



Research article

Root architecture and morphometric analysis of *Arabidopsis thaliana* grown in Cd/Cu/Zn-gradient agar dishes: A new screening technique for studying plant response to metals



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ABSTRACT

A new screening strategy using Petri dishes 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, copper and zinc at sub-toxic concentrations. Metal concentrations in the dishes were determined by anodic stripping voltammetry on digested agar samples collected along the gradient, and kriging statistical interpolation method was performed. After two weeks, all agar dishes were scanned at high resolution and the root systems analyzed. In the presence of all the three metals, primary root length did not significantly change compared to controls, excepting for zinc applied alone (+45% of controls). In metal-treated seedlings, root system total length increased due to the higher number of lateral roots. The seedlings closer to the agar sectors including metals showed a marked curvature and a higher root branching in comparison to those further away from the metals. This behavior, together with an observed increase in root diameter in metal-treated seedlings could be interpreted as compensatory growth, and a thicker roots could act as a barrier to protect root from the metals. We therefore propose that the remodeling of the root architecture in response to metals could be a pollution 'escaping strategy' aimed at seeking metal-free patches.

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1. Introduction

Phytoremediation is an important tool to remove metal(loid)s and other contaminants from polluted sites (Vangronsveld et al., 2009). Real progress in this research field depends on an in depth-analysis of the plant responding mechanisms to metal action, both at molecular and cellular levels (Hassan and Aarts, 2011). Cadmium (Cd), copper (Cu) and zinc (Zn) are common metals of polluted soils and are known to induce stress effects in all plant

species also at low tissue concentration (Kabata-Pendias and Mukherjee, 2007). However, while Cu and Zn are part of or act as cofactors of many cell macromolecules (homeostatic concentrations in plant tissues are from 6 to 12 $\mu\text{g g}^{-1}$ DW for Cu and from 30 to 200 $\mu\text{g g}^{-1}$ DW for Zn, according to Marschner, 2012), plants do not usually encounter elevated levels of Cd under normal environmental conditions and have no metabolic requirement for this metal. Levels of 1–5 μM of Cd in the soil solution are sufficient to retard plant root growth (Sanità di Toppi and Gabrielli, 1999; Cuyppers et al., 2010). Furthermore, Cd is a major environmental contaminant that enters human food via accumulation in crop plants and is considered as being one of the most ecotoxic metals that exhibits adverse effects on a wide range of biological processes in both animals and plants (Cuyppers et al., 2010).

The molecular, biochemical, physiological and morphological characteristics of the model plant *Arabidopsis thaliana* (*Arabidopsis*) are strongly affected by the exposure to Cd, Cu and Zn (Smeets et al., 2009; Semane et al., 2010; Watanabe et al., 2010; Sofo et al., 2013).

Abbreviations: *Arabidopsis*, *Arabidopsis thaliana*; IAA, indoleacetic acid; ROS, reactive oxygen species; SE, standard error; SIMR, stress-induced morphogenic response.

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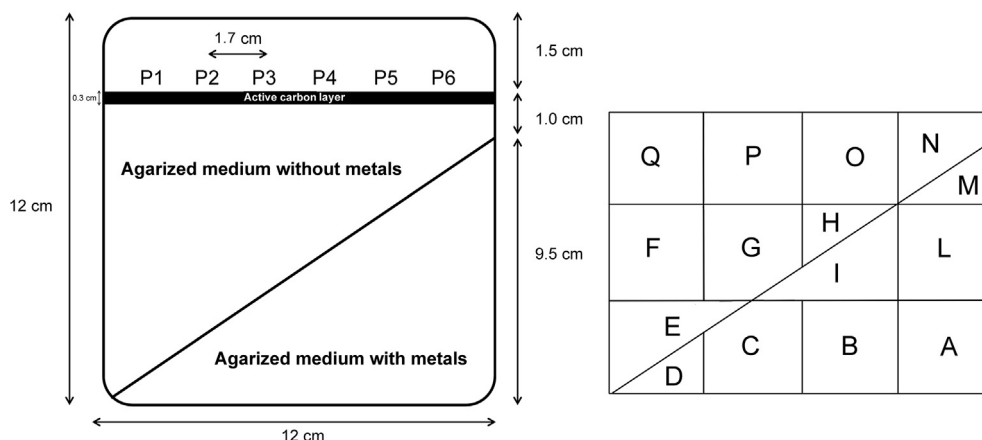


Fig. 1. (Left) Scheme of the agar dishes used. Plant position from left to right = P1–P6; with P1 being the most distant from the metal(s). (Right) Division of the agar surface (sector areas reported in Table 1).

