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Plant architecture, auxin homeostasis and phenol content in *Arabidopsis thaliana* grown in cadmium- and zinc-enriched media

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ABSTRACT

A screening strategy using micropropagation glass tubes with a gradient of distances between germinating seeds and a metal-contaminated medium was used for studying alterations in root architecture and morphology of *Arabidopsis thaliana* treated with cadmium (Cd) and zinc (Zn) at the concentration of 10–20 μM and 100–200 μM, respectively. Metal concentrations in plant shoots and roots were measured by quadrupole inductively coupled plasma mass spectrometry. After 21 days from germination, all plants in the tubes were scanned at high resolution and the root systems analyzed. The localization of indole-3-acetic acid (IAA) in the primary root and lateral root apices was monitored using *DR5:GUS*, *LAX3:GUS* and *AUX1:GUS* Arabidopsis transgenic lines. Total phenol content in leaves was measured spectrophotometrically. Shoot and root dry weight and leaf area did not change in Zn-exposed plants and significantly decreased in Cd-exposed plants, compared to control plants. Cadmium induced a reduction of root length, of mean number of roots and of total root surface. Both Cd- and Zn-exposed plants showed a reduced specific root length. This morphological behavior, together with an observed increase in root diameter in metal-exposed plants could be interpreted as compensatory growth, and the observed thicker roots could act as a barrier to protect root from the metals. In comparison with the apical localization of the IAA signal in the control plants, Zn generally reinforced the intensity of IAA signal, without affecting its localization. In Cd-exposed plants, IAA localization remained apical but weaker compared to control plants. Total phenols decreased in plants exposed to Zn and Cd. Therefore, we propose that the remodelling of the root architecture and the production of some secondary metabolites, such as IAA and phenols could be two responses of plants subjected to metal stress. This knowledge can open the way to future phytoremediation strategies of contaminated sites.

1. Introduction

Plant survival under abiotic stress conditions requires morphological, physiological and biochemical adaptations. Among abiotic stresses, understanding the adaptation of plants towards toxic metal exposure in soil and water, and in response to airborne metal pollution, is of key importance. The majority of these metals get accumulated in plants and can either directly or indirectly find their way into the food chain. In particular, cadmium (Cd) and zinc (Zn) are usually found in many industrial polluted soils and both are able to induce morphological, physiological and biochemical changes in many plant species, even at sub-toxic tissue concentrations (Sanità di Toppi and Gabbrielli, 1999; Kabata-Pendias and Mukherjee, 2007; Cuypers et al., 2010). While Zn is present in or acts as cofactor of many cell macromolecules, Cd is highly toxic for plants, as it is not an essential nutrient.

The normal concentration of zinc for plant growth ranges from 30 to

Abbreviations: Arabidopsis, *Arabidopis thaliana*; AUX1, AUXIN RESISTANT1; Cd, cadmium; CE, catechol equivalents; DW, dry weight; FW, fresh weight; IAA, indole acetic acid; GUS, recombinant *E. coli* β-glucuronidase protein; LAX3, LIKE AUXIN RESISTANT3; LR, lateral root; PR, primary root; ROS, reactive oxygen species; SIMR, stress-induced morphogenic response; Zn, zinc

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200 μg g^{-1} dry weight (DW), and a tissue concentration > 400 μg g^{-1} DW is toxic for most of the plant species (Marschner, 2012). Regarding Cd, soil levels starting from 0.5 to 1.0 mg kg^{-1} can negatively affect plant growth and development (Sanità di Toppi and Gabbrielli, 1999; Cuypers et al., 2010). In addition, Cd is a major ecotoxic contaminant that it is translocated from polluted soils to crop plants and finally to food, with adverse effects on a wide range of biological processes in humans (Cuypers et al., 2010).

Arabidopsis thaliana (Arabidopsis), a model plant species, is sensitive to the exposure to Cd and Zn from a morphological, physiological, biochemical and molecular point of view (Semane et al., 2010; Sofo et al., 2013; Bochicchio et al., 2015; Gielen et al., 2016). An important question is how to study these processes at a small scale, as in the case of Arabidopsis, using an easy, cheap, replicable and reliable technique. A first attempt was made by Bochicchio et al. (2015), who observed the appearance of a 'stress-induced morphogenic response' (SIMR) in the roots of Arabidopsis plants exposed to sub-lethal concentrations of Cd and Zn in metal-contaminated agarized Petri dishes. The authors evidenced the SIMR as an increase in the number of lateral roots (LRs) and a decrease in primary root (PR) length. The stimulation of lateral meristems is mediated by the increase of auxin/cytokinin ratio, and governed by indoleacetic-3-acid (IAA), the most important auxin, and hormonal genetic regulation (Sofo et al., 2013; Vitti et al., 2013). In Arabidopsis, the remodelling of the root architecture in response to Cd and Zn also includes the increase in root diameter either as a consequence of metal-induced xylogenesis and as an expansion of the cortical parenchyma cells (Vitti et al., 2013; Bochicchio et al., 2015). Moreover, it is known that the level of plant secondary metabolites with free radical-scavenging properties, such as phenols, is regulated for facing abiotic and biotic stresses experienced by the plants in the surrounding environment (Haribabu and Sudha, 2011). This notwithstanding, the SIMR responses in the shoots of metal-exposed Arabidopsis plants, the content of phenolic compounds, and the localization and transport of IAA in plants facing metal stress are unknown.

