

743 Pejic B, Vukcevic M, Kostic M, Skundric P (2009) Biosorption of heavy metal ions
744 from aqueous solutions by short hemp fibers: Effect of chemical composition. J
745 Hazard Mat 164: 146-153

746 Pichtel J, Salt CA (1998) Vegetative growth and trace metal accumulation on
747 metalliferous wastes. J Environ Qual 27: 618-624

748 Pilon-Smits E (2005) Phytoremediation. Annu Rev Plant Biol 56: 15-39

749 Poschenrieder CH, Barceló J (2004) Water relations in heavy metal stressed plants.
750 In: Prasad MNV (ed) Heavy metal stress in plants: from molecules to ecosystems, 2nd
751 edn. Springer-Verlag, Berlin, pp 249-270

752 Poorter H, Bühler J, van Dusschoten D, Climent J, Postma JA (2012) Pot size
753 matters: a meta-analysis of the effects of rooting volume on plant growth. Funct Plant
754 Biol 39: 839-850

Přibyl P, Cepák V, Zachleder V (2005) Cytoskeletal alterations in interphase cells of the green alga *Spirogyra decimina* in response to heavy metals exposure: I. The effect of cadmium. Protoplasma 226: 231-240

Reubens B, Poesen J, Danjon F, Geudens G, Muys B (2007) The role of fine and coarse roots in shallow slope stability and soil erosion control with a focus on root system architecture: a review. Trees-Struct Funct 21: 385-402

Robinson BH, Banuelos G, Conesa HM, Evangelou MW, Schulin R (2009) The phytomanagement of trace elements in soil. Crit Rev Plant Sci 28: 240-266

Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, Del Rio LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 52: 2115-2126

Santibáñez C, Verdugo C, Ginocchio R (2008) Phytostabilization of copper mine tailings with biosolids: Implications for metal uptake and productivity of *Lolium perenne*. Sci Total Environ 395: 1-10

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Godbold DL, Polle A (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. Plant Physiol 127: 887-898

Schwartz C, Morel JL, Saumier S, Whiting SN, Baker AJ (1999) Root development of the zinc-hyperaccumulator plant *Thlaspi caerulescens* as affected by metal origin, content and localization in soil. Plant Soil 208: 103-115

Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. Trend Plant Sci 14: 43-50

Sresty TVS, Rao MKV (1999) Ultrastructural alterations in response to zinc and nickel stress in the root cells of pigeonpea. Environ Exp Bot 41: 3-13

Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A., Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lie D, Mench M (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ Sci Pollut R 16: 765-794

Van Soest PV, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583-3597

Verbruggen N, Hermans C, Schat H (2009) Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 181: 759-776

Yang X, Baligar VC, Martens DC, Clark RB (1995) Influx, transport, and accumulation of cadmium in plant species grown at different Cd2+ activities. J Environ Sci Heal B 30: 569-583

Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory Manual for Physiological Studies of Rice, 3rd edn. The International Rice Research Institute, Manila.

Zhu J, Ingram PA, Benfey PN, Elich T (2011) From lab to field, new approaches to phenotyping root system architecture. Curr Opin Plant Biol 14: 310-317

Zhu XF, Lei GJ, Jiang T, Liu Y, Li GX, Zheng SJ (2012) Cell wall polysaccharides are involved in P-deficiency-induced Cd exclusion in *Arabidopsis thaliana*. Planta 1-9

799 Zobel RW, Kinraide TB, Baligar VC (2007) Fine root diameters can change in
800 response to changes in nutrient concentrations. Plant Soil 297: 243-254