The great importance of the responses of *Arabidopsis* to metals is due to the fact that it belongs to the family of Brassicaceae (Cruciferae), whose cultivated species are known to be good accumulators of toxic metals, allocating large amounts of most toxic metals to above-ground organs (Vangronsveld et al., 2009; Sofo et al., 2012). Furthermore, many genera of Brassicaceae (e.g., *Brassica*, *Alyssum*, *Arabis*, *Arabidopsis*, *Berkheya*, *Bornmuellera*, *Cardamine*, *Cochlearia*, *Peltaria*, *Pseudosempervivum*, *Stanleya*, *Streptanthus*, and *Thlaspi*) are well known for their use in toxic metals-remediation strategies (Sofo et al., 2012).

Field responses of plants are complex because of many factors: toxicity of metals is mediated by texture and soil status (Selim and Sparks, 2001), and by synergistic behavior of different chemicals. Also, the spatial distribution of pollutants in soils can be patchy even at a small scale and the distance of living organisms from hotspots of toxic chemicals may affect their survival rate and their effectiveness in bioremediation (Salminen and Haimi, 1999; Mukherjee et al., 2014). Plant characteristics relevant to field behavior therefore encompass morphological and architectural responses to metal distributions where many issues play a role, including the distance of roots from the areas containing high metal concentrations. 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.

The present study aims at elucidating the alterations in root architecture and morphology in *Arabidopsis* seedlings exposed to Cd, Cu and Zn, supplied either alone or in triple combination, as the presence of more than one metal is the common situation in polluted soils. For this purpose, a new screening strategy using Petri dishes (12 × 12 cm) with a gradient of distances between germinating seeds and a metal-contaminated medium was used and described here in detail. As a model plant, *A. thaliana* could be important to gain insights into adaptive evolution of ecologically important traits at the basis of metal homeostasis, detoxification and metal tolerance in plants that could be used for phytoremediation purposes.

2. Materials and methods

2.1. Experimental design and plant material

Square (12 × 12 cm) Petri dishes (Greiner 688102; Sigma–Aldrich®) 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 micro-nutrient and vitamins (Sigma–Aldrich®), pH 5.8. Metals were added to the agar medium in the form of 10 μM CdSO₄, 5 μM CuSO₄, 150 μM ZnSO₄, alone or in triple combination (Cd/Cu/Zn). After the medium solidified, the gel was cut diagonally in half (as shown in Fig. 1) under sterile conditions and the upper half discharged. Then, melted metal-free medium was poured into the dish and filled the empty space. After the second solidification step, top agar (1.5 cm from the upper border) was removed to allow shoot development. For simulating the dark conditions of the soil, the top agar surface was covered with a thin layer (approximately 3 mm) of agarized medium + active carbon (Fig. 1). Dishes without metals in both upper and lower agar sections were prepared in the same way and were kept as controls. Other dishes without metals and with a uniform agar surface (not divided in two halves) was prepared to be sure that the physical interface between the two agar sections could not affect root development. Agar average thickness after solidification was 0.4 cm, with an average density of 1.24 g/cm³.

Seeds of *A. thaliana* (L.) Heynh. (Columbia ecotype; Col-0) were sterilized using 0.1% Na-hypochlorite + 1 drop Tween® 80 (Sigma–Aldrich®, St. Louis, MO, USA), 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. Six sterilized seeds were placed on agar surface, just below the upper border, placed to 1.5-cm from each other by means of sterilized toothpick, and let germinate. Dishes were sealed with parafilm, with a 1-cm interruption in the central part of the lower side for promoting gas exchange. Excepting for the upper part (above the active carbon layer), the rest of the dish surface was covered with a dark foil. 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 of 300 μmol m⁻² s⁻¹ at top agar level. Before the main experiment, dishes containing metals in both the upper and lower part were used to test the toxicity of metals at different concentrations, in order to choose the most suitable ones.

2.2. Metal content measurements and kriging interpolation

Metal concentration in the dishes' sectors was determined by anodic stripping voltammetry (ASV) on a dropping mercury electrode (model 757 VA Computrace; Metrohm Inc., Riverview, FL, USA). Agar gel was divided into 15 sectors according to the metal gradient (Fig. 1). Each agar section was cut by means of a plastic knife, weighed and placed in a glass tube. One milliliter of concentrated HNO₃ (65%, Sigma–Aldrich, TraceSelect® grade) was