On this basis, the present study aims at analysing the alterations in shoot and root morphology, auxin levels and phenol content in Arabidopsis plants exposed to the ranges of concentrations of Cd (10–20 μM) and Zn (100–200 M) commonly found in metal-polluted soils (Vangronsveld et al., 2009; Sofo et al., 2012). For this aim, a screening technique (i.e., plant growing in micropropagation glass tubes with a gradient of distances between germinating seeds and a metal-contaminated medium) was here used. As Arabidopsis is a model plant, this species could be important for a better understanding of the fundamental aspects of metal homeostasis, detoxification and metal tolerance in plants. In addition, the analysis the responses of Arabidopsis to Cd- and Zn-metal stress would allow to understand the bases of plant adaptive responses to toxic metals and could open the way to future phytoremediation of contaminated sites.

2. Materials and methods

2.1. Experimental design and plant material

Transparent micropropagation tubes (20 cm high; radius = 1.2 cm) with polypropylene transparent caps were filled with metal (Cd or Zn) + melted agarized medium [1% bacteriological agar (LP0011; Oxoid Ltd., Cambridge, UK), 0.5% sucrose and 1/4 strength Murashige and Skoog liquid medium without micronutrients (Sigma-Aldrich®), pH 5.8]. Metals were added to the agar medium in concentrations of 20 μM $CdSO_4$ or 200 μM $ZnSO_4$, and 15 mL of this melted agar were added for each tube. After solidification, other 15 mL of the agarized medium containing 10 μM $CdSO_4$ or 100 μM $ZnSO_4$ were added in each tube. After the second solidification, 15 mL of melted metal-free medium were poured into the tubes. The top agar surface was covered with a thin layer of approximately 5 mm of agarized medium + active carbon for shading the lower agar sections and simulate dark soil conditions.

Table 1
Concentrations of Cd and Zn in roots and shoots of Col-0 Arabidopsis plants not exposed to metals (Control), or exposed for 21 days to 10/20 μM $CdSO_4$ (Cd) and 100/200 μM $ZnSO_4$ (Zn) in metal-gradient agar tubes. Mean values (n = 20 treatment–1 ± SD) with different letters for each metal are significantly different between the treatments at $P \leq 0.05$, according to Fisher's LSD test. The statistical analysis was performed separately for roots and shoots. DW = dry weight.

		Cd	Zn
		(μg g^{-1} DW)	
Roots	Control	0.10 ± 0.02 c	21.32 ± 5.10 b
	Cd	670.87 ± 60.11 a	25.50 ± 3.75 b
	Zn	0.15 ± 0.05 b	756.19 ± 31.86 a
Shoots	Control	0.09 ± 0.03 b	18.21 ± 5.10 b
	Cd	421.77 ± 52.29 a	18.03 ± 5.98 b
	Zn	0.14 ± 0.05 b	181.44 ± 25.75 a

An empty space of approximately 10 cm from the upper border of top agar remained or allowing shoot development. Tubes without metals were prepared in the same way described above and considered as control. Other tubes without metals and with a uniform agar surface were prepared to be sure that the physical interface between the three agar sections could not affect root development. Agar density after solidification, was on average 1.2 g cm^{-3}.

Seeds of *Arabidopsis thaliana* (L.) Heynh. (Col-0 ecotype) and *DR5:GUS, LAX3:GUS* (LIKE AUXIN RESISTANT3) and *AUX1:GUS* (AUXIN RESISTANT1) transgenic lines were sterilized with 0.1% Na-hypochlorite + 1 drop of Tween® 80 (Sigma-Aldrich®, St. Louis, MO, USA), rinsed four times in sterile distilled water for 5 min, and then placed on moist filter paper in a Petri dish at 4 °C for 24 h in the dark. Col-0 ecotype was the background wild type (wt) of all the three transgenic lines. Sterilized seeds Col-0 (one per tube) were placed on agar surface and let germinate, and tubes sealed with parafilm. Excepting for the part of the tube above the active carbon layer, the rest of the tube surface was covered with a dark foil, in order to simulate the dark conditions of soil. The tubes were incubated vertically in a growth chamber for 21 days at 20 °C, with a 16-h photoperiod and a photosynthetic photon flux density of 300 μmol m^{-2} s^{-1} at top agar level. Three tubes not covered with dark foil were used for a time-lapse video clip (Video S1).

2.2. Metal content

All glassware containers used for metal content measurements were cleaned by washing with 10% ultra-pure grade HNO_3 for at least 24 h, and then rinsed copiously with ultra-pure water before use. Shoot and root samples were digested in a HNO_3:H_2O_2 solution (5:1 v/v) using a high-performance microwave digestion unit (MLS-1200 Mega, Milestone Inc., CT, USA). The metal concentration was determined by means of quadrupole inductively coupled plasma mass spectrometry, ICP-MS (Elan DRC II, Perkin-Elmer SCIEX, CT, USA), according to Huang et al. (2004). High purity He (99.9999%) and H_2 (99.9995%) were used, in order to minimize the potential problems caused by unidentified reactive contaminant species in the cell. The reference wavelengths for each metal were chosen avoiding interferences with the other elements analyzed. Blanks (only HNO_3 and H_2O_2) and a standard stock solution of 50 ppm for each element were analyzed for reference purposes. Reagent blanks containing ultra-pure water were additionally analyzed in order to control the purity of the reagents and the laboratory equipment. Results were expressed as μg of metal for g of dry weight (DW).

