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Mobilization of Cu and Zn by root exudates of dicotyledonous plants in resin-buffered solutions and in soil

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Abstract

It has been frequently suggested that root exudates play a role in trace metal mobilization and uptake by plants, but there is little in vivo evidence. We studied root exudation of dicotyledonous plants in relation to mobilization and uptake of Cu and Zn in nutrient solutions and in a calcareous soil at varying Cu and Zn supply. Spinach (*Spinacia oleracea* L.) and tomato (*Lycopersicon esculentum* L.) were grown on resin-buffered nutrient solutions at varying free ion activities of Cu (pCu 13.0–10.4) and Zn (pZn 10.1–6.6). The Cu and Zn concentrations in the nutrient solution increased with time, except in plant-free controls, indicating that the plant roots released organic ligands that mobilized Cu and Zn from the resin. At same pCu, soluble Cu increased more at low Zn supply, as long as Zn deficiency effects on growth were small. Zinc deficiency was observed in most treatment solutions with pZn ≥ 9.3, but not in nutrient solutions of a smaller volume/plant ratio in which higher Zn concentrations were observed at same pZn. Root exudates of Zn-deficient plants showed higher specific UV absorbance (SUVA, an indicator of aromaticity and metal affinity) than those of non-deficient plants. Measurement of the metal diffusion flux with the DGT technique showed that the Cu and Zn complexes in the nutrient solutions were highly labile. Diffusive transport (through the unstirred layer surrounding the roots) of the free ion only could not explain the observed plant uptake of Cu and of Zn at low Zn^{2+} activity. The Cu and Zn uptake by the plants was well explained if it was assumed that the complexes with root exudates contributed 0.4% (Cu) or 20% (Zn) relative to the free ion. In the soil experiment, metal concentrations and organic C concentrations were larger in the solution of planted soils than in unplanted controls. The SUVA of the soil solution after plant growth was higher for unamended soils, on which the plants were Zn-deficient, than for Zn-amended soils. In conclusion, root exudates of dicotyledonous plants are able to mobilize Cu and Zn, and plants appear to respond to Zn deficiency by exuding root exudates with higher metal affinity.

Introduction

Plant roots release organic components into the rhizosphere, which results in higher concentrations of organic ligands in the rhizosphere than in the bulk soil. These root exudates have been implicated in many processes, such as metal mobilization, uptake of nutrients (e.g., P, Fe, Cu) and toxic elements (e.g., Cd), detoxification of Al, and mineral weathering, but there is little in vivo evidence for their role in these processes, which relies for a large part on speculation (Jones, 1998).

Root exudates are important for the Fe nutrition of Poaceae (Strategy II plants). Grasses excrete phytosiderophores (PS), that have a high affinity for Fe(III). The Fe(III)-PS complexes can be absorbed intact by Stragegy II plants (Ma and Nomoto, 1996). Some evidence has been found that PS also play a role in nutrition of trace metals others than just Fe. Chaignon et al. (2002) observed higher uptake of Cu by wheat under Fe-deficient conditions, coinciding with a higher PS release. Several studies showed that Zn deficiency increases PS exudation (e.g., Zhang et al., 1989). Hoffland et al. (2006) also reported enhanced exudation of low-molecular weight organic acids, mainly citrate, by rice under Zn-deficient conditions. Cakmak et al. (1996) found that enhanced PS-release was more pronounced for Zn-efficient than for Zn-inefficient genotypes of wheat. Direct uptake of the Zn-PS complex has been observed for maize in double-labeled ($^{65}Zn/^{14}C$) experiments (von Wirén et al., 1996). However, other studies showed that enhanced PS-release was only significantly induced by Fe deficiency, and not by deficiency of other trace metals (Gries et al., 1998; Pedler et al., 2000).

