Subtoxic levels of some heavy metals cause differential root-shoot structure, morphology and auxins levels in *Arabidopsis thaliana*

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A B S T R A C T

Contamination of soil by heavy metals severely affects plant growth and causes soil pollution. While effects on plant growth have been investigated for metals taken individually or in groups, less is known about their comparative effects. In this study *Arabidopsis thaliana* seedlings were grown for 14 days in Petri dishes containing medium contaminated by six common heavy metals (Hg, Cd, Pb, Cu, Ni and Zn), at the minimum concentrations defined as toxic by the most recent EU legislation on contamination of agricultural soils. (a) Root structure and morphology, (b) metal composition and translocation, and (c) the levels of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were analyzed. Metals accumulated more in roots than in shoots, with concentrations that differed by several orders of magnitude depending on the metal: Cd (ca. 700 × and ca. 450 × in roots and shoots, respectively), Hg (150 ×, 80 ×), Ni (50 ×, 20 ×), Cu (48 ×, 20 ×), Zn (23 ×, 6 ×), and Pb (9 ×, 4 ×). Responses were significant for at least nine of the ten root parameters (with the exception of Hg), and five of the six shoot parameters (with the exception of Zn). Cu and Zn induced respectively the strongest responses in root hormonal (up to ca. 240% the control values for IBA, 190% for IAA) and structural parameters (up to 210% for main root length, 330% for total lateral root length, 220% for number of root tips, 600% for total root surface, and from 2.5° to 26.0° of root growth angle). Regarding the shoots, the largest changes occurred for shoot height (down to 60% for Ni), rosette diameter (down to 45% for Hg), leaf number (up to 230% for Zn) and IBA (up to 240% for Pb and Cu). A microscope analysis revealed that shape and conformation of root hairs were strongly inhibited after Cd exposure, and enhanced under Hg and Pb. The results could have positive applications such as for defining toxicity thresholds (in phytoremediation) and acceptable concentration levels (for policies) for some of the most common heavy metals in agricultural soils.

1. Introduction

Metals can significantly influence plant physiological and biochemical status (Goolsby and Mason, 2015; Xiao et al., 2020). Regarding their relation with plants, metals can be classified into two categories: 1) essential metals, which are necessary in many physiological processes, and for which an excess or deficiency may cause harmful effects on plant cells; and 2) non-essential metals, which are toxic even at relatively low concentrations. The absorption of metals, their translation to different plant tissues, as well as the degree of their tolerance, depend on the metal species, ion bioavailability, plant species and their metabolism (Appenroth, 2010; Kopittke et al., 2010; Xiao et al., 2020). The

absorption of metals from soil and their translocation and accumulation among the plants' organs in *Arabidopsis thaliana* L. (*Arabidopsis*) have been extensively studied, and the resistance, avoidance, and toxic mechanisms have been elucidated (Kajala et al., 2019; Chen et al., 2020; Simiele et al., 2021). Most studies on the efficiency of metal absorption by *Arabidopsis* seedlings were focused on soil-to-plant transfer factors, which differ from species to species and are mainly governed by the type of metal absorbed (Schat et al., 2000; Xiao et al., 2020). However, the accumulation of potentially toxic metals in *Arabidopsis* also depends on their concentration and bioavailability in the growth medium (Kolbert et al., 2020; Zhu et al., 2021).

Most importantly, the effects of different toxic metals on the

morphology of *Arabidopsis* are mediated by phytohormones, particularly auxins, such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (Bochicchio et al., 2015; Fattorini et al., 2017a). Piacentini et al. (2020) found that Cd affects the quiescent center identity and auxin localization in the lateral and adventitious root apices, the auxin levels and the expression of some genes involved in auxin metabolism, auxin efflux and influx carriers. Indeed, root architectural traits (e.g., in terms of branching, root hairs density and root mean diameter) and the production of some secondary metabolites, such as auxins, are two responses of *Arabidopsis* seedlings subjected to metal stress (Sofo et al., 2017). The expression levels of the genes involved in auxins metabolism have been found to be regulated by exposition to Cd, Cu and Zn, alone or in combination (Vitti et al., 2013). It is also known that a crosstalk involving ethylene signaling, IAA-influx, and IBA-to-IAA conversion exists between ethylene and IAA in the formation of adventitious roots in *Arabidopsis* (Veloccia et al., 2016).

The concurrent effects of several metals on root morphology and elemental composition of *Arabidopsis* have been the focus of some recent studies (Deckers et al., 2020; Hendrix et al., 2020; Amaral dos Reis et al., 2021; Piacentini et al., 2021). Conversely, the comparative influence of the different metals applied alone at sub-toxic levels has not been investigated yet. On this basis, this work aims at assessing the structural and morphological responses of roots, and the elemental and hormonal levels in *Arabidopsis* seedlings grown in an agarized medium containing separately six common heavy metals, namely Cd, Pb, Cu, Hg, Ni and Zn, known to differ in toxicity and negative impacts on the environment and human health (WHO, 1996; Tóth et al., 2016). The results were compared with those of seedlings grown in a metal-free agar medium.

In particular, this study analyzes: (a) root structure and morphology, (b) metal composition and translocation, and (c) IAA and IBA levels in *Arabidopsis* seedlings grown in the presence of subtoxic levels of different heavy metals. Considering that many Brassicaceae, like *Arabidopsis*, can successfully extract toxic heavy metal ions from soil, the results at laboratory scale could have positive applications also in the field, such as for phytoremediation purposes. Moreover, this study could also be useful for re-determining the level of toxicity of heavy metals in agricultural soils.

2. Materials and methods

2.1. Experimental design and plant material

Square (12 × 12 cm) Petri dishes (Greiner 688102; Sigma-Aldrich, St. Louis, MO, USA) were filled with melted 35 mL metal + agarized medium. This latter was composed of 1% bacteriological agar (LP0011; Oxoid Ltd., Cambridge, UK) supplemented with 0.5% sucrose, and 1/4 strength Murashige and Skoog liquid medium without micronutrient and vitamins (product code: M0654-1L; Sigma-Aldrich): 412.5 mg L^{-1} NH_4NO_3, 83.0 mg L^{-1} $CaCl_2$, 45.0 mg L^{-1} $MgSO_4$, 475.0 mg L^{-1} KNO_3, 42.5 mg L^{-1} KH_2PO_4. Single metals were added to the agar medium as 2 µM $HgCl_2$, 5 µM $CdCl_2$, 120 µM $PbCl_2$, 500 µM $CuCl_2$, 500 µM $NiCl_2$, and 550 µM $ZnCl_2$. The concentrations of the individual metals used were calculated based on the permissible limits for heavy metals indicated by the European legislation (Tóth et al., 2016), also suggested by World Health Organization (WHO, 1996) (Supplementary Table S1). The final metal concentrations in the agar, calculated on a weight/weight basis, were the following: 0.32 µg g^{-1} Hg, 0.45 µg g^{-1} Cd, 20.05 µg g^{-1} Pb, 23.67 µg g^{-1} Ni, 25.62 µg g^{-1} Cu, and 29.00 µg g^{-1} Zn (Supplementary Table S1).

For preparing the agarized medium, we adapted the method of Bochicchio et al. (2015). The solidified gel was cut transversally in two sections (3- and 9-cm high) under sterile conditions and the upper half discarded (Supplementary Fig. S1). Then, melted metal-free medium was poured into the dish, filling the empty space. After solidification, the top agar (1.5 cm from the upper border of the dish) was removed to allow a better shoot development. Dishes without metals, in both the upper and lower agar sections, were prepared in the same way and kept as controls. Other dishes without metals and with a uniform agar surface (not divided in two sections) were prepared to be sure that the physical interface between the two agar sections would not affect root development. Agar average thickness after solidification was 0.4 cm, with an average density of 1.24 g cm^{-3}.

