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REGULAR ARTICLE

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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Abstract
Backgrounds and aims The phytostabilization potential of plants is a direct function of their 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 1 month. Root biomass, root length density (RLD) and diameter were subsequently quantified as a function of depth. 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 whereas *T. repens* was unable to restrict heavy metal translocation. Cadmium and Zn reduced rooting depth and RLD but induced thick shoot-borne roots in *L. perenne*. Cd-induced root swelling was related to lignification occurring in the exodermis and parenchyma of central cylinder. Hemicelluloses and lignin did not play a key role in root metal retention. Cadmium slightly reduced mean root cell viability whereas Zn increased this parameter in comparison to Cd.
Conclusions Even though plant species like *Lolium perenne* and *Trifolium repens* may appear suitable for a phytostabilization scheme based on their shoot metal tolerance, exposure to toxic heavy metals drastically impairs their root distribution. This could jeopardize the setting up of phytostabilization trials. The metal-induced alterations of root system properties are clearly metal- and species-specific. At sites polluted with multiple metals, it is therefore recommended to first test their impact on the root

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system of multiple plant species so as to select the most appropriate species for each site.

Keywords Heavy metal contamination · Root distribution · Root diameter · Lignin and structural polysaccharides · *Lolium perenne* · *Trifolium repens*

Introduction

Urbanization, industrialization and the use of heavy-metal containing inputs 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 appropriate 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). 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, based on soil exploration and nutrients uptake (Hodge et al. 2009; Šmilauerová and Šmilauer 2002). 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; Domínguez 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, hemicelluloses and pectins, may constitute the main metal accumulation compartment in roots (Deiana et al. 2001; 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.

Materials and methods

Column experiment–root distribution with depth

The experiment was performed in a phytotron under fully controlled environmental conditions (16 h photoperiod, 24 °C day, 22 °C night, relative humidity 80 %, light intensity 100 $\mu mol.m^{-2}.s^{-1}$). 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 at the same stage of development, 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 (Yoshida et al. 1976). These columns were previously tested in order to develop an experimental device which allowed for studying the 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 other 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), provided in a single application. 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. All the columns received the Yoshida's solution for 2 weeks after seedlings transplantation, and then the treatments were applied for 4 weeks. The control treatment consisted of Yoshida's nutrient solution. For the other treatments, Cd ($CdCl_2.2.5H_2O$) or Zn ($ZnSO_4.7H_2O$) were added to this nutrient solution to achieve the following concentrations: for *Lolium perenne*, Zn 500 μM, Zn 1,000 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, weight determined 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 PCV 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 24 px mm^{-1}. 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 each images the following steps were performed: (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 an 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). Some fine roots with diameter lower than 0.08 mm may not be detected because of the resolution of the scanner. As a consequence, total root length may be underestimated.

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 weight determined. 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 weight recorded, 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 the same as for the first pot experiment, with 10 replicates 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 hemicelluloses) were assessed according to Van Soest et al. (1991). Briefly, the crushed plant sample was exposed successively to a neutral detergent solution during 1 h at 100 °C to obtain by filtration the NDF fraction (neutral detergent fibers; containing cellulose, hemicelluloses and lignin), then to an acid detergent solution during 1 h at 100 °C to get the ADF fraction (acid detergent fibers; with cellulose and lignin), and then to sulfuric acid 72 % during 3 h to obtain the ADL fraction (acid detergent lignin). The ADL fraction was incinerated at 550 °C during 3 h, 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 gave the percentage of hemicelluloses.

For *Lolium perenne*, some apices of first order roots (sections of 3 cm-length apices of both seminal and shoot-borne 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 performed on freshly harvested *Lolium perenne* apex roots, through the reduction of 2,3, 5-triphenyltetrazolium chloride (TTC) into formazan (Lutts et al. 2004), with eight replications per treatment. Approximately 50 mg of fresh tissue were excised, quickly

rinsed in deionized water containing 0.05 % Tween 20 and incubated in darkness in tubes containing 5 ml of 0.5 % TTC in 50 mM K_2HPO_4 pH 7.0 for 15 h. Samples were then filtered (Whatman n°4), rinsed with deionized water and incubated in 3 ml ethanol 94 % at 80 °C during 5 min under gentle agitation (80 rpm). After centrifugation at 5, 000 g during 1 min, extracted formazan was quantified spectrophotometrically at 487 nm (Shimadzu UV Spectrophotometer UV-1800). The viability index is defined as the absorbance measured per gram of fresh tissue.

For the root systems stored into FAA solution, cross-sections were performed on random first order roots at 1 cm below the crown. Selected roots were dehydrated in a graded ethanol series, embedded in paraffin and sectioned at 5 μm with a rotary microtome. Serial cross-sections were stained with safranine-fast green and observed with a light microscope Leica DM500 equipped with a camera Leica ICC50. Analysis of root diameter and lignification percentage was assessed thanks to image analysis software ImageJ. For the lignification percentage, a macro was developed to convert the color image into a binary one based on a fixed threshold, which only selects lignin-colored pixel, and to compare the binary area to the total root area.