Strategy I plants, the dicots and non-grass monocots, do not release PS, but obtain Fe through acidification of the rhizosphere and reduction of Fe(III) to Fe(II) at the plasma membrane before uptake by a specific transporter. Some studies have suggested that exudation of organic acids (especially citrate) plays a role in mobilizing Fe under Fe-

deficient conditions, but direct evidence is lacking (Parker et al., 2005). There is even less evidence that non-PS root exudates increase bioavailability of metals other than Fe. Only a few studies support for this hypothesis. High uptake of Cd by tobacco plants could be related to a large Cd binding affinity of their root exudates (Mench and Martin, 1991). Keller and Römer (2001) found that increased Zn uptake under P-deficient conditions was related to higher exudation rates of organic acids, and higher Zn concentrations in the soil solution. Zhang et al. (1991) on the other hand found that the root exudates of dicotyledonous species did not enhance Zn mobilization from a synthetic resin or a calcareous soil, in contrast with the exudates of barley and wheat.

Most root exudation studies have been performed in nutrient solutions. Artifacts may be associated with the use of solution culture, since roots are morphologically and physiologically different from those of plants grown in soils (Jones, 1998). The effect of root exudates on metal mobilization and uptake needs to be validated in soil-grown plants. However, assessing these effects in the complex soil system poses experimental challenges, since the effects of root exudation cannot be directly disentangled from other plant-related processes, such as nutrient uptake and pH changes (Parker et al., 2005). Moreover, collection and analysis of exudates components is much easier in solution culture than in soils.

In this study, we assessed the role of root exudates in metal mobilization and uptake by dicotyledonous plants in resin-buffered nutrient solutions (Checkai et al., 1987) and in soils. Both in the solution and soil system, the Cu^{2+} and Zn^{2+} activity was varied. Resin-buffered solutions offer the advantage over chelator-buffered solutions that no assumptions are required about the availability of chelated metals (Degryse et al., 2006a). In the resin-buffered solutions, Cu^{2+} and Zn^{2+} activities are buffered with a solid phase, as is the case in a soil, and complexation of Cu and Zn with root exudates will therefore increase the metal concentration in solutions. This experimental set-up thus allowed assessment of the effects of root exudation on metal mobilization at varying metal supply. Metal mobilization by root

exudates does not prove *per se* that these complexes with root exudates affect the plant uptake. We therefore also assessed whether plants response (yield, uptake) was more related to free ion activity or to total metal concentration in solution. Free ion activities and total solution concentrations are often positively correlated under similar experimental conditions. To avoid this covariation, we conducted solution experiments in solutions of different volume.

Materials and methods

Solution experiments

The effect of root exudation by two dicotyledonous species was assessed in solutions buffered with a chelating resin (Chelex-100, 100–200 mesh) at varying Cu^{2+} and Zn^{2+} activities (see below). All nutrient solutions were composed of (in mM) 2 $Ca(NO_3)_2$, 0.5 $MgSO_4$, 1.2 KNO_3, 0.1 KH_2PO_4, and (in μM) 25 NaCl, 15 H_3BO_3, 0.07 $(NH_4)_6Mo_7O_{24}$, 25 FeHBED (Fe-N,N'-bis (2-hydroxybenzyl)-ethylenediamine-N,N'-diacetate). The pH of the solutions was buffered with 2 mM MES (2-(N-morpholino)ethanesulfonic acid) at pH 6.1 (except in Experiment 3; see below). The FeHBED stock solution was prepared according to Chaney (1988) from HBED (acid form) and $FeCl_3$ in a 30% molar excess over HBED.

Chelex resin was used to buffer the metals (Cu, Zn, Mn) in solution. Preliminary experiments had shown the following relationships between metal loading on the Chelex and metal activities of Cu and Zn maintained in solution (2mM $Ca(NO_3)_2$; pH=6.1):

$pCu = -1.66 \log(Z_{Cu}/Z_{Ca}) + 10.12$ (R^2=0.89, n=16, pCu between 13.0 and 10.4)

$pZn = -1.65 \log(Z_{Zn}/Z_{Ca}) + 7.09$ (R^2=0.89, n=16, pZn between 10.0 and 6.6)

where Z_{Cu}, Z_{Zn} and Z_{Ca} are the equivalent fractions of Cu, Zn and Ca on the resin. The Chelex was loaded with metals by equilibrating the resin with a solution of $CuSO_4$, $ZnSO_4$, $MnSO_4$ and $Ca(NO_3)_2$ in appropriate concentrations, and subsequently rinsing the resin