2.3. Plant morphology

Tubes were scanned at a resolution of 1200 DPI using a STD4800 Image Acquisition System. After scanning, plant shoots and roots were

Table 2
Shoot and root morphometric parameters of Col-0 Arabidopsis plants not exposed to metals (Control), or exposed for 21 days to 10/20 μM $CdSO_4$ (Cd) and 100/200 μM $ZnSO_4$ (Zn) in metal-gradient agar tubes. Mean values (n = 20 treatment^{-1} ± SD) with different letters for each parameter are significantly different between the treatments at $P \leq 0.05$, according to Fisher's LSD test. DW = dry weight.

	Leaf area	Specific leaf area	Total root length	Average root diameter	Lateral roots	Total root surface	Specific root length	Root/shoot area ratio
	(cm^2)	(cm^2 mg^{-1} DW)	(cm)	(μm)	(number)	(cm^2)	(cm mg^{-1} DW)	
Control	1.50 ± 0.33 a	1.16 ± 0.36 a	60.73 ± 19.86 a	80 ± 4 c	195 ± 11 a	1.55 ± 0.52 a	223.97 ± 22.97 a	1.08 ± 0.13 b
Cd	0.11 ± 0.05 b	0.43 ± 0.10 b	5.46 ± 1.35 b	105 ± 5a	30 ± 9 b	0.19 ± 0.05 b	62.33 ± 19.04 c	1.53 ± 0.24 a
Zn	1.44 ± 0.42 a	0.95 ± 0.19 a	50.22 ± 18.77 a	95 ± 5 b	239 ± 10 a	1.45 ± 0.44 a	148.83 ± 15.43 b	1.00 ± 0.19 b

Table 3
Shoot and root dry weights, and root/shoot weight ratio of Col-0 Arabidopsis plants not exposed to metals (Control), or exposed for 21 days to 10/20 μM $CdSO_4$ (Cd) and 100/200 μM $ZnSO_4$ (Zn) in metal-gradient agar tubes. Mean values (n = 20 treatment^{-1} ± SD) with different letters for each parameter are significantly different between the treatments at $P \leq 0.05$, according to Fisher's LSD test. DW = dry weight.

	Root DW	Shoot DW	Root/shoot weight ratio
	(mg)	(mg)	
Control	0.33 ± 0.18 a	1.44 ± 0.61 a	0.20 ± 0.03 b
Cd	0.10 ± 0.04 b	0.27 ± 0.15 b	0.32 ± 0.04 a
Zn	0.38 ± 0.07 a	1.63 ± 0.66 a	0.23 ± 0.06 b

analyzed by WinRhizo Arabidopsis V2009c (Regent Instruments Inc., Chemin Sainte-Foy, Canada) (Fig. S1) to determine total root length, average root diameter, number of LRs, and total root surface. Leaves were scanned to determine the area. The dry mass of roots and shoot were determined after harvesting separately and weighed after oven drying at 70 °C until constant weight. Root/shoot weight ratio, root/leaf area ratio, specific leaf area (leaf area per unit mass) and specific root length (root length per unit mass) were then calculated.

2.4. Histological and histochemical analysis

Plants of all the GUS-lines were processed for β-glucuronidase (GUS) staining, and incubated at 37 °C in the dark either for 30 min (*DR5:GUS* and *LAX3:GUS*) or 45 min (*AUX1:GUS*). Finally, the GUS buffer was replaced by 70% ethanol until observation. The observations were carried out with a Leica DMRB microscope (Leica Microsystems GmbH, Wetzlar, Germany), in bright field, and pictures were made with LEICA DC 500 camera and the software LEICA IM1000 Image Manager.

2.5. Phenolic content

Total phenolic content of the plants was determined by the Folin-Ciocalteu colorimetric method using catechol as a standard, according to Ojha and Chatterjee (2012). Sample was homogenized in 80% aqueous ethanol and mixture was centrifuged at 10,000*g* for 15 min and the supernatant was saved. The reaction was carried out in 96-well plates. Each reaction solution contained 16 μL of the supernatant obtained diluted to 320 μL with distilled water. 60 μL of Folin-Ciocalteu's phenol reagent were added, then the solution was mixed by inversion and left for 3 min. Then, 200 μL of a 20% sodium carbonate was added and solution mixed. After dark incubation for 60 min, absorbance was measured at 650 nm.

2.6. Statistics

The statistical analysis was performed by Sigmastat 3.1 SPSS Inc. software (SPSS Inc., Quarry Bay, Hong Kong). One-way analysis of variance (ANOVA) for each parameter was carried out with metal treatment as factor. Means were statistically analyzed by Fisher's LSD test at $P \leq 0.05$.