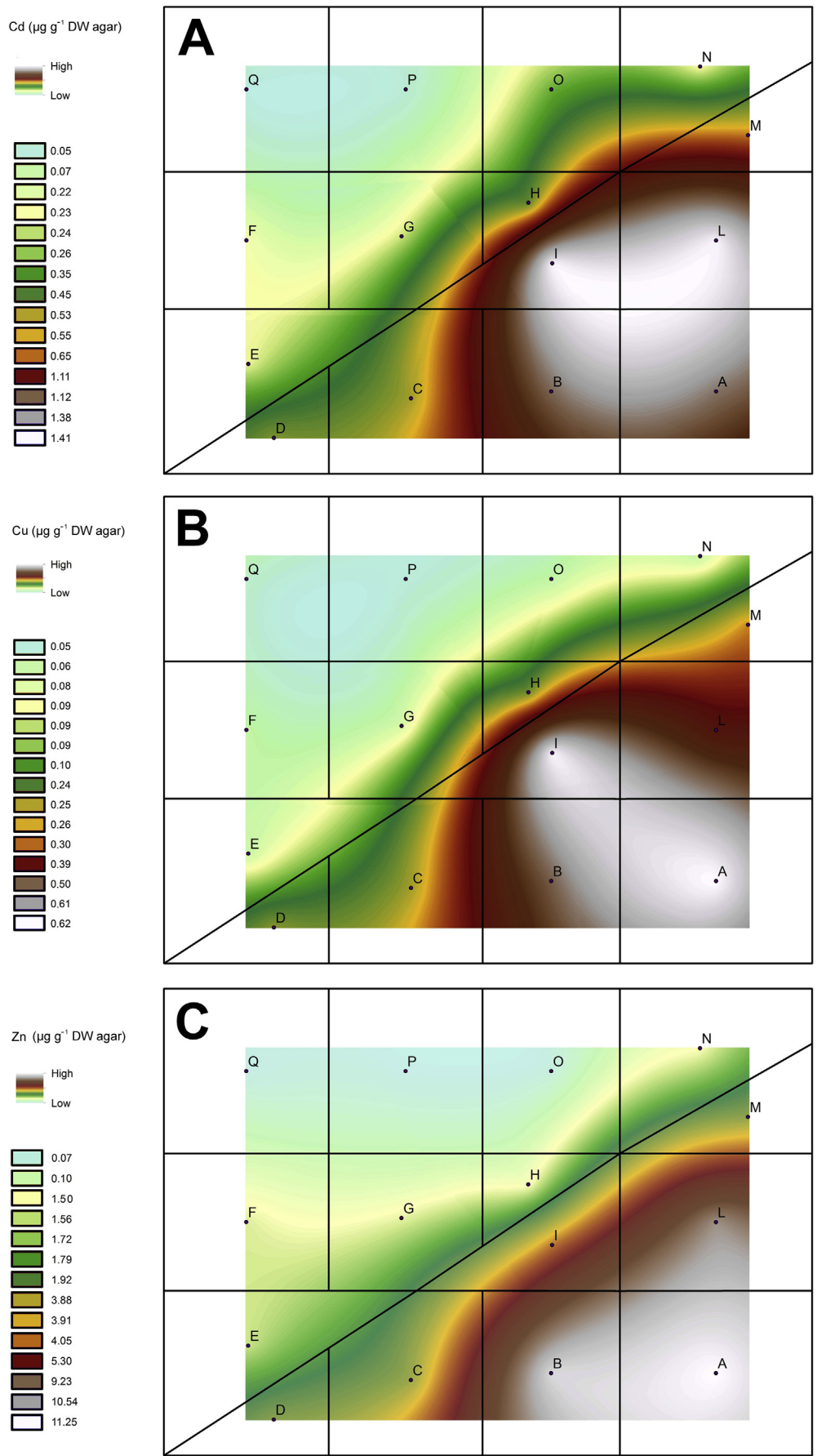


Fig. 2. Kriging statistical interpolation ($n = 5$ treatment $^{-1}$) of (A) Cd, (B) Cu and (C) Zn concentrations in the agar dishes. Agar sectors were depicted in Fig. 1. • = center point. Kriging elaboration values are showed at the right of each table.

Table 1

Mean concentrations (\pm SE; $n = 5$ treatment⁻¹) of Cd, Cu and Zn in the different sectors of the agar dishes shown in Fig. 1.

| Agar sector | Sector area (cm ²) | Cd ($\mu\text{g g}^{-1}$ DW agar) | Cu | Zn |
|-------------|-----------------------------------|---------------------------------------|-----------------|------------------|
| A | 7.5 | 1.13 \pm 0.10 | 0.63 \pm 0.05 | 11.25 \pm 2.41 |
| B | 10.5 | 1.11 \pm 0.10 | 0.50 \pm 0.04 | 10.55 \pm 1.75 |
| C | 7.3 | 0.55 \pm 0.04 | 0.27 \pm 0.02 | 4.05 \pm 0.82 |
| D | 2.8 | 0.54 \pm 0.09 | 0.26 \pm 0.05 | 3.88 \pm 0.40 |
| E | 6.6 | 0.24 \pm 0.03 | 0.10 \pm 0.02 | 1.92 \pm 0.23 |
| F | 7.5 | 0.23 \pm 0.04 | 0.09 \pm 0.03 | 1.80 \pm 0.09 |
| G | 5.6 | 0.27 \pm 0.06 | 0.11 \pm 0.03 | 1.72 \pm 0.18 |
| H | 1.9 | 0.45 \pm 0.06 | 0.25 \pm 0.05 | 1.98 \pm 0.15 |
| I | 4.6 | 1.41 \pm 0.12 | 0.62 \pm 0.07 | 5.31 \pm 1.02 |
| L | 8.8 | 1.38 \pm 0.12 | 0.40 \pm 0.02 | 9.24 \pm 1.05 |
| M | 3.5 | 0.66 \pm 0.08 | 0.31 \pm 0.04 | 3.92 \pm 0.34 |
| N | 7.0 | 0.22 \pm 0.06 | 0.09 \pm 0.02 | 1.56 \pm 0.25 |
| O | 7.5 | 0.35 \pm 0.05 | 0.09 \pm 0.02 | 0.29 \pm 0.03 |
| P | 8.4 | 0.20 \pm 0.02 | 0.05 \pm 0.00 | 0.19 \pm 0.08 |
| Q | 7.5 | 0.20 \pm 0.02 | 0.06 \pm 0.01 | 0.13 \pm 0.03 |