several times with a 2 mM MES (pH 6.1)/ 2mM $Ca(NO_3)_2$ solution. To check the Cu^{2+}, Zn^{2+} and Mn^{2+} activities at which the resin buffered the solution, a subsample of the resin was equilibrated for 3 days in a solution with 2 mM MES (pH 6.1), 2mM $Ca(NO_3)_2$ and 0.2 mM NTA (nitrilotriacetic acid) (Degryse et al., 2006a). The free metal activities were then calculated with GEOCHEM-PC from the equilibrium metal concentrations. One gram of metal-loaded moist resin was added to each 0.9-L nutrient solution (or 0.3 g resin to 0.25-L solutions in Exp. 3), and the solutions were equilibrated for three days. Treatment solutions were prepared in triplicate, except for some treatments of Exp. 2 (see further) and control (plant-free) solutions that were made in duplicate.

Seeds of spinach, *Spinacia oleracea* L. (Géant d'Hiver), or tomato, *Lycopersicon esculentum* L. (Pyros F1), were surface sterilized for 15 minutes in 0.04 M NaOCl, rinsed with deionized water, and germinated for 6 or 7 days at 25 °C between sheets of moist tissue continuously wetted with metal-free nutrient solution. Two seedlings of each species were transferred to each container. The plants were grown in a growth chamber with a 16-8 h day-night cycle, day-night temperatures of 20-16 °C, a photosynthetic photon flux density (PPFD) of 450 μmol m^{-2} s^{-1}, and relative humidity at 60%. The nutrient solution containers were wrapped with Al foil and continuously aerated. The pH was measured daily and adjusted to 6.1 ± 0.1 and water was added when necessary. Nutrients (KH_2PO_4, $MgSO_4$, KNO_3) were added to each container, except to the plant-free control solutions, at 12 DAT (days after transplanting) and every 2 or 3 days thereafter. The nutrient solutions were sampled at 5 or 6 occasions during the plant growth, filtered and acidified to pH=1 before analysis. The plants were taken from the nutrient solution between 20 and 22 DAT, and transferred to 100 mL of deionized water for collection of root exudates. The plants were left on this solution for about 5 hours (from 3 hours after the onset of the light period). The roots were rinsed and blotted, and root and shoot fresh weight was recorded. The plant material was dried (2 days at 65 °C), weighed, and digested with hot concentrated HNO_3.

Elemental concentrations in the digests and the nutrient solutions were determined with ICP-AES (Perkin Elmer 3300 DV). The root exudate solutions were analyzed for dissolved organic carbon (DOC; Thermalox carbon analyzer), UV absorbance at 254 nm (Perkin-Elmer, Lambda 20 spectrophotometer, quartz cells) and organics acids (lactate, acetate, formate, malate, succinate, malonate, tartrate, oxalate and citrate) with anion chromatography (Dionex, ICS 2000, AS18 column).

To evaluate the lability of the metal complexes in the nutrient solution, DGT (Diffusive Gradients in Thin Films) analysis was performed on the nutrient solutions for selected treatments at the end of the plant growth experiment (Exp. 2; see below). The DGT device was placed into the stirred solution, and removed after ca. 3 days. The resin gel was retrieved and eluted with 1 M HNO_3. The DGT measured concentration of Cu and Zn, c_{DGT}, was calculated from the concentration in the eluent according to Zhang and Davison (1995).

Three solution experiments were carried out with similar set-up, but different treatments:

Experiment 1. Both tomato and spinach were grown, in separate containers. Treatments included a series of varying Zn^{2+} activity (pZn 10.1, 9.7 and 8.0) at constant Cu^{2+} activity (pCu~11.6), and a series of varying pCu (12.3, 11.6 and 10.8) at constant pZn (~8.6).

Experiment 2. This experiment was similar to Experiment 1, but only tomato was used and more treatments were included. These included a series of varying Zn^{2+} activity (pZn 9.6, 9.3, 8.8, 7.7 and 6.6) at nearly constant pCu (~11.7), and a series of varying Cu^{2+} activity (pCu 13, 12.4, 11.8, 11.2 and 10.4) at pZn ~9. The treatment solutions with the lowest and the middle metal activities were made in triplicate, the others in duplicate.