Seeds of *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype; Col-0) were sterilized using 0.1% NaClO + 1 drop of Tween® 80 (Sigma-Aldrich), then rinsed four times in sterile distilled water for 5 min, before imbibition on moist filter paper in a Petri dish at 4 °C for 24 h in the dark. Three sterilized seeds were placed on each agar surface, just below the upper border, placed 1.5-cm from each other, by means of a sterilized toothpick, and let germinate. After placing the seeds, dishes were sealed with parafilm, with a 1-cm interruption in the central part of the bottom side, for promoting gas exchange. The dishes with seeds were placed vertically in a growth chamber for 14 days at 20 °C, with a 16-h photoperiod and a photosynthetic photon flux density at the top of the agar of 350 µmol m^{-2} s^{-1}.

2.2. Morphological parameters

Fourteen days after germination, all agar dishes were scanned at high resolution (5400 DPI) by Epson Perfection V600 Photo scanner (Epson Inc., Los Alamitos, CA, USA) and the whole root systems analyzed for each plant species by SmartRoot (https://smartroot.github.io/), a semi-automated root image analysis freeware software, running under the ImageJ platform (Lobet et al., 2011). For each metal treatment, the following root morphological measurements were carried out: main root length, total lateral root length, lateral/main root length, number of root tips, average root diameter, total root surface area, and root angle. Also, shoot height and rosette diameter were measured, and leaves counted.

The meristematic, elongation and maturation zones of the roots were observed, directly inside the agarized dishes, with a light microscope (Nikon Eclipse E600; Tokyo, Japan), and photographed at 4, 10× and 20 × magnifications with a digital camera (Nikon DXM1200) equipped by a software for image analysis (Nikon ACT-1).

2.3. Metals determination

Shoot and root samples were then carefully washed either with distilled water or with 1 M HCl, followed by 1 M Na_2 EDTA, to remove the metals bound to cell wall components (Rascio et al., 2008). The samples were dried at 80 °C until constant weight and then pulverized. Approximately 0.2 g of plant tissue samples were completely digested by HNO_3–HCl–$HClO_4$–HF for about 3 h on a hot plate. The residue was solubilized with 2% HNO_3 and then diluted to the desired volume. The concentrations of Hg, Cd, Pb, Cu, Ni and Zn were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES; PerkinElmer Optimal 8000, USA). The precision and accuracy of the metal concentration were determined using certified reference materials.

2.4. Auxin quantification

The auxins IAA and IBA were extracted from 200 mg-aliquots of shoots and roots samples ground into powder with liquid nitrogen. To each tissue sample, 3.0 ml of extraction solvent (2-propanol/H_2O/HCl 37%; 2:1:0.002, v/v/v) were added. The tubes were shaken at a speed of 150 rpm for 20 min at 4 °C. To each tube, 3.0 ml of dichloromethane were added. Then, the samples were shaken for 30 min at 4 °C and centrifuged at 15,000*g* for 5 min. After centrifugation, 1.0 mL of the solvent from the lower phase was transferred into a screw-cap vial, and the solvent mixture was concentrated using an evaporator with N_2 flow. Finally, the samples were re-dissolved in 60 µL methanol and stored at −20 °C before quantitative analysis. The quantitative determinations of IAA and IBA were carried out by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS), according to

Veloccia et al. (2016). Pure standards of IAA and IBA were used for quantification (Duchefa Biochemie B.V., Haarlem, The Netherlands). The internal standards used were [^{2}H$_5$] IAA and [^{2}H$_9$] IBA (OlChemIm Ltd, Olomouc, Czech Republic; crystalline form, purity >97% for HPLC).

2.5. Statistics

Ten dishes per metal, including control, were used, for a total of 30 seedlings per treatment. Statistical analysis was performed in the R statistical environment (https://www.r-project.org/). Differences in plant responses to the given metals were tested by means of ANOVA and Post-Hoc Tukey tests. While the occurrence of significant relationships between plant responses and the range of individual metal concentrations, after partitioning and accumulation into the root/shoot organs, was assessed by linear regression models.

3. Results and discussion

3.1. Metals concentration, partitioning and accumulation

Metals accumulated in plant organs, always with higher values in roots than in shoots, but with concentrations that differed by several orders of magnitude depending on the metal (Fig. 1A). As reported, the highest concentrations in the agar were, in descending order, Zn, Ni and Cd, Pb, Cd and Hg. Conversely, metals that accumulated most in plants were Cd (ca. 700 × and ca. 450 × in roots and shoots, respectively, compared to the levels in the agar) and Hg (150 ×, 80 ×), followed by Ni (50 ×, 20 ×), Cu (48 ×, 20 ×), Zn (23 ×, 6 ×), and Pb (9 ×, 4 ×) (Fig. 1B).

Compared to other studies with the same time of exposition to metals, the levels of Cd, Cu and Zn taken up were comparable to those previously found by other authors (Sofo et al., 2013; Hendrix et al., 2020; Amaral dos Reis et al., 2021). Interestingly, Cd and Hg, even if at low concentration in the agar, were the most absorbed and translocated,