added to the tube and the agar was left to dissolve at room temperature. One hundred microliters of the digested sample were added to 7 ml of deionized water in the measurement cell and additional 100 μl of 3 M KCl solution were added to the mixture as background electrolyte. The solution was then flushed with N₂ under continuous stirring for 300 s. The voltammetric parameters employed for the analyses were set according to Metrohm Application Bulletin 231 and DIN 38406 part 16 (Metrohm Inc.). Quantification was performed by using the method of standard additions. Three replicate measurements were made for each sample.

On the basis of metal measurements of the sections selected in the dishes, kriging statistical interpolation method, that allows to measure the average error and the standardized error variance through cross-validation, was performed using the software ArcGIS ArcMap version 10.1 (ESRI Inc., Redlands, CA, USA). In order to taking into account the possible effects of dish borders on metal gradients, the areas within 1 cm from the borders were not considered in kriging interpolation (Fig. 2).

2.3. Root growth observations

After 14 days from germination, all agar dishes were scanned at high resolution (600 DPI) by STD4800 Image Acquisition System and the whole root systems analyzed by WinRhizo Arabidopsis V2009c (Regent Instruments Inc., Chemin Sainte-Foy, Canada). For each plant, the following root morphological measurements were made: total length of the root system, total surface area, average root diameter, number of root tips, main root length, total length of lateral roots, and root growth measured in sexagesimal angle respect to gravity direction. The values of these parameters were averaged for 120 plants ($n = 120$; 20 dishes per treatment). The

Table 2

Root morphometric parameters and root bending of Arabidopsis seedlings not exposed to metals (Control), or exposed for 14 days to 10 μM Cd, 5 μM Cu, 150 μM Zn, and Cd/Cu/Zn in triple combination in metal-gradient agar dishes. Mean values ($n = 120$ treatment⁻¹ \pm SE) with different letters for each parameter are significantly different between the treatments at $P \leq 0.05$, according to Fisher's LSD test.

| Treatment | Total root length (cm) | Total root surface area (cm ²) | Average root diameter (mm) | Root tips (Number) | Main root length (cm) | Lateral root length (cm) | Lateral/total length ratio (%) | Root bending (°) |
|-----------|---------------------------|---|-------------------------------|-----------------------|--------------------------|-----------------------------|-----------------------------------|---------------------|
| Cd | 8.68 \pm 1.21 c | 0.37 \pm 0.05 c | 0.11 \pm 0.02 b | 29 \pm 4 c | 3.65 \pm 0.46 b | 5.02 \pm 1.03 b | 57.9 b | 13 \pm 3 b |
| Cu | 11.80 \pm 2.03 b | 0.54 \pm 0.10 b | 0.17 \pm 0.02 a | 48 \pm 3 b | 3.87 \pm 0.40 b | 7.92 \pm 1.39 b | 67.2 a | 23 \pm 5 a |
| Zn | 18.70 \pm 1.64 a | 0.94 \pm 0.10 a | 0.11 \pm 0.02 b | 60 \pm 6 a | 7.29 \pm 0.82 a | 11.41 \pm 2.09 a | 61.0 a | 30 \pm 5 a |
| Cd/Cu/Zn | 7.50 \pm 1.17 c | 0.30 \pm 0.02 c | 0.13 \pm 0.03 b | 28 \pm 4 c | 3.94 \pm 0.47 b | 3.56 \pm 0.98 c | 47.5 b | 25 \pm 3 a |
| Control | 5.30 \pm 0.83 d | 0.25 \pm 0.02 d | 0.07 \pm 0.01 c | 19 \pm 2 d | 3.49 \pm 0.25 b | 1.81 \pm 0.34 d | 34.1 c | 4 \pm 1 c |

same parameters were compared among metal treatments for plants germinated in the same position (from left to right: P1 to P6; with P1 being the most distant from the metal(s) ($n = 20$).