Statistical analysis

Data of plant growth and development related parameters, metal concentrations, root system distribution and diameter, lignin and structural polysaccharides 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).

Results

Column experiment–modification of shoot biomass and root distribution

The addition of heavy metals into the nutrient solution led to a decrease in the shoot biomass of Cd 50 μM and Zn 1,000 μM 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.

Figure 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}; Cd 25 μM, 0.38^{B} g.dm^{-3}; Zn 500 μM, 0.20^{BC} g.dm^{-3}; Cd 50 μM, 0.09^{C} g.dm^{-3}; Zn 1, 000 μM, 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: Cd 25 μM, 35 cm; Cd 50 μM, 30 cm; Zn 500 μM, 25 cm; Zn 1,000 μM, 20 cm. These observations suggest that heavy metals drastically impaired root elongation. Nevertheless, RWDs were also

Table 1 Dry biomass [g per plant] and Cd and Zn concentrations [mg.kg^{-1}] in shoots of *Lolium perenne* and *Trifolium repens* grown for 28 days in columns filled with quartz and irrigated daily with different contaminated nutrient solutions (*Lolium perenne*: control (C), Cd 25 μM, Cd 50 μM, Zn 500 μM, Zn 1,000 μM; *Trifolium repens*: control (C), Cd 5 μM, Cd 10 μM, Zn 50 μM, Zn 100 μM)

	Dry biomass [g]	Cd [mg.kg^{-1}]	Zn [mg.kg^{-1}]
Lolium perenne			
C	0.535^{A} [0.139]	n.d.	72^{B} [19]
Cd 25	0.490^{AB} [0.129]	103^{B} [17]	102^{B} [11]
Cd 50	0.232^{BC} [0.040]	297^{A} [70]	94^{B} [5]
Zn 500	0.317^{ABC} [0.088]	n.d.	2212^{A} [111]
Zn 1000	0.192^{C} [0.046]	n.d.	2503^{A} [193]
Trifolium repens			
C	0.250^{A} [0.080]	n.d.	46^{B} [5]
Cd 5	0.200^{A} [0.035]	42^{B} [7]	54^{B} [2]
Cd 10	0.132^{A} [0.018]	98^{A} [5]	62^{B} [8]
Zn 50	0.134^{A} [0.009]	n.d.	655^{A} [79]
Zn 100	0.171^{A} [0.034]	n.d.	916^{A} [174]

Values are means of three replicates, standard errors between brackets. Means sharing a common letter are not significantly different at $p<0.05$. n.d. = values below the detection limit

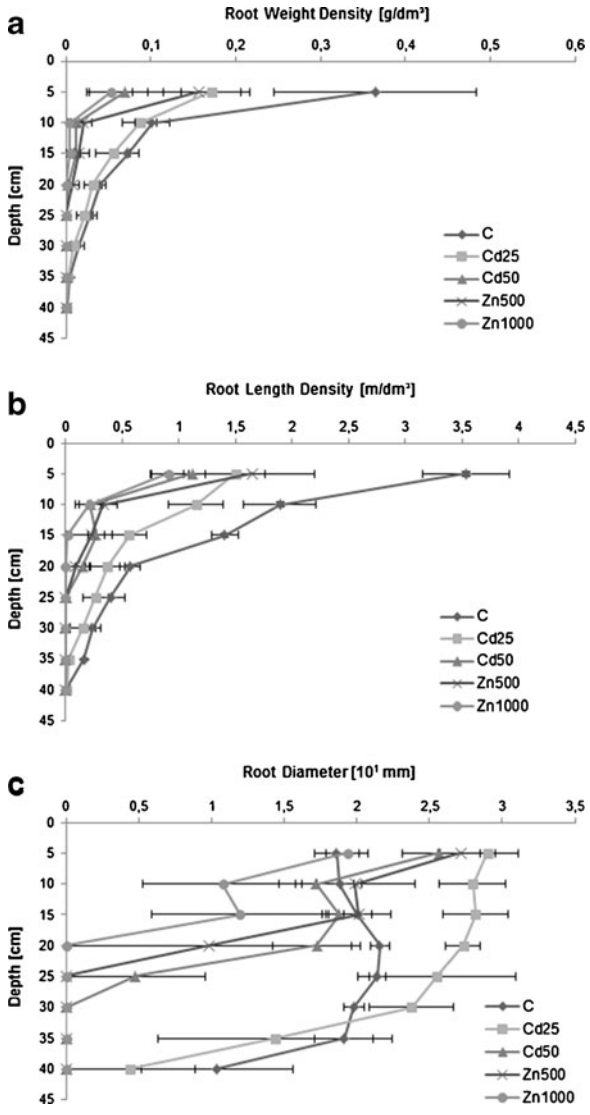

Fig. 1 Changes in root distribution and morphology with depth [cm] for *Lolium perenne* grown for 28 days in columns filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions: control (C), Cd 25 μM, Cd 50 μM, Zn 500 μM, Zn 1,000 μM. **a** root weight density [g.dm^{-3}], **b** root length density [m.dm^{-3}], **c** root diameter [10^{1} mm]. Values are means of three replicates and *horizontal bars* are standard errors