likely because in *Arabidopsis* these two metals are transported through the root cortex both via the symplast and apoplast and there is an efficient compartmentalization mediated by glutathione (GSH) and phytochelatins (PCs) (Deckers et al., 2020; Hendrix et al., 2020). Singh et al. (2012) observed heavy metal accumulation and distribution pattern in different vegetable crops. They found that crop species also showed remarkable difference in the metals, Zn, Cd and Ni concentration in various plant parts. In a report of Karatas et al. (2006), it was shown that higher concentrations of heavy metals (Cu, Cr, Mn, Ni, Pb and Zn) were found in root and seeds of wheat plants. Similar results were obtained by Souri and HatamianTesfamariam (2019), who showed that plant species and plant growth stage influence heavy metals accumulation in tissues of basil and garden cress. Reisinger et al. (2008) analyzed how overexpression of γ-glutamyl synthetase (ECS) and glutathione synthetase (GS) genes affect tolerance and accumulation of heavy metals and metalloids supplemented individually, in agar medium (seedlings) and in hydroponics (mature plants) in *Brassica juncea*. It was found that ECS and GS transgenics plants have an increased capacity to withstand and accumulate a variety of metal/loids (especially As, Cd, and Cr) as well as mixed-metal combinations (AS, Cd, Zn/As, Pb and Zn), compared to wild type plants. This improved metal tolerance and accumulation of ECS and GS transgenics could be attributed to increased PCs synthesis, which was supported by increased GSH availability as a substrate (Reisinger et al., 2008). In tobacco plants, the overexpression of *Arabidopsis* phytochelatin synthase gene (*PCS1*) enhances Cd tolerance and accumulation, but not translocation to the shoots. From this report, it can be inferred that the availability of GSH is directly related to the increase in Cd tolerance and accumulation in *PCS1* overexpressing plants, whereas overexpression of phytochelatin synthase did not improve long-distance root-to-shoot Cd transport (Pomponi et al., 2006). The same role of GSH in increasing Hg accumulation in *Arabidopsis* was observed by Sobrino-Plata et al. (2014), who concluded that in this species GSH is required for adequate metal tolerance.
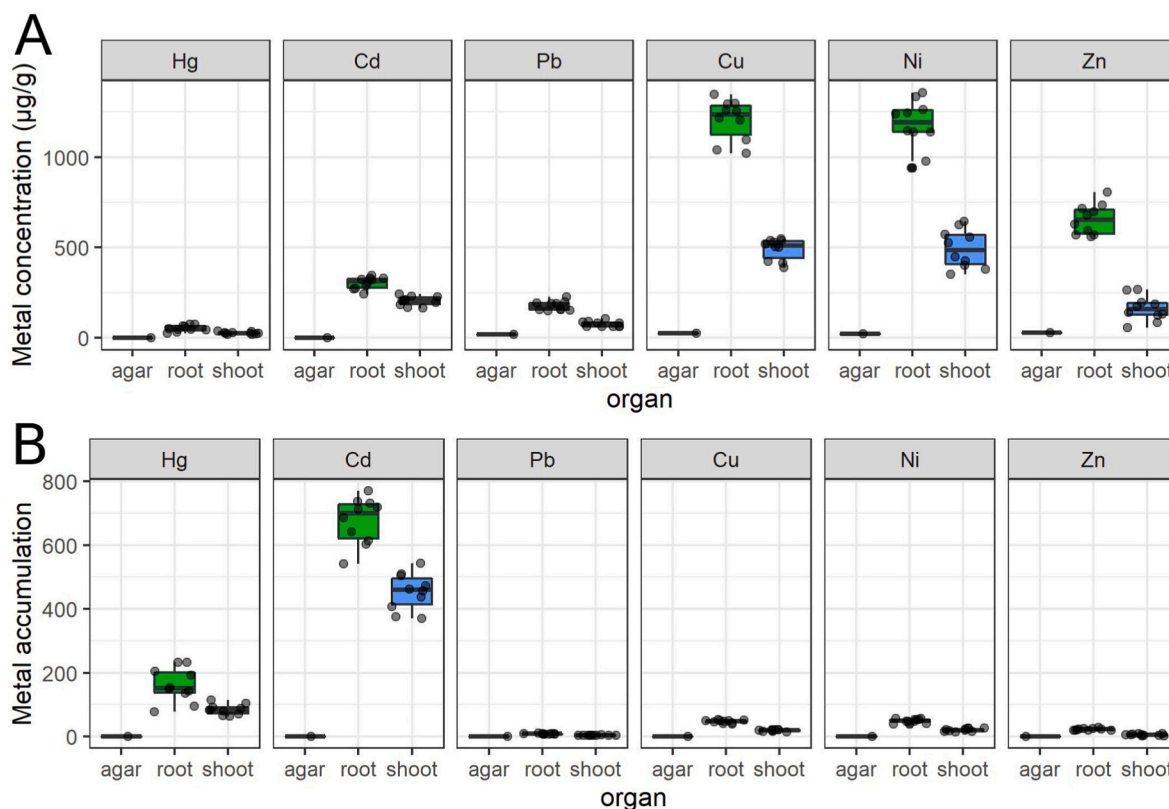

Fig. 1. (A) Metals concentrations in organs and (B) rate of accumulation compared to agar concentration in roots and shoots of *Arabidopsis* seedlings.

3.2. Single metals induce distinct plant responses

A PCA on the root physiological responses (Supplementary Fig. S2AB) explained most of the variability (75%) by the first two principal components and well discriminated responses depending on the different metals present in the agar. The first principal component represented the number of root tips and their total lateral length, while the second component represented IAA and IBA values (Supplementary Fig. S2A). High total lateral root length, IAA and IBA values were associated to high lateral-to-main root lengths ratios and high root angles, while low IAA and IBA values were related to high mean root diameters, root length and total root surface (Supplementary Fig. S2A).

The strongest responses were induced by Cu and Zn, with Cu more related to the responses of the first PCA component (IAA and IBA), while only moderately to all the others, and Zn mostly related to the second component (root angle, main root length, mean root diameter, number of root tips, total lateral root length and root surface) (Supplementary Fig. S2B). From the PCA results, it appears that metal concentration, more than the type of metal itself, caused a wider range of responses in *Arabidopsis* plants. In fact, Cu and Zn were the most concentrated in the agar (25.62 and 29.00 µg g^{-1}, respectively).

Similarly, a PCA on shoot responses split them into structural (first component, 39% of the variability) and hormonal ones (IAA/IBA and IAA, second component, 25% of the variability) (Supplementary Fig. S2CD), but provided a weaker discrimination of individual metals, as associated to specific responses.

3.3. Differences among metals responses

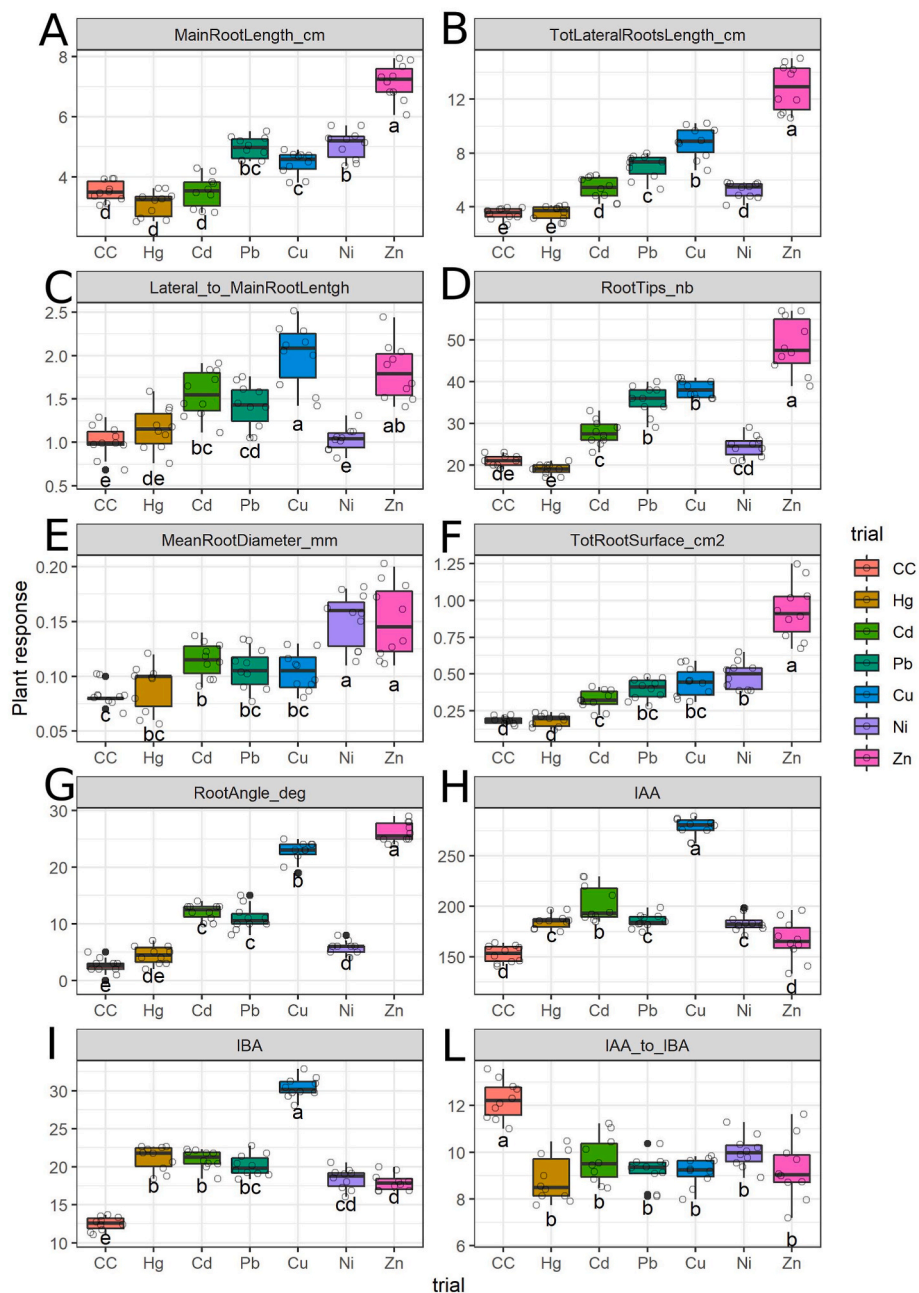Plant responses largely depended on the metal experienced, each of