2.4. Statistics

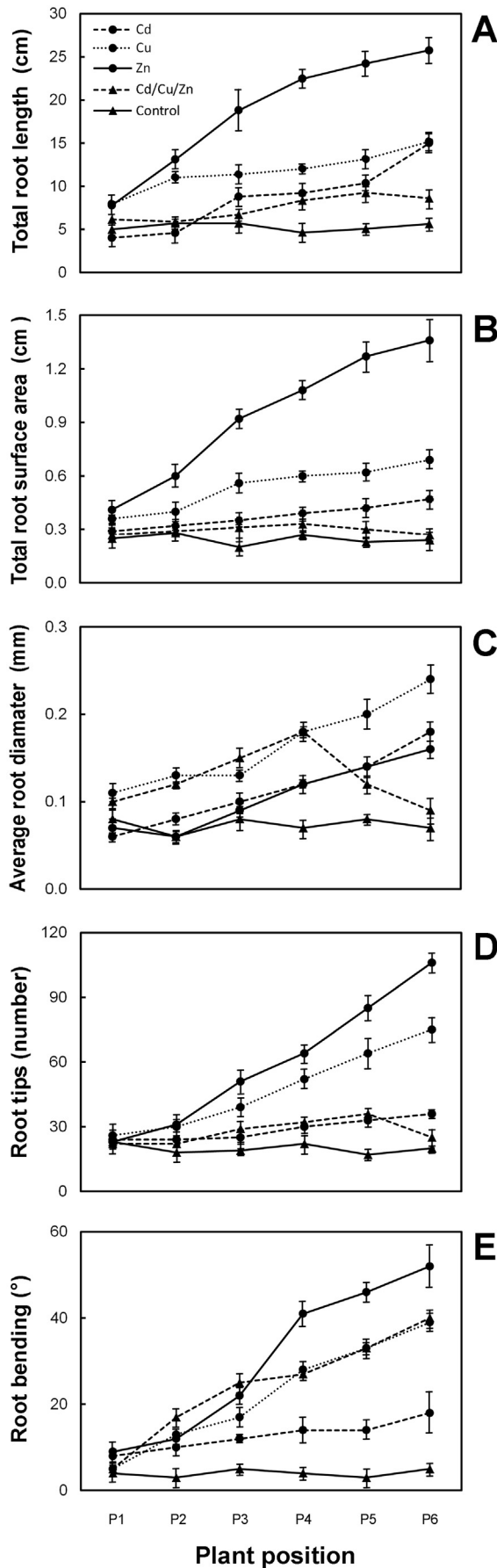
Twenty dishes per treatment were used, for a total of 120 seedlings per treatment. The number of replicates (n) for each measured parameter is specified throughout the text, and in the table and figure captions. The statistical analysis of data was carried out using the Sigmasat 3.1 SPSS Inc. software (SPSS Inc., Quarry Bay, Hong Kong). Analysis of variance (ANOVA) of root and shoot parameters was performed with metal treatments as factors. Means were statistically analyzed by Fisher's LSD test at $P \leq 0.05$.

3. Results and discussion

3.1. Metal gradients

The border between metal-free agar sections and those containing metal(s) was not sharp and the concentration of metals were constant in the lower agar, as the heat of the poured not-agarized medium inevitably caused a partial melting of the agar sections at the borderline between the upper and lower parts, with consequent partial metal diffusion (Fig. 2). The technique here used successfully allowed to create well defined metal concentrations, with low metal levels in the lower left sectors and high metal levels in the upper right sectors of the agar (Table 1). The metal concentrations here adopted were chosen on the basis of metal levels normally present in the soils contaminated by Cd/Cu/Zn, where the three metals are often found in combination, and can be defined as sub-lethal/toxic for this species (Smeets et al., 2009; Vangronsveld et al., 2009; Sofu et al., 2013). The effects of each single metal on Arabidopsis plants were comparable and plants managed to germinate and grow under the chosen metal stress conditions. The physical interface between the upper and lower agar sections did not affect root development. Watanabe et al. (2010) devised a screening strategy using agar plates with a stratified gradient of Cd concentrations, in order to isolate Arabidopsis mutants that displayed altered reactions to this metal, but without considering the possible vertical diffusion of cadmium within the agar gel. On the other hand, trials carried out in contaminated soils, even under controlled conditions, could be affected by a plethora of variables and the study of root samples could be destructive and/or difficult to perform.

A powerful method based on a statistic model of the phenomenon and not on the model of the interpolation function is kriging (Chiles and Delfiner, 1999). The use of such interpolators, included in the so-called "regionalized variables theory", allows to get an estimate of the characteristics of a stochastic field in the points where it has not been measured (Lloyd, 2011). On the basis of the punctual measured concentrations in the 15 agar sectors, the



results of kriging interpolation method are shown in Fig. 2. These results confirm the presence of a well-defined metal gradient within the dishes.