severely affected in the top layer. At this level, the different treatments are ranked as follows: C, 0.36^{A} g.dm^{-3}; Cd 25 μM, 0.17^{B}; Zn 500 μM, 0.16^{B}; Cd 50 μM, 0.07^{C}; Zn 1,000 μM, 0.05^{C}. Although Cd 25 μM and Zn 500 μM exhibited similar values in the top layer, the RWD between the two treatments differed in the deeper layers: RWD for Zn 500 μM decreased strongly from the first to the second layer, whereas RWD for Cd 25 μM 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}; Cd 25 μM, 4.05^{B}; Zn 500 μM, 2.3^{BC}; Cd 50 μM, 1.77^{C}; Zn 1,000 μM, 1.13^{C}. These values, as well as Fig. 1b, show that RLD followed the same trend as RWD, except for Cd 25 μM. 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 Cd 25 μM, Cd 50 μM and Zn 500 μM, but not for Zn 1,000 μM. However, a higher mean root diameter was only significant for the first 5-cm soil layer in the case of Cd 50 μM and Zn 500 μM treatments, and the root diameters decreased strongly in the deeper layers in comparison to control. This was not the case for Cd 25 μM: the mean root diameters were higher than those of control plants until 35 cm depth.

Figure 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}; Zn 100 μM, 0.26^{AB}; Zn 50 μM, 0.18^{B}; Cd 5 μM, 0.18^{B}; Cd 10 μM, 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}; Zn 100 μM, 2.40^{A}; Zn 50 μM, 2.40^{A}; Cd 5 μM, 2.29^{A}; Cd 10 μM, 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 40 cm depth, but this was not the case for the two Zn treatments. Roots of Zn 100 μM exhibited a higher mean diameter in the first 15 cm, but below this depth the values decreased faster than the other treatments.

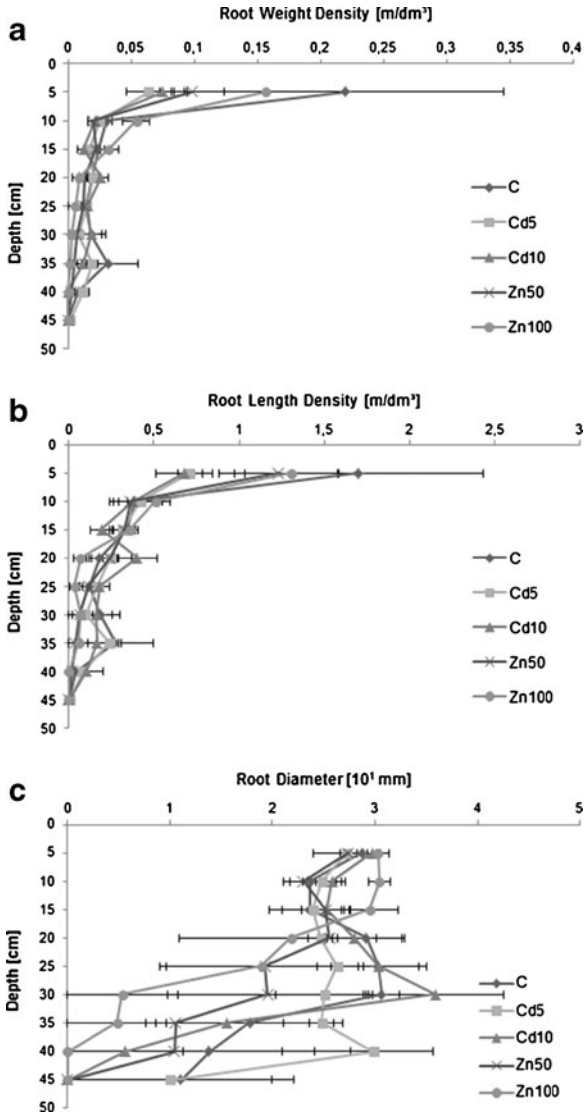

Fig. 2 Changes in root distribution and morphology with depth [cm] for *Trifolium repens* grown for 28 days in columns filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions: control (C), Cd 5 μM, Cd 10 μM, Zn 50 μM, Zn 100 μM. **a** root mass density [g.dm^{-3}], **b** root length density [m.dm^{-3}], **c** root diameter [10^{1} mm]. Values are means of three replicates and *horizontal bars* are standard errors

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 and b). 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), Cd 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 and e). 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 500 μM and Zn 1,000 μM 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*.

Pot experiment–lignin and structural polysaccharides

The analysis of the contents of lignin, hemicelluloses and cellulose were performed for the whole root systems of the two plant species (Fig. 4). Structural polysaccharides and lignin fractions differed strongly between the controls of the two plant species, especially for cellulose and hemicelluloses. Indeed, their contents were clearly higher in *Lolium perenne* in comparison to *Trifolium repens*. Zinc excess in nutrient solution decreased cellulose, hemicelluloses and lignin contents in *Lolium perenne* roots. These modifications were proportional to the exogenous Zn concentrations. Cadmium also decreased the root cellulose and hemicelluloses contents, but lignin remained unaffected. Zn 50 μM and Cd 5 μM 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 Zn 100 μM and Cd 10 μM.