3. Results and discussion

3.1. Metal content

After 21 days, metal-exposed plants accumulated both Cd and Zn in roots, that were partially translocated to shoots, where they were found in lower concentrations (Table 1). Sofo et al. (2013) and Vitti et al. (2013) found comparable values of absorbed and translocated Cd and Zn in Arabidopsis plants grown in similar metal-stress conditions. Interestingly, Cd was more translocated to shoots than Zn (Table 1), and this was evident analysing the shoot/root Cd ratios (0.63 in Cd-exposed plants and 0.24 in Zn-exposed plants). The differences in shoot/root ratios for Cd and Zn could be due to the efficient Cd transport through the root cortex and its translocation *via* the xylem occurring in Arabidopsis, compared to other species (Vangronsveld et al., 2009; Cuypers et al., 2010). The low Zn translocation ratio here measured (0.24) is comparable to that (0.15) observed by Sofo et al. (2013), who exposed Arabidopsis plants to 150 μM $ZnSO_4$ for 14 days.

3.2. Plant growth and morphology

During the experimental period, shoot colour turned from light green in the control to reddish-yellowish-green in Zn-exposed plants, to yellowish-green in Cd-exposed plants (Video S1). Moreover, after 21 days, control and Zn-exposed plants reached flowering, whereas Cd-exposed ones did not (Video S1). The macroscopic observations of roots after 21 days from germination showed that both Cd- and Zn-exposed plants succeeded in PR growth beyond the contact surface with the high concentration of metal (20 and 200 μM for Cd and Zn, respectively). A significant decrease in leaf area and total root surface were recorded, with a significant 1.4-fold increase in root/shoot area ratio in Cd-exposed plants, compared to control ones (Table 2). Also, both shoot and root dry weight decreased in the Cd-exposed plants, with a significant increase in root/shoot weight ratio (about 1.6-fold) compared to the control (Table 3).

Different types of mild abiotic stresses experience by plants, including Arabidopsis, are able to generate a 'stress-induced morphogenic response' (SIMR), whose cellular messengers are mainly reactive oxygen species (ROS) and phytohormones (Potters et al., 2007; Zolla et al., 2010; Blomster et al., 2011). Similarly, in an arsenic (As) hyperaccumulator plant, i.e., the fern *Pteris vittata*, the exposure to Cd and/or As induced an increase in frond formation, that was interpreted as a morphogenetic response of the plant to defend against the stress effects (Ronzan et al., 2017).

In the present experiment, Arabidopsis root morphology was strongly and significantly influenced by metal stress, with more marked effects in the Cd treatment than in Zn one (Table 2). Firstly, Cd caused a significant decrease in total (PR + LRs) root length, compared to control and Zn-exposed plants (Table 2). Control plants had a mean total root length of 60.73 cm plant^{-1}, whereas the values in the plants exposed to Cd was 5.47 cm plant^{-1} (Table 2). This decrease was also due to a statistically lower number of LRs (Table 2). These results were partly in contrast with those of Bochicchio et al. (2015), who used lower Cd concentration in their plates and for a shorter time, probably