Experiment 3. Using MES-buffered solutions has the advantage that pH is more stable. However, the use of MES (formula: $C_6H_{13}NO_4S$) prevented measuring the increase in DOC concentrations in the nutrient solution resulting from root exudation, because of the large organic C concentrations associated with the MES (2 mM MES~ 144 mg C L^{-1}). Therefore, a third experiment was carried out without MES buffer. The pH was adjusted more

frequently, by the end of the experiment two or three times a day. A smaller volume of nutrient solution (0.25 L) was used in this experiment. The DOC concentrations in the nutrient solutions were determined at 15 DAT and at harvest (20 DAT). There were four treatments in a 2×2 factorial design, with pZn ~9.8 or ~8.9 and pCu ~13 or ~11.7.

Soil experiment

A calcareous topsoil from Guadelajara (Spain, 40°37'02" N, 3°09'07" W) with relatively low metal content was used for the experiment (Table 1). The soil was sampled in the spring of 2003, sieved to 4 mm, dried at 25°C and stored air-dry. Prior to the experiment, the soil was sieved to 2 mm.

There were 4 metal treatments: no added metal, +Cu (50 mg Cu kg^{-1}), +Zn (100 mg Zn kg^{-1}) or +Cu/Zn (50/100 mg kg^{-1}). The soils were either unamended with Fe, or amended with 0.025 mmol FeHBED kg^{-1}. Adding Fe in chelated form prevents that Fe precipitates, and the ligand HBED was selected because it does not affect the speciation of other metals such as Cu and Zn. These +Fe treatments were included to alleviate possible Fe deficiency, which might obscure effects of Cu and Zn status on root exudation. The treatments were imposed by adding metal salt solutions ($ZnSO_4$ or $CuSO_4$) or distilled water to the soil, so that a moisture content of 25% (w:w) was obtained. The soils were well mixed, equilibrated for one day, and subsequently leached with 2 pore volumes of a dilute salt solution (1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$ and 0.2 mM KNO_3) to remove excess salt. The soils were partly air-dried for one day and either destilled water (-Fe) or a FeHBED solution (0.025 mmol FeHBED kg^{-1}; +Fe treatments) was added. The final moisture content was 22% (w:w). Three days later, soil solution was isolated (by centrifugation, see below) and analyzed for elemental composition with ICP-AES.

For each treatment, 5 pots were filled with each ca. 50 g of moist soil, of which 3 replicates served to grow plants and 2 served as plant-free controls. These 'pots' were disposable syringes without plunger wrapped with Al foil and with quartz wool on the bottom, which allowed for easy soil solution collection at the end of the experiment. A small volume of soil was used, so that all of the soil could be considered to be rhizosphere soil. Seeds of tomato (*Lycopersicon esculentum* L. cv. Pyros F1) were surface sterilized for 15 minutes in 0.04 M NaOCl, rinsed with deionized water, and germinated for 2 days at 25 °C between sheets of moist tissue. Three germinated seeds were planted per pot, and the soil surface was covered with polyethylene beads. The plants were thinned to one plant per pot after a few days. The plants were grown in a growth chamber under the same conditions as for the solution experiments. Evapotranspiration was compensated daily, or by the end of the experiment 2 or 3 times a day, with distilled water or a fertilizer solution. The fertilizer solution, which consisted of 6 mM KNO_3, 5 mM NH_4NO_3, 3.4 mM $MgSO_4$, 9 mM $Ca(NO_3)_2$ and 2 mM KH_2PO_4, was added as (presumably) needed based on normal plant contents and estimated growth rate (Ingestad, 1982).

The plants were harvested at 28 DAT. The shoot of each plant was removed, weighed and dried (2 days at 65 °C). A subsample of the soil was taken from 5 selected treatments for the DGT analysis (see below). The syringes were then placed in centrifuge tubes and centrifuged for 30 min (3000g). The soil solution was filtered over a 0.45 µm membrane filter, and analyzed for elemental composition (ICP-AES), dissolved organic and inorganic C concentrations (Thermalox TOC analyzer) and UV absorbance at 254 nm. After centrifuging the soils, roots were removed by handpicking, cleaned with distilled water, and dried. Both shoots (replicates separately) and roots (replicates pooled) were digested with hot concentrated HNO_3, and analyzed with ICP-AES.