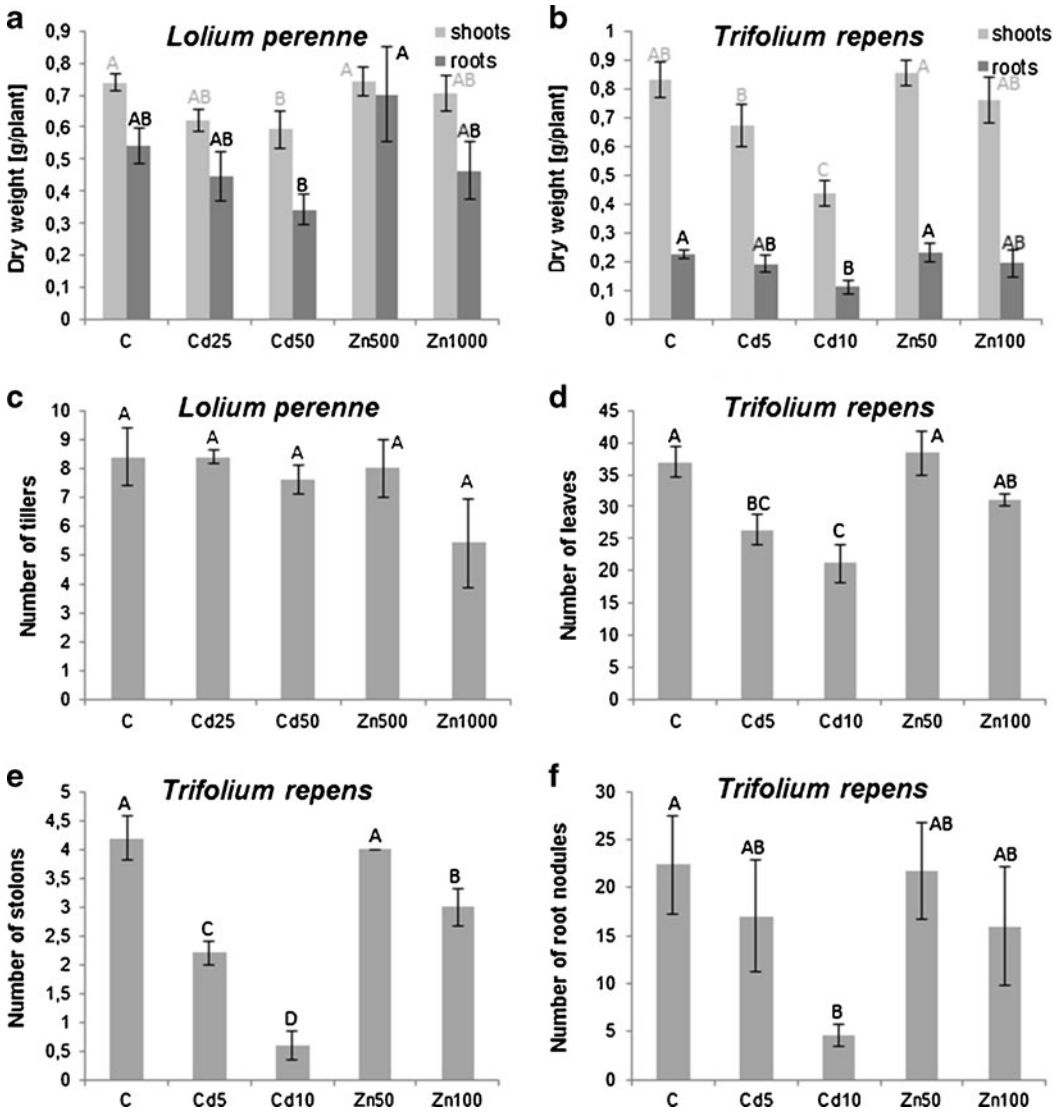

Fig. 3 Growth and development-related parameters of *Lolium perenne* and *Trifolium repens* grown for 28 days in pots filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions (*Lolium perenne*: control (C), Cd 25 μM, Cd 50 μM, Zn 500 μM, Zn 1,000 μM; *Trifolium repens*: control (C), Cd 5 μM, Cd 10 μM, Zn 50 μM, Zn 100 μM). **a** and **b** shoot and root dry weights [g per plant]; **c** number of tillers for *Lolium perenne*; **d** number of leaves for *Trifolium repens*; **e** number of stolons for *Trifolium repens*; **f** number of root nodules for *Trifolium repens*. Values are means of five replicates, standard errors into brackets. Means sharing a common letter are not significantly different at $p < 0.05$