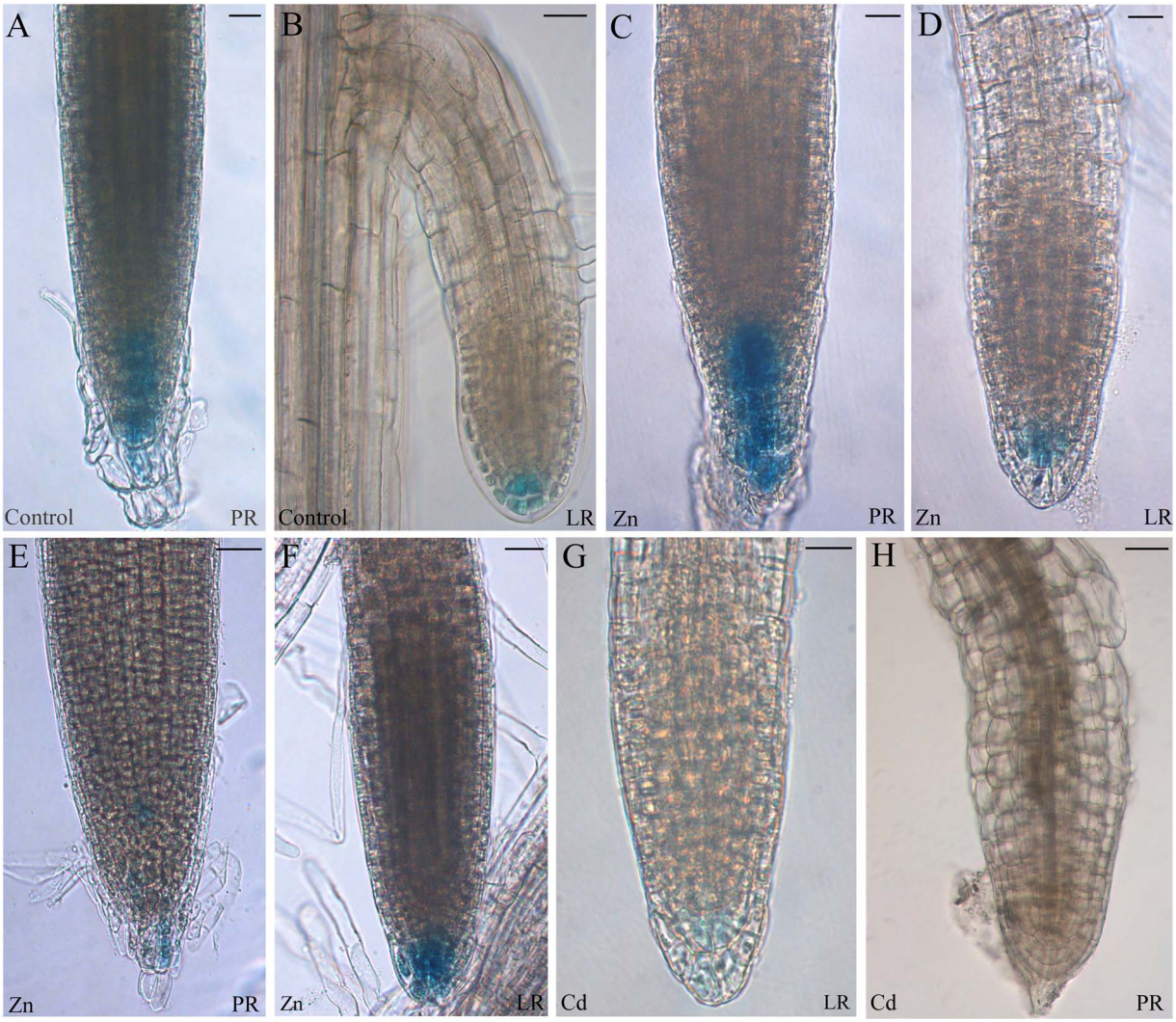

Fig. 1. Histochemical GUS staining analysis of *DR5:GUS* Arabidopsis of primary (PR) and lateral roots (LRs) of plants not exposed to metals (control = A, B), grown in the presence of 100/200 μM $ZnSO_4$ (C–F) or 10/20 μM $CdSO_4$ (G,H). A,B = PR (A) and LR (B) showing GUS signal in apical region. C-D = PR (C) and LR (D) grown in agar areas with 100 μM $ZnSO_4$ showing a reinforced GUS signal in apical region. E,F = PR (E) and LR (F) grown in agar areas with 200 μM $ZnSO_4$ with a decreased signal in PR apex and unchanged in LR. G = LR grown in agar areas with 10 μM $CdSO_4$ with a very weaker GUS expression. H = PR grown in agar areas with 20 μM $CdSO_4$ showing altered root morphology and absence of GUS signal. Bars = 20 μm (C–G); 50 μm (A-B, H).

without reaching the threshold level of Cd toxicity for plants. The high root/shoot weight ratio in the plants exposed to toxic Cd levels (Table 3) could also be a pollution 'escaping strategy' aimed at seeking metal-free patches.

Considering that there were no statistical differences in total root length between control and Zn-exposed plants, and that the number of LRs was higher in the Zn treatment (Table 2), the inception of LRs was associated with a reduction in PR growth. The changes in total root length (PR + LRs) due to metal exposure were accompanied by similar differences in total root surface and specific root length (Table 2). Similar differences, at lower Cd concentrations and exposure times were observed by Gielen et al. (2016). In general, a reduction in root length due to decreased root elongation and lateral root formation is reported as consequence of metal stress and specifically Cd toxicity (Gallego et al., 2012; Lux et al., 2011) and Zn (Ivanov et al., 2003), although mechanisms vary with single metal species. Secondly, Cd, and in a lesser extent Zn, caused a significant increase in average root diameter, compared to control plants (Table 2). This, in turn, was one of the causes of the drop in total root surface area in Cd-exposed plants, that was reduced from 1.55 cm^2 $plant^{-1}$ to 0.19 cm^2 $plant^{-1}$, in the control vs Cd-exposed plants (Table 2). As evidenced by several authors (Ďurčeková et al., 2007; Magalhães et al., 2010; Lux et al., 2011), changes in root development due to xylogenesis, premature endodermis differentiation, and expansion and lignification of cortical and stelar tissues could be the causes of the observed increase in average root diameter between metal-exposed and control plants (Table 2). Precocious endodermis differentiation, cellular hypertrophy and cell wall thickening by lignin deposition in the cortex were also reported in tobacco plants exposed to Cd (Zanella et al., 2016).

Generally, Cd influenced root morphology more than Zn (Table 2). On the other side, Zn caused a higher lateral root proliferation (Table 2). Terzano et al. (2008) found that Zn accumulates at the level of the root endodermis and pericycle, where it can induce LR induction. The effects of metals on shoot and root growth are usually complex: besides direct toxicity a number of indirect processes are triggered by the fact that roots are the first organ to contact the toxic metal. In general, toxic metal concentrations are reported to reduce root growth more than shoot growth, and this results in a lower root/shoot ratio, although a wide variation is found with plant species and growth conditions (Barceló and Poschenrieder, 1990). The reduction in root growth, though, deeply affects processes such as water uptake and transport, stomatal closure, hormone synthesis and translocation, which may be then responsible for other morphological changes not directly caused by metal toxicity (Rucińska-Sobkowiak, 2016). This is