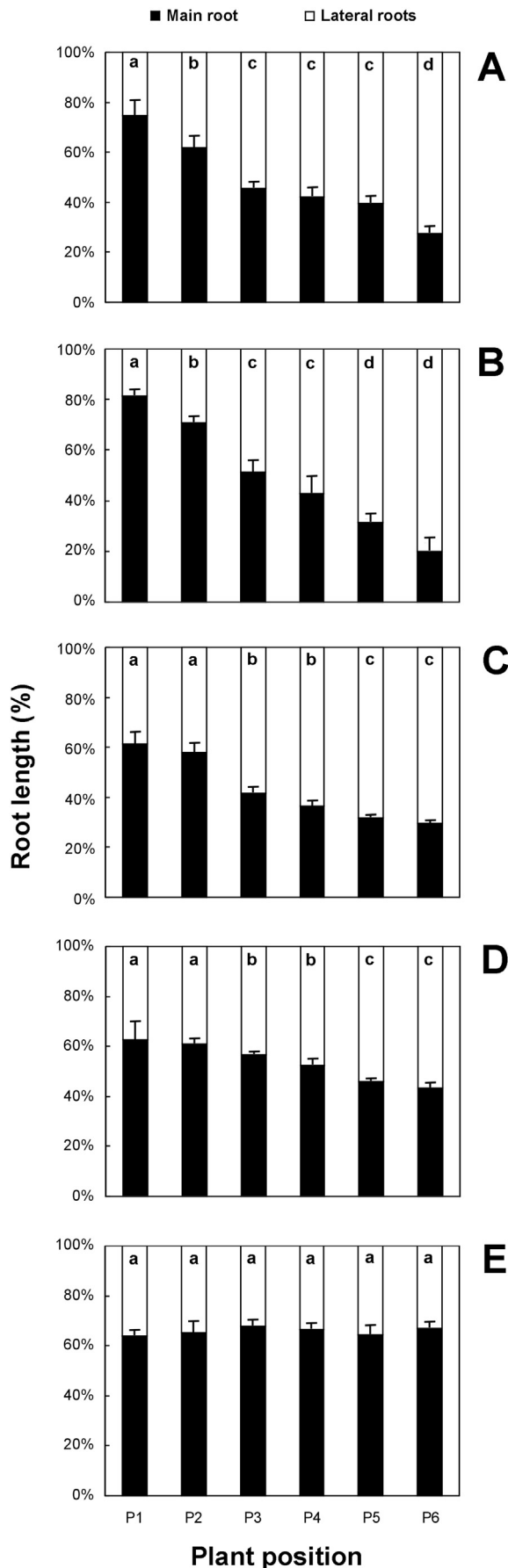
3.2. Effects of metals on general root morphology

The responses of plants to sub-lethal/toxic abiotic stress conditions are often similar among different species. Indeed, a generic 'stress-induced morphogenic response' (SIMR), mediated by common molecular processes such as increased reactive oxygen species (ROS) production and altered phytohormone balance (Potters et al., 2007). SIMR is characterized by a blockage of cell division in the main meristematic tissues, an inhibition of elongation and a redirected outgrowth of lateral organs (Potters et al., 2009). In Arabidopsis, this response has been reported in plants exposed to mild/medium level of different types of abiotic stresses, such as salinity (Zolla et al., 2010), ozone (Blomster et al., 2011), UV-B (Potters et al., 2007), some nutrient deficiencies (Potters et al., 2009; Zolla et al., 2010), and metals (Potters et al., 2007), but rarely quantified in accurate controlled conditions.

In our experiment, root morphology was heavily affected by the exposure to the metals both alone and in triple combination, with more marked effects in the Zn treatment (Table 2). Firstly, single or combined exposure to Cd, Cu, Zn lead to significant increases in total root length, compared to metal-free controls (Table 2). Indeed, control seedlings presented a mean ($n = 120$) root total length of 5.30 cm plant⁻¹, whereas the values in the metal-exposed seedlings were 8.68, 11.80, 18.70 and 7.50 cm plant⁻¹, for Cd, Cu, Zn, and Cd/Cu/Zn treatments, respectively. These increases were due to higher root branching, measured by the number of root tips and lateral root length (Table 2), that indeed statistically increased in all metal treatments with respect to controls (Table 2). Secondly, Cd, Cu and Zn, alone or in combination, have caused intense and significant increases in average root diameter, compared to controls, with statistical differences within them (Table 2). This, together with higher total root length, in turn caused rises of total root surface area, with the highest value in Zn treatment (0.94 cm²) and the lowest in metal-free controls (0.25 cm²) (Table 2). The observed increase in average root diameter could be the consequence of changes in root development, involving xylogenesis, premature endodermis differentiation, and lignification of cortical and stelar tissues (Schützendübel et al., 2001; Dürčeková et al., 2007; Lux et al., 2011). In all the treatments examined, Zn influenced root morphology more than the other two metals. This was due not only to the higher Zn concentrations in the medium, compared to those of Cd and Cu, but could be partly due to the fact that this metal, once absorbed by Arabidopsis root, is slowly translocated to the shoot and so accumulates in the root, where it exerts its effects, as observed by Sofu et al. (2013). The results of other authors from our group (Terzano et al., 2008) give support to this hypothesis, as Zn seems to accumulate at the level of root endodermis and pericycle. In this latter, Zn could trigger lateral root elongation by altering root auxin distribution or hormonal balance, as observed by Giehl et al. (2012) for iron.