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*. Figure 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 Zn 500 μM, Zn 1,000 μM and Cd 50 μM showed a proliferation of short and thick shoot-borne roots, which was not observed for Cd 25 μM and control

Table 2 Cd and Zn concentrations [mg.kg^{-1}] in shoots and roots of *Lolium perenne* and *Trifolium repens* grown for 28 days in pots filled with quartz and irrigated daily with different contaminated nutrient solutions (*Lolium perenne*: control (C), Cd 25 μM, Cd 50 μM, Zn 500 μM, Zn 1,000 μM; *Trifolium repens*: control (C), Cd 5 μM, Cd 10 μM, Zn 50 μM, Zn 100 μM)

	Cd [mg.kg^{-1}]		Zn [mg.kg^{-1}]		Translocation factor [-]	
	Shoots	Roots	Shoots	Roots	Cd	Zn
Lolium perenne						
C	n.d.	n.d.	134^{C} [18]	157^{B} [25]	/	1.056^{A} [0.316]
Cd 25	86^{B} [15]	1279^{B} [261]	146^{C} [24]	190^{B} [27]	0.069^{A} [0.005]	0.819^{A} [0.146]
Cd 50	135^{A} [24]	2299^{A} [388]	129^{C} [9]	296^{B} [77]	0.062^{A} [0.013]	0.566^{AB} [0.160]
Zn 500	n.d.	n.d.	953^{B} [131]	5258^{A} [777]	/	0.200^{B} [0.041]
Zn 1000	n.d.	n.d.	1403^{A} [99]	7174^{A} [1949]	/	0.262^{B} [0.063]
Trifolium repens						
C	n.d.	n.d.	140^{C} [18]	391^{C} [60]	/	0.396^{A} [0.074]
Cd 5	49^{B} [4]	260^{B} [42]	240^{C} [82]	287^{C} [41]	0.199^{A} [0.021]	0.877^{A} [0.332]
Cd 10	82^{A} [17]	568^{A} [76]	130^{C} [16]	222^{C} [28]	0.160^{A} [0.038]	0.659^{A} [0.154]
Zn 50	n.d.	n.d.	835^{B} [81]	1743^{B} [116]	/	0.498^{A} [0.075]
Zn 100	n.d.	n.d.	1201^{A} [120]	2652^{A} [455]	/	0.488^{A} [0.059]

Values are means of five replicates, standard errors between brackets. Means sharing a common letter are not significantly different at $p < 0.05$. n.d. = values below the detection limit

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 showed a tendency 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.

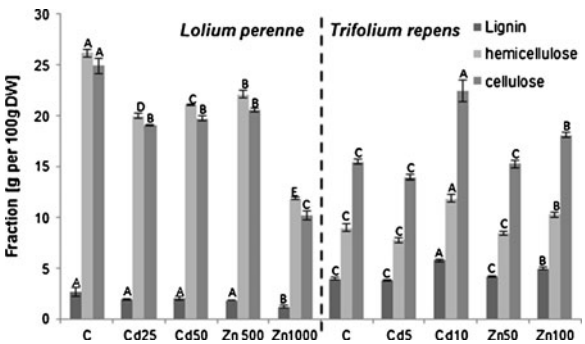

Fig. 4 Lignin, hemicellulose and cellulose fractions [g per 100 g DW] of roots of *Lolium perenne* and *Trifolium repens* grown for 28 days in pots filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions. The different treatments were: for *Lolium perenne*, control (C), Cd 25 μM, Cd 50 μM, Zn 500 μM, Zn 1,000 μM; for *Trifolium repens*, control (C), Cd 5 μM, Cd 10 μM, Zn 50 μM, Zn 100 μM. Values are means of three replicates and *vertical bars* are standard errors

The Cd and Zn contaminated roots were compared to the apices of the roots of control plants in terms of cell viability for *Lolium perenne* (Fig. 6). Zn 1,000 μM contaminated root cells led to higher cell viability than the control and Cd-treated roots.