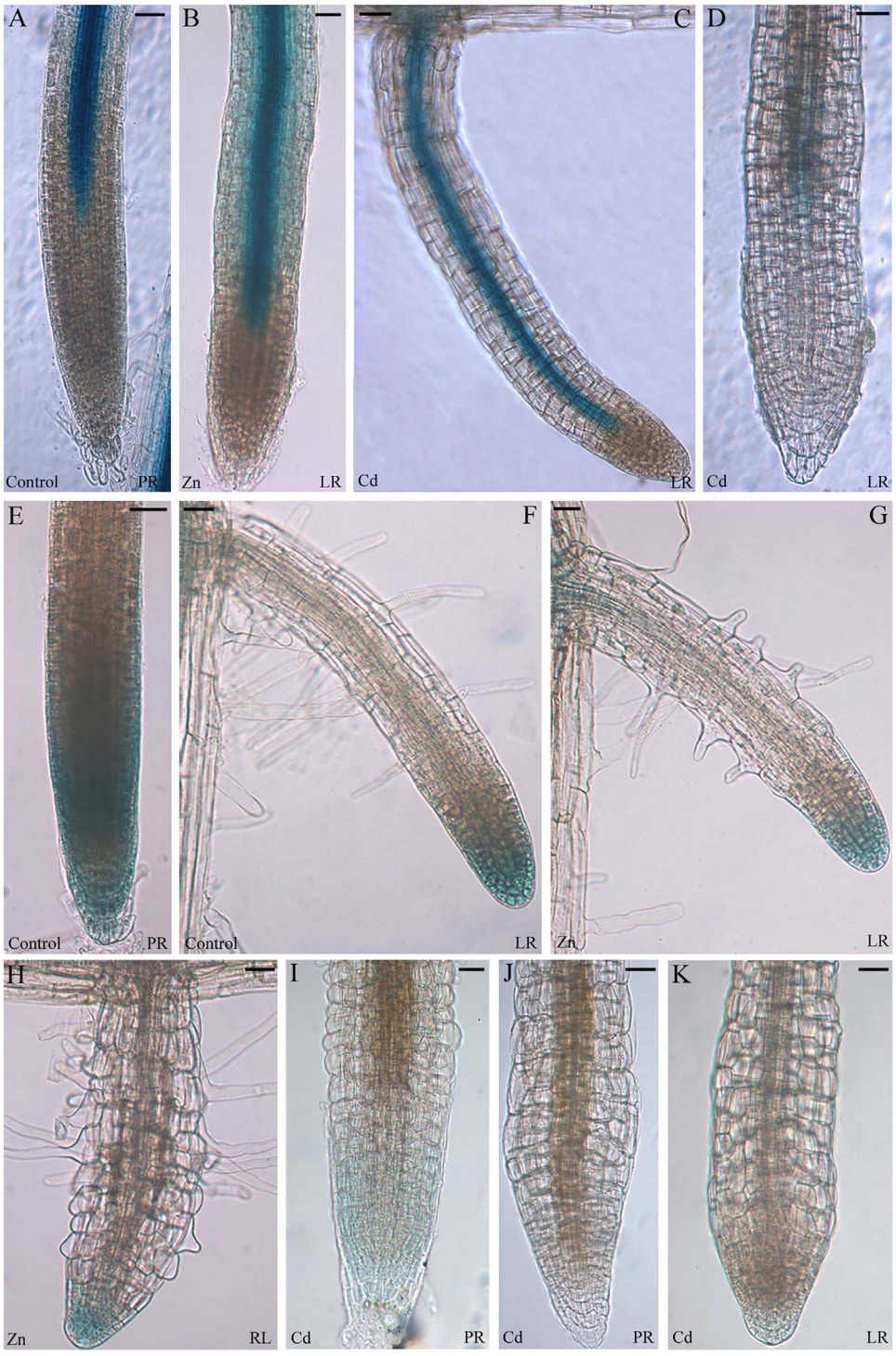

Fig. 2. GUS expression analysis of *LAX3:GUS* (A-D) and *AUX1:GUS* (E-J) Arabidopsis lines non exposed (control = A, E-F) and exposed to metals (B-D, G-J). A = *LAX3* expression in PR showing a strong signal in the vascular strand. B = *LAX3* expression in LR grown in agar areas with 100 μM $ZnSO_4$ with a GUS signal in the vascular strand. C = *LAX3* expression in LR grown in agar areas with 10 μM $CdSO_4$ showing GUS signal in the vascular strand. D = *LAX3* expression in LR grown in agar areas with 20 μM $CdSO_4$ showing an altered morphology and reduced GUS signal. E-F = PR and LR of control plants expressing *AUX1* in all tip tissues. G = LR grown in agar areas with 100 μM $ZnSO_4$ with tip expression of *AUX1*. H = morphologically altered RL grown in agar areas with 200 μM $ZnSO_4$ showing reduced *AUX1* signal. I = PR grown in agar areas with 10 μM $CdSO_4$ with weaker tip *AUX1* expression. J-K = PR (J) and LR (K) grown in agar areas with 20 μM $CdSO_4$ with altered morphology and without *AUX1* expression. Bars = 30 μm (B, F-I, K); 50 μm (A, C-E, J).

more likely if morphological changes such as a reduction in length and an increase in diameter, as found in our case, reduce the root-soil contact (lower total root surface) and increase the radial resistance to water flow (Barceló and Poschenrieder, 1990). Therefore, a higher reduction in shoot rather than root and a high root/shoot area ratio, as in plants exposed to Cd in our study (Table 2), can therefore be interpreted as an indirect response to metal mediated by the impairment of water and hormone relations. A lower specific root length (Table 2), as found here in Cd-exposed plants, is also an indicator of a low conversion of photosynthetates allocated to roots into absorbing surfaces, resulting in low water uptake efficiency and reduced contact of the roots with toxic metals (Rucińska-Sobkowiak, 2016).