Fig. 2. Boxplots of root responses to metal concentrations in *Arabidopsis* seedlings. CC indicates control. Circles indicate individual measurements. Letters indicate results of ANOVA Post-Hoc Tukey test.

which induced significantly different responses in at least nine over ten of the root parameters, and in all shoot parameters (Fig. 2). Out of the 96 metal-plant responses, the only non-significantly different from the control were: the effects of Hg on all root responses, excluding auxin parameters (Fig. 2HIL) and on leaves number (Fig. 3C); of Cd on main root length (Fig. 2A); of Pb and Cu on mean root diameter (Fig. 2E); of Cu on shoot IAA (Fig. 3E); of Zn on shoot IAA, shoot height and rosette diameter (Figs. 2H and 3AB); of Ni on lateral to main root length, number of root tips and shoot IAA (Fig. 2CD and 3D). Auxins (root IAA, IBA, IAA/IBA; shoot IAA/IBA) differed mostly in respect to the control, and only to a minor extent across groups of metals (shoot IBA and IAA for Cd, Zn, Ni were lower than for Hg, Pb, Cu) (Fig. 2HIL and 3DEF). An exception was root Cu, in relation to which IAA and IBA were higher than all other metals (Fig. 2HI). IBA increased more than IAA and consistently for all metals, so that the IAA/IBA ratio remained almost constant for both roots and shoots (Figs. 2L and 3F).

Considering all metals together, most of the structural variables, and especially those related to absorption (total lateral root length, number of root tips, total root surface and, in a lesser extent, root angle), increased with the mean metal concentration in the roots. For four of the metals (Hg, Pb, Cd, Zn), responses in all structural variables systematically increased for increasing concentrations in the plant organs (Supplementary Figs. S3 and S4). Concerning the other two metals, Cu induced generally higher or equal responses than Ni, but their higher concentrations in the organs did not correspond to systematically higher/lower responses in respect to the previously mentioned metals (Supplementary Figs. S3 and S4).

As anticipated by the PCA results (Supplementary Fig. S2B), root Cd, Pb and to some extent Ni, induced similar responses (root angle, IAA, IBA, lateral/main root length, mean root diameter, number of root tips, total lateral root length, total root surface), with Cd showing a somewhat larger variability in some responses (IAA, lateral-to-main root length), and Ni having stronger effects on mean root diameter, and lower effects on root angle (Fig. 2 and Supplementary Fig. 3). When considering the plant response as a function of the individual concentration,

relationships were only rarely significant (Table 1): negatively between Ni, mean root diameter and total root surface (Supplementary Fig. S3EF); positively between Ni and root angle (Supplementary Fig. S3G), and between Hg and main root length (Supplementary Fig. S3A). The latter result may be due to the fact that legislation provides a very low threshold for this element. Indeed, despite the average main root length, as affected by Hg at legal concentrations, is not

Table 1
Linear relationships between metal concentrations in the root/shoot exposed to metal concentrations and plant response variables. Only significant relationships are reported. Statistical differences are indicated as follows: * = *p*-value < 0.05; ** = *p*-value < 0.01; *** = *p*-value < 0.001; **** = *p*-value < 0.0001.

| Organ	Independent variable	Response variable	*p*-value	
Root				
	Hg	Main root length		
	Intercept	2.2372	0.000254	***
	Slope	0.016492	0.037563	*
	Ni	Mean root diameter		
	Intercept	3.10E-01	0.000359	***
	Slope	−1.35E-04	0.015841	*
	Ni	Total root surface		
	Intercept	1.1524792	6.28E-05	***
	Slope	−0.0005614	0.00231	**
	Ni	Root angle		
	Intercept	−2.318304	0.3761	
	Slope	0.006805	0.0115	*
Shoot				
	Pb	IAA/IBA		
	Intercept	14.64359	7.97E-05	***
	Slope	−0.07044	0.0253	*
	Cu	IAA/IBA		
	Intercept	18.926166	0.00141	**
	Slope	−0.024156	0.01693	*
	Hg	Shoot height		
	Intercept	4.29931	6.98E-06	***
	Slope	−0.03554	0.048	*
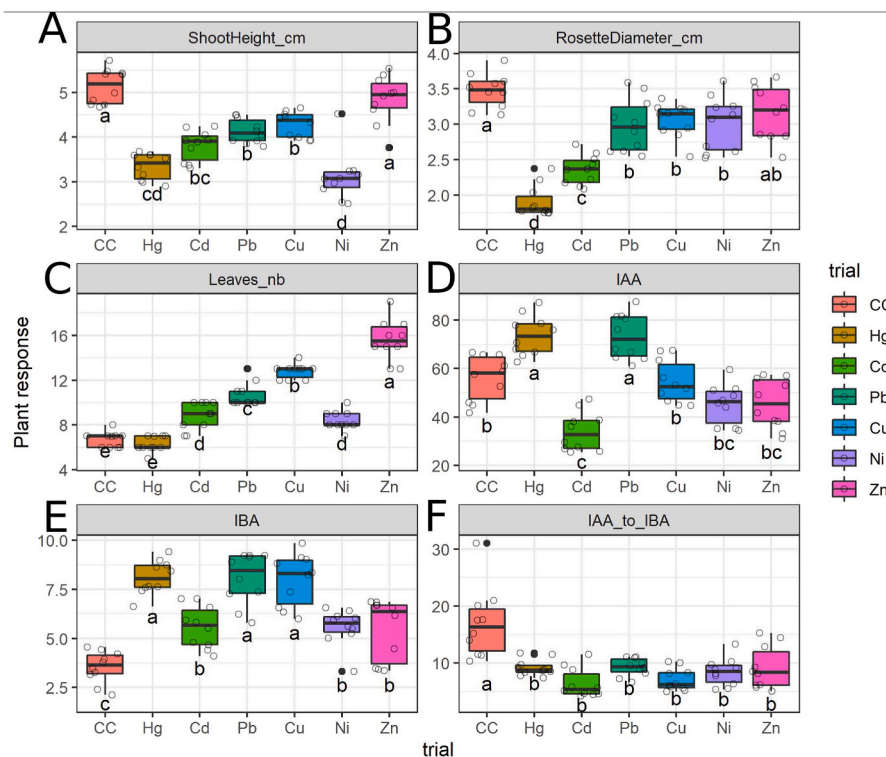
Fig. 3. Boxplots of shoot responses to metal concentrations in *Arabidopsis* seedlings. CC indicates control. Circles indicate individual measurements. Letters indicate results of ANOVA Post-Hoc Tukey test.

significantly different from the control, minimal increases in this metal result in significant increases in root responses (Table 1; Supplementary Fig. S3A).

Compared to control plants, Hg induced higher root and shoot IAA and IBA, while caused lower shoot height, rosette diameters, and root and shoot IAA/IBA (Figs. 2 and 3). Moreover, Hg did not significantly affect the other variables. Wild *Arabidopsis* is a species not particularly tolerant to Hg (Sun et al., 2018) and this can explain the observed plant responses. The observed low IAA/IBA (Fig. 2A) due to the abundance of IBA (Fig. 2I), that is the IAA precursor (Veloccia et al., 2016), is likely the cause of the non-significant effect of Hg on root branching (Fig. 2L). On the other side, although at low concentrations, Hg is highly toxic to plants (Appenroth, 2010) and so inhibited shoot development (Fig. 3). This was likely due to the high level of Hg accumulation in roots and translocation into the shoot (Fig. 1).