Discussion

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

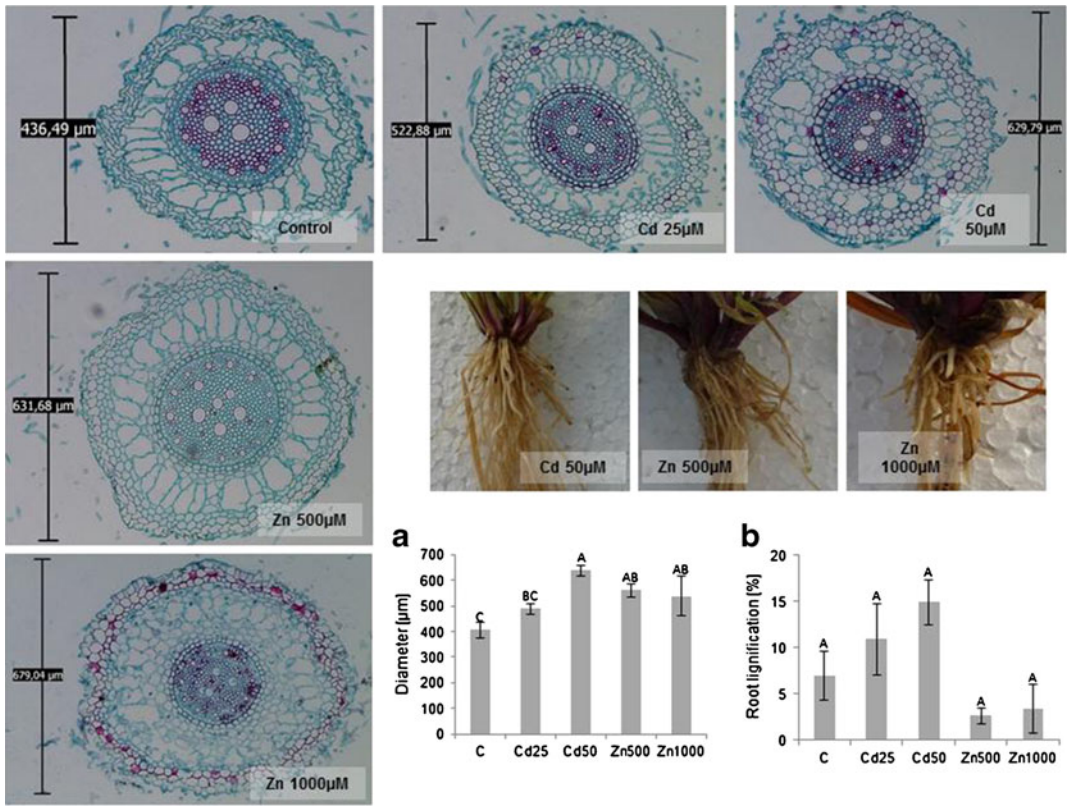

Fig. 5 Representative cross-sections through roots of *Lolium perenne* 1 cm below the crown and stained with safranine–fast green, after 28 days of growth in pots filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions [control (C), Cd 25 µM, Cd 50 µM, Zn 500 µM, Zn 1,000 µM]. The top of the root systems are also shown, as well as measurements of cross-sections, with **a**: root diameter [µm], **b**: root lignification [%]. Values are means of five replicates and means sharing a common letter are not significantly different at $p<0.05$. *Vertical bars* are standard errors

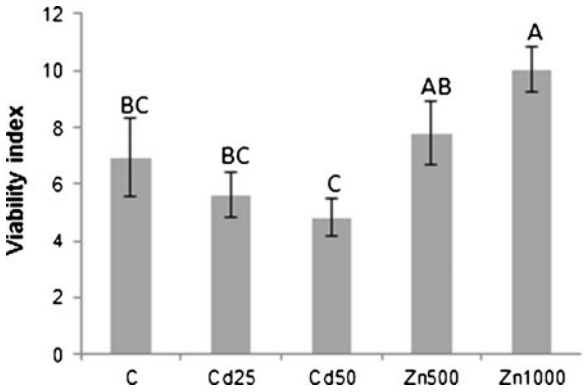

Fig. 6 Cell viability index of random selected fresh roots of *Lolium perenne* after 28 days of growth in pots filled with quartz and irrigated daily with Cd or Zn-containing nutrient solutions: control (C), Cd 25 µM, Cd 50 µM, Zn 500 µM, Zn 1,000 µM. Values are means of eight replicates and means sharing a common letter are not significantly different at $p<0.05$. *Vertical bars* are standard errors

et al. (1995) observed the same patterns for white clover and perennial ryegrass with Cd hydroponic experiments. Moreover, for the same endogenous metal concentrations 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 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) conducted pot and field experiments with soils polluted by Cd, Zn and Pb and with *Lolium perenne* and *Trifolium repens*. They reported a higher sensitivity to metal-induced oxidative stress in *Lolium perenne* than in *Trifolium repens*. This may suggest 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*.

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 50 μM in comparison to control and could provide an important plant cover to limit pollutant dispersion by water and wind erosion. However, Cd and Zn decreased both the rooting depth and the RLD for this plant species (Fig. 1). The root systems of grass species present seminal roots, arising from the primordial laid down in the embryo, and shoot-borne roots, which arise from the basal nodes of the main shoot and tillers. These first order roots can behave very differently (Dawson et al. 2004). At the time of transplantation, only seminal roots were present. After transplantation, root systems were allowed to grow during 2 weeks before the application of the treatments. During this period, seminal roots continue to grow and the first shoot-borne roots appeared (correlated to the shoot development). At the end of the experiment, the comparison of the RWD and RLD profiles for the control and Cd 25 μM treatments indicated that Cd 25 μM affected mainly the growth and lateral ramification of shoot-borne roots. On the contrary, Zn 500 μM impaired not only shoot-borne roots growth and branching, but also those of seminal roots, and these effects were magnified with higher metal concentrations. However, metal stresses did not affect the initiation of shoot-borne roots, but only their growth and ramification, as observed in the photos of Fig. 5.