Effects of metals on plant shoots include a reduction of weight and leaf area as found in our experiment, and specifically effects of Cd have been documented in *Pisum vulgaris* (Barceló and Poschenrieder, 1990), and of Zn on *Nicotiana tabacum* (Bazihizina et al., 2014). The authors of these studies commented this in terms of a major effect of metals on plant water relations. Effects of Cd stress on specific leaf area, or its inverse specific leaf weight in accordance with our data, have been reported by Sharma and Agrawal (2006) in carrots, but the effect varies with plant age/time of exposure. This is linked to variations in leaf anatomy like the reduction in intercellular spaces in the mesophyll as shown by Tran et al. (2013) for *Pisum sativum* or to increases in leaf thickness, and is a general index of a reduction of efficiency of

Table 4
Total phenol content in Col-0 Arabidopsis plants (shoot + root) not exposed to metals (Control), or exposed for 21 days to 10/20 μM CdSO4 (Cd) and 100/200 μM ZnSO4 (Zn) in metal-gradient agar tubes. Mean values (n = 20 treatment–1 ± SD) with different significantly different between the treatments at $P \leq 0.05$, according to Fisher's LSD test. CE = catechol equivalents; FW = fresh weight.

	Total phenols
	(mg CE g^{-1} FW)
Control	11.42 ± 2.10 b
Cd	1.78 ± 0.58 b
Zn	1.25 ± 0.36 a

conversion of photosyntates into photosynthetically active surfaces. Its reduction is classically linked to water shortage stress across ecosystems or management (Cunningham et al., 1999; Liu and Stützel, 2004) and its relations with metal toxicity deserve further inquiry to clarify water relations of metal stressed plants.

3.3. IAA localization and transport in roots

Many authors identified the major chemical messengers and signal-transduction pathways that influences auxin-dependent lateral root initiation in Arabidopsis (Peleg and Blumwald, 2011; Baster et al., 2013; De Smet et al., 2015). Particularly, the up-regulation of some genes involved in IAA biosynthesis, and the increase in auxin content and auxin/cytokinin ratio are all factors of key importance in determining morphological root changes in plants experiencing metal stressors, as evidenced by Sofo et al. (2013) and Vitti et al. (2013). *DR5:GUS* system, the well- known reporter of auxin-induced gene expression, allows to monitor auxin distribution during root formation. LIKE AUXIN RESISTANT3 (LAX3) and AUXIN RESISTANT1 (AUX1) are IAA influx carriers essential for the root formation. In Arabidopsis, they are required for root quiescent centre organization in PR (Ugartechea-Chirino et al., 2010). Moreover, AUX1 and LAX3 are also involved in LR formation (Swarup et al., 2008). LAX3 is required for defining the IAA maximum and maintenance in the adventitious root tip (Della Rovere et al., 2013). AUX1 controls organ identity by mediating auxin–cytokinin interaction in Arabidopsis (Kakani et al., 2009). A role for LAX3 in auxin/cytokinin distribution during adventitious root development was also reported (Della Rovere et al., 2013).

The localization of IAA in the PR apices and in those of the LRs was monitored after 21 days from germination by the use of the *DR5:GUS* transgenic line (Fig. 1). In comparison with the apical localization of the IAA signal in the PR and LRs of the control plants (Fig. 1A and B), Zn seemed to reinforce the intensity of the signal, without affecting its localization, i.e., the apical auxin maximum, in the PR and LRs growing in the agar with 100 μM of the ion (Fig. 1C and D). In the agar-zone at a Zn concentration of 200 μM, the IAA signal was strongly reduced in the apex of PR (Fig. 1E), whereas it remained unchanged in the LR apices (Fig. 1F). In the plants exposed to 10 μM Cd, the IAA localization remained apical in the roots, but weaker than control and Zn-exposed plants (Fig. 1G). Moreover, the PR and LR apical morphology was strongly altered at 20 μM Cd, in concomitance with the disappearance of the auxin signal (Fig. 1H).

The expression pattern of the *LAX3* auxin influx carrier was monitored by the use of the *LAX3:GUS* line (Fig. 2A–D). No significant change occurred in the LAX3 expression pattern in PR and LRs exposed to the metals. In fact, the main part of the PR apex and of the apex of the LRs showed no expression, as in the control (Fig. 2A–C). The expression remained restricted to the vascular differentiating region and was reduced in the PR of the Cd-exposed plants (Fig. 2D). The expression pattern of the *AUX1* auxin influx carrier was analyzed by the use of the *AUX1:GUS* line (Fig. 2E–K). In the PR and LRs of the control plants the signal was extended to all the apical dome (Fig. 2E and F). In the presence of 100 μM Zn, the signal was similar to the control (Fig. 2G), whereas at 200 μM the LRs showed an altered morphology, with a weak, and spatially reduced, apical signal (Fig. 2H). The exposure to Cd caused a strong reduction of the gene expression already at 10 μM in the PR apical meristem (Fig. 2I). Moreover, the signal progressively disappeared, in concomitance with the appearance of strong morphological alterations, either in the PR and LR root apices (Fig. 2J and K).