Cd induced higher total lateral root length, lateral to main root length, number of root tips, mean root diameter, total root surface, root angle, leaf number, root IAA and IBA, shoot IBA, while caused significantly lower length of shoot and rosette diameter, root IAA/IBA, shoot IAA and IAA/IBA (Figs. 2 and 3). On the other side, Cd did not significantly affect the main root length. As Hg, Cd was strongly absorbed by roots and translocated in the shoot (Fig. 1). As pointed out by Piacentini et al. (2020, 2021), in *Arabidopsis* these Cd-induced changes in root morphology are caused by a hormonal unbalance, mainly governed by the auxin levels (IAA and IBA), as also observed in this study (Fig. 2). Indeed, *Arabidopsis* seedlings exposed to Cd stress resemble, in terms of the modifications of root and shoot growth, plants altered in phytohormone metabolism (Sofo et al., 2012). Similarly to this study, Liu et al. (2011) conducted an experiment to evaluate the response of the gene phytochelatin synthase 1 (*PCS1*) in enhancing the accumulation and tolerance of individual heavy metal, such as Cd, and observed that accumulation of Cd was higher in roots and shoots of transgenic plants compared to the wild type, but translocation of Cd from root to shoot was unaffected. In addition, these data indicate that *PCS1* expression might increase Cd accumulation and, as a result, cadmium-induced oxidative stress. The authors concluded that the generation of PCs could produce a transitory drop in the cytosolic GSH pool, and the oxidative response could be exacerbated by Cd and decreased GSH concentrations.

It is known that Cu and Zn at low concentrations are essential to plants (30–200 µg of Zn g^{-1} dry weight, and 6–12 µg of Cu g^{-1} DW) (Marschner, 2012). This notwithstanding, in *Arabidopsis* excessive levels of Cu and Zn (>400 µg of Zn g^{-1} DW and >35 µg of Cu g^{-1} DW), typical for polluted soils, cause toxicity symptoms and altered morphogenesis (Yemets et al., 2021). In this study, Cu induced higher main root length, total lateral root length, lateral to main root length, number of root tips, total root surface, root angle, leaf number, root and shoot IBA (Figs. 2 and 3). On the other side, Cu caused lower shoot height, rosette diameter, root and shoot IAA/IBA, while it did not significantly affect the mean root diameter and shoot IAA (Figs. 2 and 3). Interestingly, Zn caused responses overlapped to those induced by Cu. Indeed, *Arabidopsis* seedlings exposed to Zn had higher main root length, total lateral root length, lateral to main root length, number of root tips, mean root diameter, total root surface, root angle, root IBA, shoot IBA, and leaf number, whereas they showed lower root and shoot IAA/IBA (Figs. 2 and 3). Conversely, Zn did not significantly affect the root and shoot height, rosette diameter and IAA (Figs. 2 and 3).

Pb induced higher main root length, total lateral root length, lateral to main root length, number of root tips, total root surface, root angle, leaf number, root and shoot IAA and IBA, while it caused lower shoot height, rosette diameter, root and shoot IAA/IBA. Conversely, Pd did not significantly affect the mean root diameter. These responses are in accordance to what was found by Corpas and Barroso (2017) and Zhu et al. (2019), who also observed that rhizosphere acidification by *Arabidopsis* roots favours Pb uptake and a role of peroxisomal enzymes in plant remodelling.

Finally, Ni induced higher main root length, total lateral root length, mean root diameter, total root surface, root angle, leaf number, root IAA and IBA, shoot IBA, while it induced lower shoot height, rosette diameter, and root and shoot IAA/IBA (Figs. 2 and 3). On the other side, Ni did not significantly affect the lateral to main root length, number of root tips and shoot IAA (Figs. 2 and 3). The role of nickel in plants is nowadays considered essential, as it acts as a cofactor of urease, a necessary enzyme for avoiding urea accumulation and the toxic levels of ammonium as a result of urea breakdown (Souri, 2010; Merlot, 2020). If in excess, Ni becomes toxic for plants, partly due to the overproduction of reactive oxygen and nitrogen species and the consequent increase in oxidative stress signaling. Similarly to our study Kolbert et al. (2020) found that, in *Arabidopsis*, 50 µM Ni promoted lateral root formation and modified auxin, cytokinin and ethylene levels, resulting in altered root growth.

Sethy and Ghosh (2013) and Asati et al. (2016) revealed that the accumulation of heavy metals such as Pb, Ni, Cd, Cu, Co, Cr and Hg are significant environmental pollutants that have harmful effects on plants, reducing productivity and posing a serious threat to agro-ecosystems. Baek et al. (2012) studied the effects of heavy metals accumulation on plant growth characteristics and pigment contents in *Arabidopsis thaliana* using essential (Cu, Mn and Zn) and non-essential (Pb and Hg) metals. This experiment suggested that according to the metal types, increasing metal concentrations resulted in a steady decrease in root and shoot length, a drop in chlorophyll, an increase in anthocyanins, and a variation in carotenoid content. Guo et al. (2008) also confirmed that overexpression of glutamate-cysteine ligase 1 (*GSH1*) increases the accumulation and tolerance of As and Cd in *Arabidopsis*. Additionally, John et al. (2012) observed the toxicity of heavy metals on plant growth in *Brassica juncea*. This study confirmed that exposure of heavy metals such as Pb and Cd exhibited a decline in overall plant growth, chlorophyll content and carotenoid content but Cd was found to be more detrimental than Pb treatment. Although Cd was more readily absorbed than Pb, but higher concentration of Pb impeded Cd adsorption.

3.4. Microscopy analysis

The growth parameters measured on scanned dishes were compared to the microscope analysis of the seedlings (Fig. 4; Supplementary Figs. 5–11). The visual analysis confirmed the different growth patterns under diverse metal treatments. In particular, the microscope analysis allowed to measure the shape and conformation of root hairs (Fig. 4). After Cd exposure, root hairs in the maturation zone of the roots were found to be strongly inhibited or dead. The treatments with Hg and Pb had long root hairs with a chaotic arrangement, while the size and shape of the hairs of the seedlings subjected to Cu, Ni and Zn did not visually differ from those of control plants. Sofo et al. (2013) demonstrated that an increased root hairs density in *Arabidopsis* seedlings exposed to Cd/Cu/Zn for 12 days could be functionally related to the root exploration of free-metal-patches in the growing medium. Here, we also found that different metals cause a remodelling of the shape of root hairs. This, together with the increase in root diameter observed for Cd, Ni and Zn (Fig. 2E), could be considered as stress avoidance mechanisms mediated by IAA and IBA ratio. Indeed, Fattorini et al. (2017b) found that in *Arabidopsis*, IAA promotes protoxylem formation, whereas IBA induces ectopic metaxylem, with ethylene cooperation through the EIN3EIL1 network, both interacting with ethylene signaling.