The changes in root parameters above described (Table 2), were more marked in plants grown closer to the metals (P4 to P6) than in

Fig. 3. (A) Total root length, (B) total root surface area, (C) average root diameter, (D) number of root tips, (E) and root bending ($n = 20$ position⁻¹ treatment⁻¹ \pm SE) of Arabidopsis seedlings exposed for 14 days to 10 μ M Cd (●; dashed line), 5 μ M Cu (●; dotted line), 150 μ M Zn (●; continuous line), and metals in triple combination (Cd/Cu/Zn) (▲; dashed line) in metal-gradient agar dishes, and not exposed to metals (control) (▲; continuous line). Plant position from left to right = P1–P6; with P1 being the most distant from the metal(s).



those grown further away from the metals (P1 to P3) (Fig. 3 and Fig. S1). These differences due to the position in the dishes were absent in the control plates, where no significant discrepancies in root parameters among P1 and P6 were observed (Fig. 3). These trends clearly demonstrated the strong influence of metal on root morphology. In the presence of the three metals together (Cd/Cu/Zn treatment), two interesting things were observed (Table 2 and Fig. 3): firstly, the high branching effect due to Zn applied alone was not present; secondly, a stop of total root length occurred in the plants closer to the metals, compared to the treatments with metals applied alone. Particularly, the roots of the plants exposed to the three metals in combination showed a decrease in the mean diameter starting from P5, and a stop of root length development (Fig. 3A–C). These inhibitory effects were due to the fact that the three metals together, applied alone at sub-toxic concentrations, likely exceeded the threshold of toxicity for plants (Sanità di Toppi and Gabbriellini, 1999; Cuyppers et al. 2010). Furthermore, Smeets et al. (2009) observed that the combined exposure of *Arabidopsis* to Cd and Cu enhanced some of the effects that were induced when only one metal was applied to the medium. These authors demonstrated that oxidative stress due to the two metals combined was due to a down-regulation of the genes encoding for some antioxidant enzymes and to the lower rate of scavenging reactions, that do not keep pace with the increase of ROS. Similar effects of multiple metal stress on root morphology were observed by Sofo et al. (2013) in *Arabidopsis* grown hydroponically at the same Cd/Cu/Zn concentrations here used. On this basis, we hypothesize that both a threshold effect and a synergistic metal effect occurred in our plants.

3.3. SIMR strategies in roots

In *Arabidopsis* roots, SIMR-like strategies include the redifferentiation of pericycle cells and formation of lateral roots, with a crosstalk between hormones (mainly auxin) and ROS signaling (Malamy and Ryan, 2001; Potters et al., 2009; Vitti et al., 2013; Atkinson et al., 2014). Indeed, *Arabidopsis* exposed to metal stress often resemble, in terms of the redistribution of growth, plants altered in phytohormone metabolism (Pasternak et al., 2005; Kai et al., 2007), particularly as far as roots are concerned (Brunetti et al., 2011). Key components of the signal-transduction pathways that affect auxin-dependent lateral root initiation in *Arabidopsis* have been identified (Peleg and Blumwald, 2011; Baster et al., 2013; Atkinson et al., 2014). Morphological root changes could be part of an integrated hormonal response against the metal stressors, mainly governed by the auxin/cytokinin ratio. In this regard, Sofo et al. (2013) and Vitti et al. (2013) found an over 5-fold increasing of IAA level and an up-regulation of some genes involved in IAA biosynthesis in *Arabidopsis* roots exposed to Cd, Cu and Zn in combination at the same concentrations used in this work. It was observed that in *Arabidopsis* exposed to Cu levels of 30–100 μM, root hairs density was significantly increased, and an acceleration of the emergence of lateral roots occurred, with a consequent increase in the number of root tips (Pasternak et al., 2005). In carrot plants grown *in vitro*, a prolonged exposure to Cd (2–7 days) anticipated and stimulated the production of lateral root primordia,

Fig. 4. Length of main root (black columns) and of lateral roots (white columns) expressed as percentages of the total root length of *Arabidopsis* seedlings exposed for 14 days to (A) 10 μM Cd, (B) 5 μM Cu, (C) 150 μM Zn, and (D) Cd/Cu/Zn in triple combination in metal-gradient agar dishes, and (E) not exposed to metals (control). Plant position from left to right = P1–P6; with P1 being the most distant from the metal(s). Mean values ($n = 20$ position⁻¹ treatment⁻¹ ± SE) with different letters are significantly different between the plants of the same treatment at $P \leq 0.05$, according to Fisher's LSD test.

as well as the development of primary and secondary xylem (Sanità di Toppi et al., 2012).