3.4. Phenolic content

It is known that the influence of different abiotic factors on secondary metabolites, such as phenols, in plants include salt, drought, light, heavy metals, frost etc. (Ramakrishna and Aswathanarayana Ravishanka, 2011; Kapoor et al., 2014; Król et al., 2014). The metabolism of several phenols has been reported to change under metal stress, being up- or down-regulated on the basis of metal concentrations (Mourato et al., 2012). When plant experience metal stress, phenolic compounds can act as metal chelators and/or directly as ROS scavengers (Michalak, 2006). In this study, total phenol content dramatically decreased both in Cd- and Zn-exposed plants (1.78 and 1.25 mg CE g^{-1} FW, compared to 11.42 mg CE g^{-1} FW of the control), indicating that plants entered in an acute phase of metal stress after 21 days of metal exposure (Table 4).

Similar results were obtained by Haribabu and Sudha (2011), who found decreased phenolic contents in plants of *Cleome gynandra* subjected to long-term (four weeks) copper and Cd stress. Kapoor et al. (2014) found that exposure times (30 days, comparable to that used in this work) with higher Cd concentrations (up to 0.6 mM Cd) did not determine significant increases of total phenolic content in metal-exposed *Brassica juncea* plants. It seems that, as a stress response, Arabidopsis plants synthesize phenols and secrete them in the medium through the roots, where, according to Michalak (2006) and Mourato et al. (2012), they can act as metal chelators and/or directly as ROS scavengers.

4. Conclusions

The results obtained in this work highlighted the influence of Cd, and in a lesser extent of Zn, on plant architecture and the high phytotoxicity of Cd, compared to Zn. In turn, root architecture is partially remodeled by the action of IAA, and phenols produced in the presence of metals. The identification of the genes involved in metal uptake, transport and compartmentation of metals in *Arabidoosis thaliana* provides a bridge between functional genetics and evolutionary analyses also for other species of high interest in phytoremediation processes, such as the close relative *Arabidopsis halleri* and *Thlaspi caerulescens*, both able to hyperaccumulate Cd and Zn. As *Arabidopsis thaliana* is a species belonging to the family of Brassicaceae, that includes many cultivated species considered as good metal accumulators (Vangronsveld et al., 2009; Sofo et al., 2012), this knowledge can open the way to field studies regarding the phytoremediation of contaminated sites.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2017.06.008.

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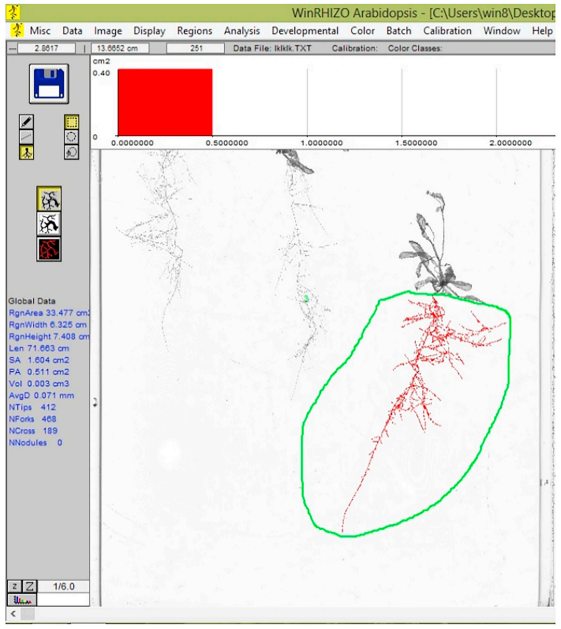
Supplementary material captions

Video S1. Arabidopsis plants (center) not exposed to metals (control), or exposed for 21 days to (left) 10/20 µM $CdSO_4$ (Cd) and (right) 100/200 µM $ZnSO_4$ (Zn) in metal-gradient agar tubes.

Figure S1. Screenshoots from WinRhizo Arabidopsis software. On the left, a control plants; on the right a Cd-exposed plant.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RgnHeight 7.408 cm
Len 71.663 cm
SA 1.604 cm2
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Vol 0.003 cm3
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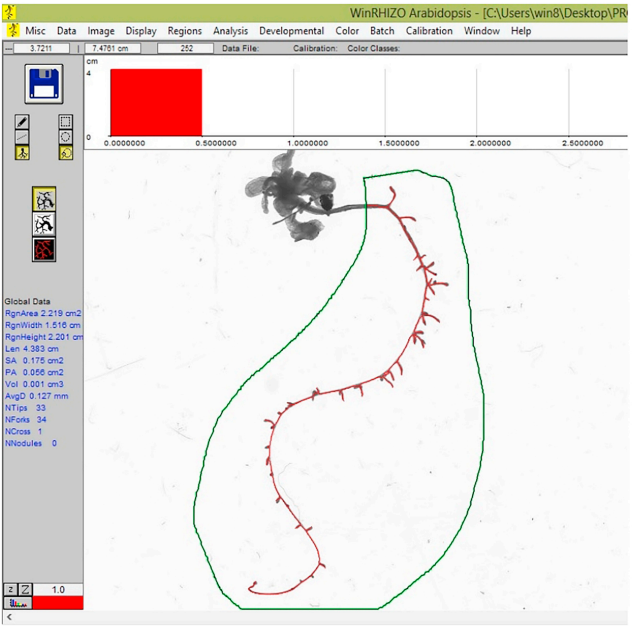
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RgnWidth 1.516 cm
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Len 4.383 cm
SA 0.175 cm2
PA 0.056 cm2
Vol 0.001 cm3
AvgD 0.127 mm
NTips 33
NForks 34
NCross 1
NNodules 0
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