4. Conclusions

The presented data demonstrates that some heavy metals induce differential response on physiological and biochemical parameters of *Arabidopsis*, with significant changes in root-shoot structure and morphology. The observed root modifications (particularly the increased root branching and diameter) could be a form of root plasticity, functionally related to avoidance mechanisms towards harmful
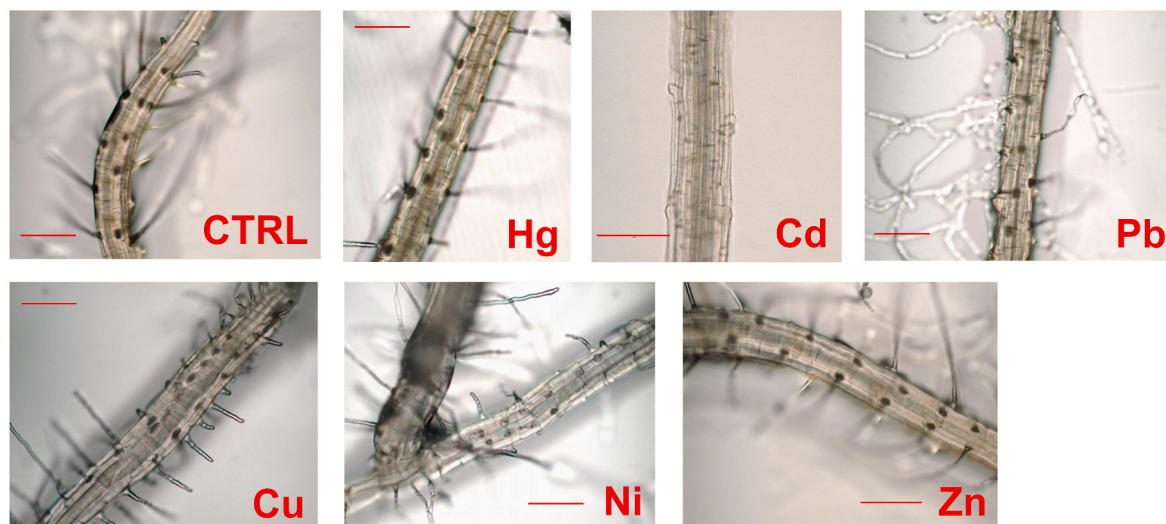
Fig. 4. Root maturation zone with root hairs in *Arabidopsis* seedlings grown under different heavy metals or without metals (Ctrl). Magnification = 20 × ; bars = 0.5 mm.

metals, maximizing the rhizosphere exploration and avoiding metals in the growing medium. In this view, each metal caused modifications in plants that can be considered necessary for proper growth and development, acclimation and adaptative responses under the contrasting environmental conditions caused by the same or similar metals. From the analysis of the results, it also appears that morphological shoot and root changes could be part of an integrated IAA/IBA hormonal balance against the metal stressors. Therefore, an intricate and complex crosstalk of metals with plant growth regulators, ROS, and other signaling molecules to mediate plant acclimation responses to metals may be expected.

The results could have positive applications in the field, such as for phytoremediation purposes and for eventually defining the toxicity threshold of some of the most common heavy metals in agricultural soils. This study could also be useful for re-determining the level of toxicity of heavy metals in agricultural soils and may also add value to improving plant nutritional value and crop productivity in metal-polluted soils. Since agar does not have chelating properties towards metal, it can be interpreted as a standard of maximum metal availability in the soil. Compared to this standard, different soils will have a higher, but variable, capacity to block metals, with a consequent variable bioavailability to plants. This blocking capacity can be considered as a soil-specific factor. Thus, the ratio 'metal availability in a particular soil/metal availability in agar' could be a useful and reliable parameter for scaling the maximum metal availability reproduced in this experiment.

Author contributions

All authors contributed to the study conception and design. Material preparation, morphological analysis, metal determination, and data collection were performed by AS and ID. Data statistical analysis and figures preparation were performed by FR. The first draft of the manuscript was written by AS, FR, NAK, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.01.027.

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Supplementary materials

Supplementary Table S1. Calculations for determining the concentrations of heavy metals used in the agar dishes. Agar average density = 1.24 g cm^{-3}; average soil weight = 1.70 kg L^{-3}. AM = atomic mass. Reference for metal thresholds: WHO (1996) and Tóth et al. (2016).