Concerning *Trifolium repens*, Cd treatments led to a significant decrease of biomass and RLD in the first centimeters of depth (Fig. 2). This suggested that the growth of the taproot was not impaired by metal stress, as rooting depths were not significantly different between the different treatments, but that the development of lateral roots was affected by application of Cd. Therefore, soil exploration by roots of *Lolium perenne* and *Trifolium repens* may progressively be limited while increasing heavy metal concentrations, which in turn could lead to nutrient deficiencies and thus affect the durability of the plant cover. Moreover, this metal-induced decrease in the total root length, especially in the top layer of soils, could reduce plant anchorage and the roots' potential to stabilize soils (Reubens et al. 2007). These observations suggested the requirement, during the development of a phytostabilization strategy, to combine plant installation with the use of adapted amendments to overcome this potential metal-induced impairment of the root distribution within the soil profile (Kumpiene et al. 2008; Lambrechts et al. 2011; Mench et al. 2010).

The reduction of root depth and RLD in *Lolium perenne* (Fig. 1) was correlated to a Cd- and Zn-induced proliferation of short and thick shoot-borne roots (Fig. 5) which contributed greatly to the observed increase of mean root diameter in the top soil layer and to the drastic decrease of root depth. The reduction of root growth may result from cell death, inhibition of cell division, a decrease in the rate of cell elongation or different processes occurring simultaneously (Delpérée and Lutts 2008; Sresty and Rao 1999). In our work, Zn treatments induced an increase in cell viability in comparison to the control (Fig. 6). Moreover, cell viability was not affected in response to Cd treatments, as observed also by Delpérée and Lutts (2008) with *Solanum lycopersicum* after contamination with Cd 250 μM during 14 days. Fusconi et al. (2007) observed with *Pisum sativum* a decrease of cell viability only for Cd 250 μM, but not for the lower Cd concentrations. They attributed the reduction of plant growth to a decrease of apex length in relation to the inhibition of mitotic activity.

However, some authors suggested that root growth inhibition could be rather attributed to metal-induced inhibition of cell elongation occurring through several mechanisms. Our work demonstrates that such inhibition was linked with an increase in root diameter (Fig. 5). Cytoskeleton, and especially microtubules, constitutes a major target of heavy metals. Přibyl et al.

(2005) showed that heavy metals could induce a disintegration of microtubules followed by a steep increase in cell width and a decrease in growth rate for green alga *Spirogyra decimina* exposed to Cd. Heavy metals could also damage cell membrane, as shown by Bidar et al. (2008) for *Lolium perenne* and *Trifolium repens*. This loss of membrane integrity would result in turgor loss and inhibition of cell expansion (Poschenrieder and Barceló 2004). However, heavy metals at lower concentrations could also act on cell elongation by decreasing cell wall extensibility without damaging cell membrane. Exposure of *Pinus sylvestris* roots to Cd concentrations exceeding cell detoxification capacity led to accumulation of H_2O_2 because of an imbalance of the redox systems (Schützendübel et al. 2001). H_2O_2 is a signaling intermediate which could increase the mechanical strength and lower the extensibility of plant cell walls, following an abnormal lignification (Ďurčeková et al. 2007; Ederli et al. 2004; Schützendübel et al. 2001). This premature xylogenesis shortened the root elongation zone and therefore reduced root growth (Ďurčeková et al. 2007; Lunáčková et al. 2003).

Cd-induced lignification was detected in the present study based on the cross-sections of ryegrass roots (Fig. 5). We demonstrate that lignification, linked to an increase in root diameter, was localized in *Lolium perenne* in the exodermis and in the parenchyma cells in the central cylinder. Lux et al. (2011) showed that root tissues which increased their size after metal exposure varied according to plant species and Cd concentration. However, the lignin contents were not significantly different between control and Cd-treated whole root systems (Fig. 4). This suggests that the short and thick shoot-borne roots represented only a small part of the whole root biomass, which included also longer and finer seminal and shoot-borne roots formed before the exposure to metal toxicity, for which only their apices were exposed to Cd-induced lignification. As shoot-borne and seminal roots could react differently to heavy metal exposure, histochemical analyses performed on identified root types could add more data about the physiological adaptation of roots to metal stress than analyses on random roots as in the present study.

Several cell wall compounds may adsorb heavy metals (Chen et al. 2013) and Cd-induced lignification is accordingly sometimes reported as a plant adaptation mechanism to tolerate heavy metals (Ederli et al. 2004; Maksimović et al. 2007). Metals can sorb to carboxylic (hemicelluloses, pectin and lignin), phenolic (lignin, waxes, fat), hydroxylic (structural polysaccharides, lignin, pectin) and carbonyl groups (lignin) (Pejic et al. 2009). In contrast, cellulose could not efficiently bind metal ions efficiently due to its unbranched structure (Zhu et al. 2012). Although hemicelluloses, pectins and lignin may bind heavy metal compounds, their sorbing efficiency depends strongly of the considered plant species and metal types. Indeed, Hu et al. (2010) obtained the highest metal binding potential for hemicelluloses from 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, hemicelluloses 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 1,000 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, the increase in root diameter could not be linked to root lignification in this case. The higher cell viability detected for Zn 1,000 μM indicated also that other mechanisms act on such increase (Fig. 6). However, to our knowledge, scientific data are missing about the Zn-induced mechanisms which increase root diameter. Cell viability measurements showed that root elongation inhibition and diameter increasing 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). However, the data in the present study were not sufficient to support such hypothesis.