Our results clearly depicted SIMR-like strategies in Arabidopsis, as in all the metal treatments lateral roots led to a percentage decrease of the main root in relation to the total root length (Table 2 and Fig. 4). Furthermore, both for the morphometric values averaged for all plant positions (P1–P6) (Table 2) and for the plants in the same position (Fig. 4), the effects of the triple combination (Cd/Cu/Zn) were milder.

3.4. Root bending

Lateral root proliferation and length is classically described as a response to very favorable conditions such as a high nitrogen concentration (Malamy and Ryan, 2001), but also to several abiotic stresses such as the presence of metals or salinity (Drew et al., 1973; Potters et al., 2007). In some cases conflicting evidence has been clarified by applying simultaneously a stress condition and a nutrient (Zolla et al., 2010) or a spatially variable condition (Linkohr et al., 2002). This has helped elucidate that in both cases of nutrients and toxic substances, root proliferation and growth responses may be viewed as a form of compensatory growth occurring in relatively favorable soil patches. Our experimental setup (Fig. 1) provides a simple tool to advance this kind of studies further, since it provides plant responses as an explicit and continuously variable function of the spatial distribution of toxic substances.

Root systems grown in the presence of metals showed visible and significant bending toward free-metal-patches in the culture medium, with angle values of 13°, 23°, 30° and 25° in Cd, Cu, Zn and Cd/Cu/Zn treatments, and a non-significant fluctuation of 4° (both at left and at the right respect to gravity direction) in control plants (Table 2). The seedlings closer to metals (P4–P6) showed a higher curvature toward the agar areas free from metals compared to P1–P3 (Fig. 3E and Fig. S1). The ability of Arabidopsis to respond to geotropism is tied to the redistribution of auxin (Muday and Rahman, 2008; Geisler et al., 2014), as this hormone regulates root growth by targeting Aux/IAA (auxin/indoleacetic acid) repressor proteins for degradation (Band et al., 2012) and the asymmetry in the auxin distribution is established and maintained by a spatio-temporal regulation of the PIN-FORMED (PIN) auxin transporter activity (Baster et al., 2013). Thus, the different gravitropic response observed in the metal-treated seedlings (Table 2 and Fig. 3E) is likely due to a modified hormonal balanced ruled by auxin.

Therefore, whereas the stimulation of lateral meristems may be interpreted as a response to toxicity (SIMR) (Zolla et al., 2010), the actual growth of laterals is a form of compensatory growth which is possible in the case of spatially variable growth medium, resulting in a higher lateral root length only in less toxic patches.

4. Conclusions

In Arabidopsis plants exposed to metals, root system's total length increased due to the higher root branching. This was confirmed by the significantly higher number of root tips in metal-treated seedlings. The seedlings closest to the areas with agar + metals showed a marked curvature and a higher root branching, as a form of stress avoidance. The observed increase in root diameter in metal-treated seedlings may be interpreted as compensatory growth, as a thicker roots could act as a barrier to protect root from the metals. The observed increase in root diameter in metal-exposed plants could be a consequence of metal-induced premature differentiation of root tissues, particularly endodermis and/or exodermis, that act as barriers to protect root from the metals. The increased lateral root formation and increase

in mean root diameter could be functionally related to metal stress avoidance mechanisms. In fact, the lateral roots could allow the Cd/Cu/Zn-stressed root system to maximize the rhizosphere exploration, in order to attempt the recruiting of as many free-metal-patches in the culture medium (and in the soil) as possible. We therefore propose that the remodeling of the root architecture in response to metals could be a pollution “escaping” strategy aimed at recruiting free-metal-patches.

Taken together these results point to a significant influence of the three metals on Arabidopsis root morphology. Consequently, a combination of Cu, Cd and Zn seems to act synergically in inducing an univocal metal-pollution response. The technique here used for studying the response of Arabidopsis to metals can be considered reliable, reproducible, and relatively cheap and fast.

Contributions

Rocco Bochicchio: dishes scanning and morphometric measurements.

Adriano Sofo: writing of the manuscript and dishes preparation.

Roberto Terzano: metal analysis.

Concetta Eliana Gattullo: metal analysis.

Mariana Amato: manuscript correction and root growth analysis.

Antonio Scopa: plant preparation and statistical analysis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2015.03.010>.

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