	mg kg^{-1} soil (EU regulation)	AM	mmol kg^{-1} soil	μmol kg^{-1} soil	μmol L^{-1} agar	μmol L^{-1} agar (subtoxic)	μmol L^{-1} agar (normalized)	μg L^{-1} agar	μg g^{-1} agar
Hg	1	200.59	0.005	5	4	1.2	2	401.18	0.32
Cd	2	112.411	0.018	18	13	4.3	5	562.06	0.45
Pb	100	207.2	0.483	483	352	117.3	120	24,864.00	20.05
Cu	120	63.546	1.888	1888	1377	459.1	500	31,773.00	25.62
Ni	120	58.6934	2.045	2045	1491	497.1	500	29,346.70	23.67
Zn	150	65.38	2.294	2294	1673	557.8	550	35,959.00	29.00
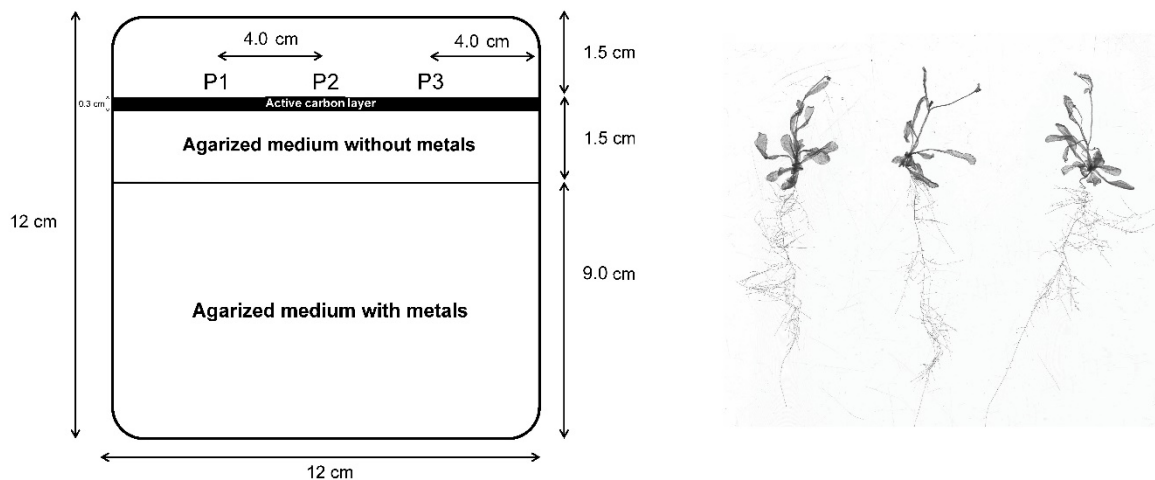
Supplementary Figure S1. (left) Scheme of the agar dishes used. Plant position from left to right = P1-P3. (right) Digitalized *Arabidopsis* seedlings (b/w 5400 DPI scanning) ready for the analysis with SmartRoot software.
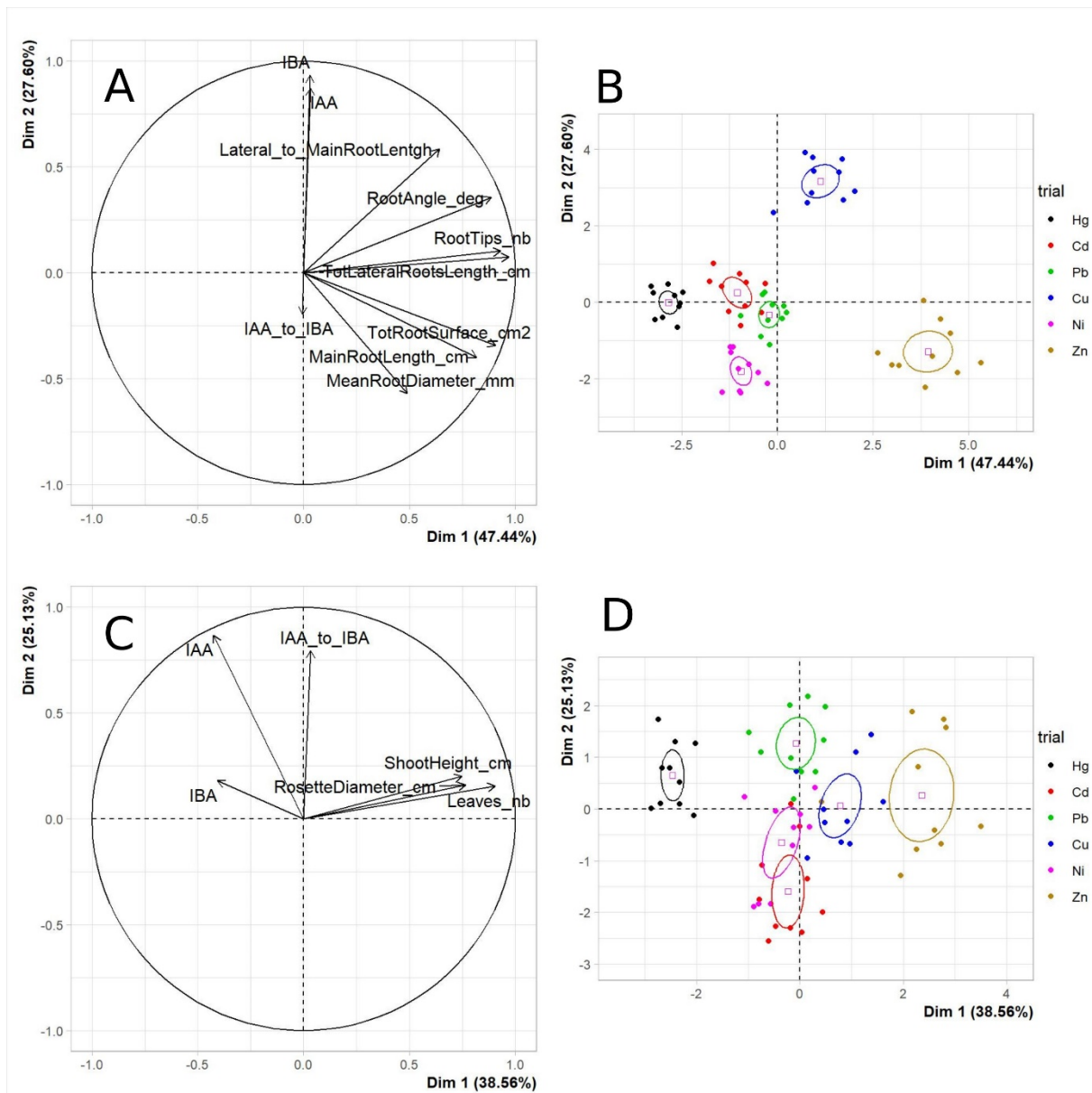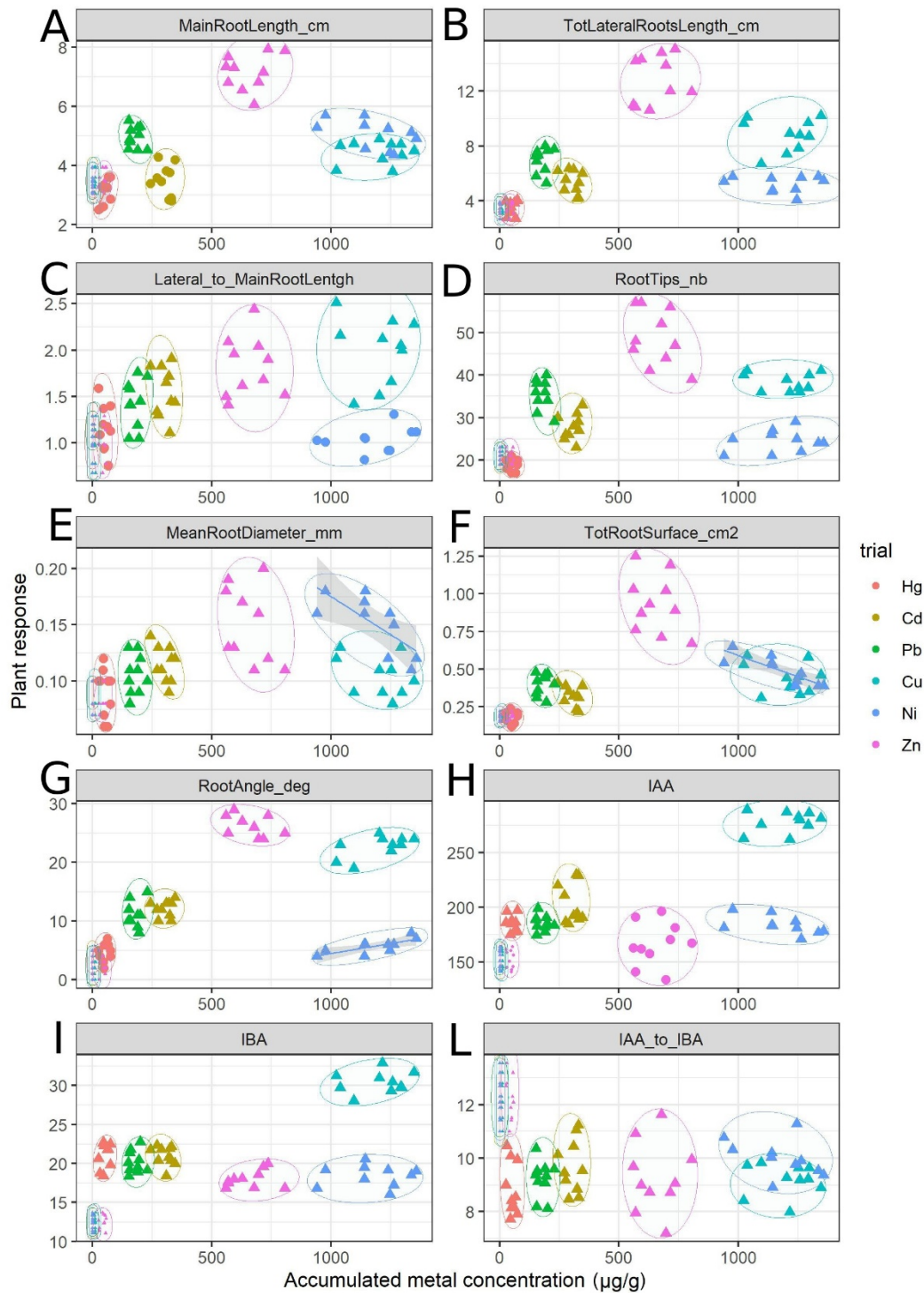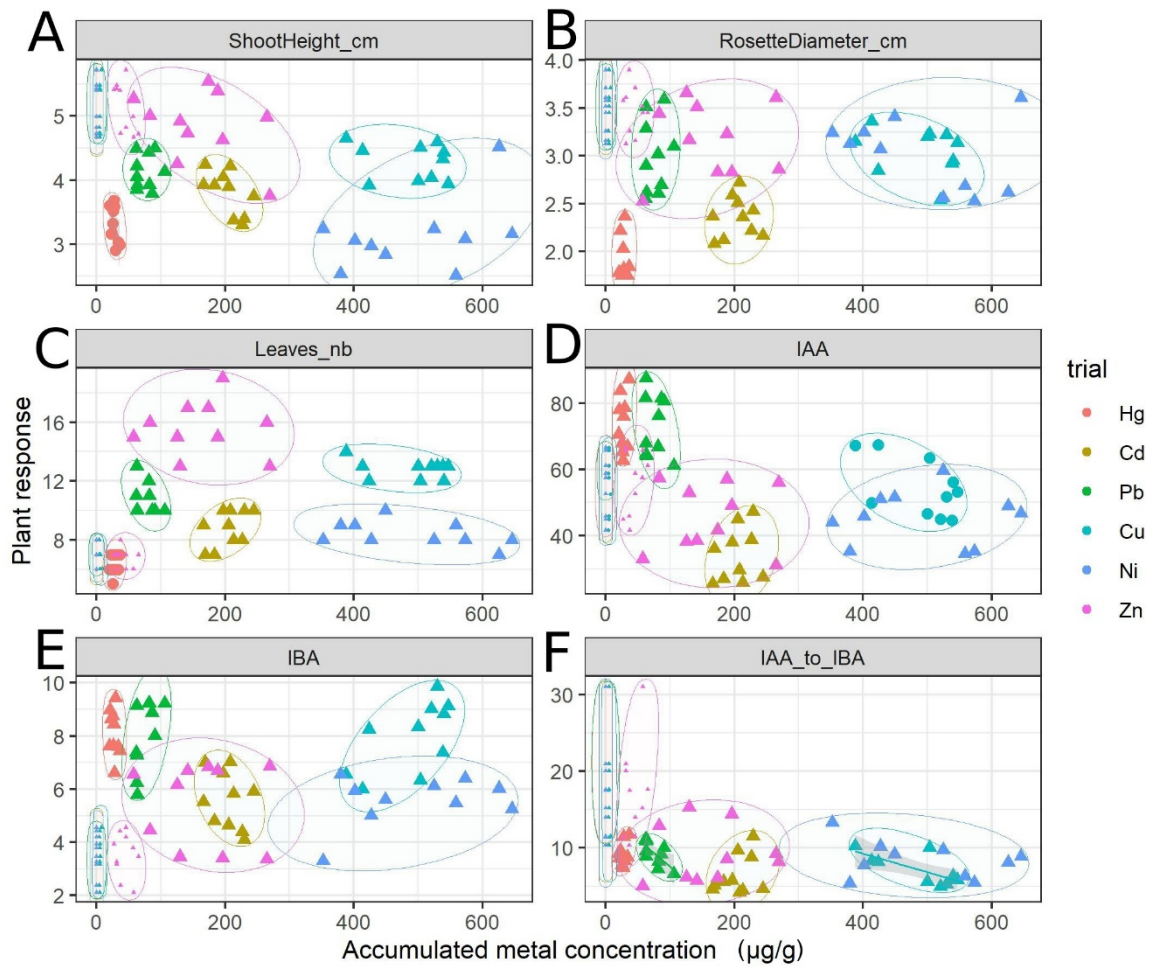
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25 **Supplementary Figure S1.** Principal component analysis of the (upper-left) root and (lower-
26 left) shoot responses and the distribution of responses depending on the metal concentration in
27 roots (upper-right) and shoots (upper-right) of *Arabidopsis* seedlings.