Advantages and limitations of the experimental devices

When designing a pot experiment, experimental factors such as pot size, rooting medium, nutrient contents, etc. should be cautiously selected (Poorter et al. 2012). The present study was performed in spiked sand substrates in order to enable root sampling without damage and to control soil metal concentrations. However, spiked soils exposed roots to dissolved salts which require a period of equilibrium and are dependent upon the particular physicochemical properties of the used substrate (Dickinson et al. 2009). Moreover, many soil physicochemical parameters affect root growth and architecture. Therefore, under real-life soil conditions, root growth and architecture may be influenced in a different manner than in sand spiked substrates. The metal concentrations in the present study were determined based on hydroponic and spiked soil experiments (Lambrechts et al. 2013). However, roots may be exposed to higher metal concentrations than those encountered in some field conditions. The assessment of metal concentrations in soil solution in the field is sensitive. Indeed, many soil parameters could affect these concentrations, and moreover it is difficult and perhaps impossible to collect in situ soil solution samples which are not altered or biased by the sampling process (Weihermüller et al. 2007). As an example, Tack et al. (1998) estimated 5–10 $\mu g\ l^{-1}$ for Cd and 300–1,000 $\mu g\ l^{-1}$ for Zn in pore water extracted from neutral and moderate metal-contaminated soils near Ghent (Belgium). These concentrations were much lower than those used in the present study; however, metal concentrations in soil solution could be higher in more acidic and polluted soils.

The pot size was adapted in the present study based on the scientific objectives: column size allowed not to limit root system setting up and was suitable for root distribution study. However, such experimental devices are costly in terms of space occupation, maintenance and harvest times, thus limiting the feasible number of treatments and replicates. Therefore, smaller pots containing the same rooting medium and metal concentrations are often used to investigate metal-induced modifications of root properties. Although the same ratio volume pot/volume irrigation was considered for pots and columns for daily irrigation, biomass production was higher in pots than in columns, especially for *Trifolium repens* (Table 1; Fig. 3). During preliminary experiments, an experimental duration of 8 weeks instead of 4 weeks was tested, among other things, and higher biomass production was observed for plants into columns in comparison to pots (results not shown), because of a limitation of root development after this duration. The difference in biomass production after 4 weeks of treatment may be linked to different water content gradient between the two containers, which is a typical and often-unrecognized artifact with pot experiments (Passioura 2006). Because of the rapid drainage of nutrient solution irrigated each morning, water content was low at the top of the column device, and increased gradually with depth. This water content gradient, as well as nutrient content gradient, were detected during preliminary tests. The addition of coarse material at the bottom of the columns to facilitate drainage and the weekly flushes with deionized water should partly have alleviated this artifact. However, the residual water content gradient may have affected the root distribution with depth of *Trifolium repens*, as observed with small peaks for RWD, RLD and root diameter (Fig. 4) at the bottom of the device. On the contrary, for the pot experiment, root medium was more saturated with water despite the presence of coarse particles at the bottom of the containers. Therefore, the higher duration of contact between the roots and the nutrient solutions for the pot experiment may lead to an increase in nutrient and metal uptake. Indeed, Cd and Zn mineralomasses were higher in shoots grown in pots. It may be assumed that the metal exposure of root systems led to the same impacts for the two experimental devices, but that these effects may be exacerbated with the pot experiment, despite the same concentrations in the contaminated nutrient solutions and the same irrigation ratios.

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 may drastically impair their root distribution within the substrate profile, leading to a reduction of their soil stabilization potential. Assessment of plant potential for phytostabilization purpose should therefore be based on both shoot and root behaviors against toxic metal exposure. In the case of *Lolium perenne*, this metal-induced reduction of root distribution was linked with a proliferation of thick and short shoot-borne roots. However, the mechanisms leading to such an increase in root diameter differed between Cd and Zn. It was linked to

Cd-induced root lignification, localized in the exodermis and parenchyma of the central cylinder. Exposure to Zn increased the root diameter without increasing root lignification, and higher cell viability was detected. These promising observations deserve more investigation, for example cross-sections on identified root types and longitudinal-sections of the root apices to detect the impact of metals on mitotic activity in the meristematic zone and impact on cell length in the elongation zone. Moreover, the composition of root cell walls was differently affected by metal exposure depending on 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. Quantification of pectins may bring new insights concerning the relation between metal retention in roots and the modifications of cell wall composition. This study demonstrates that the impacts of heavy metal toxicity on root properties differ according to the considered pollutant and plant species. At sites polluted with multiple metals, it is therefore recommended to first test their impact on the root system of multiple plant species so as to select the most appropriate species for each site.

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