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Supplementary Figure S3. Root plant responses in relation to metal concentrations in *Arabidopsis* seedlings. Small points are used for control observations, large points for the others. Ellipses and colours identify responses for individual metals. Straight lines represent linear regression models among metal concentrations and plant responses, and their confidence intervals (only significant ones are shown, for better clarity). Triangles are used for metals for which their mean concentration is significantly different across treatments, circles for non-significant differences.

Supplementary Figure S4. Shoot plant responses in relation to metal concentrations in *Arabidopsis* seedlings. Small points are used for control observations, large points for the others. Ellipses and colours identify responses for individual metals. Straight lines represent linear regression models among metal concentrations and plant responses, and their confidence intervals (only significant ones are shown, for better clarity). Triangles are used for metals for which their mean concentration is significantly different across treatments, circles for non-significant differences.

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Supplementary Figure S5. Root of *Arabidopsis* seedlings grown without metals (control; ctrl).

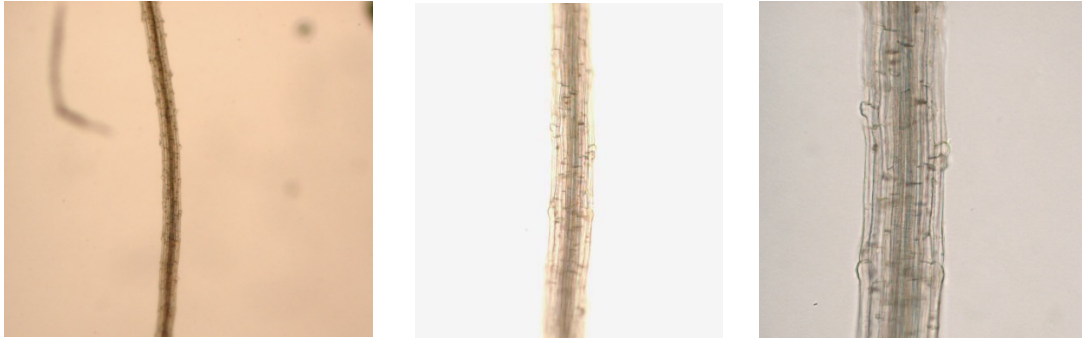Magnification (from left to right) = 4×, 10× and 20×.

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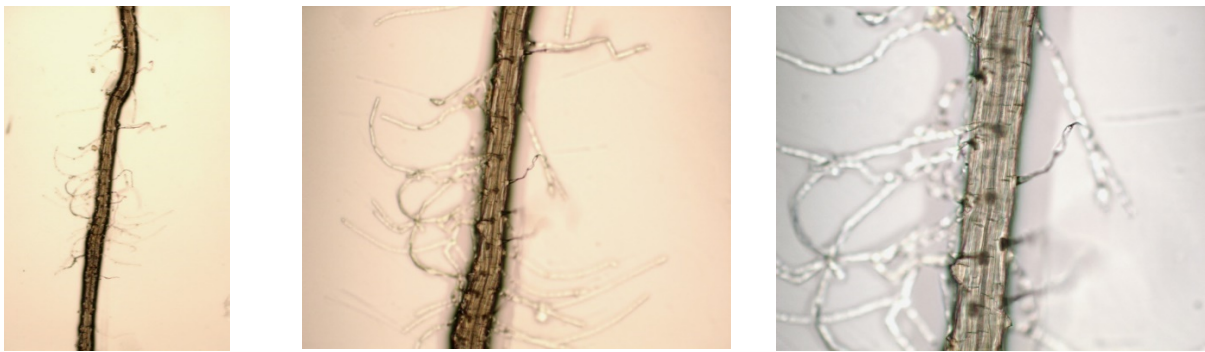58

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Supplementary Figure S6. Root of *Arabidopsis* seedlings grown under mercury (Hg).

Magnification (from left to right) = 4×, 10× and 20×.

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Supplementary Figure S7. Root of *Arabidopsis* seedlings grown under cadmium (Cd). Magnification (from left to right) = 4×, 10× and 20×.

Supplementary Figure S8. Root of *Arabidopsis* seedlings grown under lead (Pb). Magnification (from left to right) = 4×, 10× and 20×.

Supplementary Figure S9. Root of *Arabidopsis* seedlings grown under copper (Cu). Magnification (from left to right) = 4×, 10× and 20×.

Supplementary Figure S10. Root of *Arabidopsis* seedlings grown under nichel (Ni). Magnification (from left to right) = 4×, 10× and 20×.

Supplementary Figure S11. Root of *Arabidopsis* seedlings grown under zinc (Zn). Magnification (from left to right) = 4×, 10× and 20×.