For selected treatments, DGT analysis was carried out by putting a ± 2-cm thick layer of moist soil on the DGT device. The DGT device was deployed for 4 days, cleaned with milli-Q water, and the resin gel was retrieved and desorbed in 1 M HNO_3.

At the end of the experiment, the EDTA-extractable metal content was measured for all treatments. The soil was suspended in a 25 mM Na_2EDTA solution (soil:solution ratio of 1:5 kg L^{-1}), and the suspension was centrifuged (15 min., 3000g) after 2 days of equilibration on an end-over-end shaker. The supernatant was analyzed for Cu and Zn with ICP-AES.

To estimate the free ion activities of the soils, speciation calculations were made with WHAM/Model VI (Tipping, 1998). The WHAM model was used to describe the solid–solution interactions, taking into account humic acid and iron oxides as sorptive phases. Default binding parameters were used for binding on humic acid, but adjusted parameters for binding on iron oxides (Buekers, personal communication). The humic acid concentration was calculated from the organic C content of the soil, assuming 50% C in organic matter. Dithionite-extractable Fe was used to calculate the iron oxide content. Only EDTA-extractable Cu and Zn were considered to participate in the solid–solution equilibrium. Input also included the soil pH, the solution composition (Ca, Mg, K as measured and nitrate as counteranion) and free ion activities of Fe(III) and Al, calculated from the solubility products, K_{sp}, of $Fe(OH)_3$ and $Al(OH)_3$. Values for $\log K_{sp}$, expressed as $(M^{3+})/(H+)^3$, were assumed to be 2.5 for $Fe(OH)_3$ and 8.5 for $Al(OH)_3$ (Tipping et al., 2003).

Results

Solution experiments

Plant growth and deficiency symptoms.

No effect of Cu^{2+} activity on plant yield was observed in any of the experiments, indicating that no Cu deficiency occurred even at the lowest Cu^{2+} activities (pCu ~ 13).

In Experiments 1 and 2, both tomato and spinach showed clear zinc deficiency symptoms (chlorosis) and reduced growth in the treatments with low Zn activity (pZn ≥ 9.3) (Table 2). In Experiment 3, however, no Zn deficiency was observed for the treatments with low Zn activity (pZn 9.8). The shoot fresh weight was on average 8.9 g (per 2 plants), with no significant differences between treatments.

Metal mobilization in the nutrient solutions.

The Cu concentration in the nutrient solutions showed a strong and significant (P<0.0005) increase with time (Figure 1). No such increase was observed for the plant-free control solutions, indicating that this increase in solution concentration was due to exudation of organic ligands by the plant roots. Complex formation of Cu with these plant-borne organic ligands ($Cu^{2+} + L \rightarrow CuL$) resulted in an increase in soluble Cu, because the free Cu^{2+} activity was buffered by the resin.

For treatments with the same Cu^{2+} activity, the Cu mobilization was initially most pronounced in treatments with low Zn^{2+} activity (pZn ≥ 9.3). However, this increase in soluble Cu leveled off for the low Zn treatments and Cu concentrations were similar to or smaller than those at higher Zn^{2+} activity by the end of the experiment (Figure 1, Table 2). At the end of the growth period, the plants on the low Zn treatments suffered strongly from Zn deficiency, which affected the plant growth and likely the root exudation rate as well.

The Cu concentrations in solution generally increased with increasing Cu^{2+} activity, but less than proportionally, i.e. the degree of complexation (ratio of complex to free ion concentration) decreased as Cu^{2+} activity increased (Figure 2). For a single ligand, the degree of complexation is expected to remain constant as long as there is an excess ligand. The gradual decrease in degree of complexation with increasing Cu^{2+} activity suggests heterogeneity in the ligand binding sites and a saturation of the most selective binding sites.

Zinc concentrations in solution were often near or below detection limit (~0.02 μM), but an increase in Zn concentration with time could be detected nonetheless for some treatments. (e.g. Figure 3) The degree of complexation was smaller than for Cu, and decreased with increasing Zn^{2+} activity. At the lowest Zn^{2+} activities, the total Zn concentration was ca. 100-fold larger than the free ion concentration, while total Zn was only slightly larger than free Zn concentration at the highest Zn^{2+} activity, indicating negligible complexation. At the same Zn^{2+} activity, Zn concentrations were higher for the low Cu treatments (Table 2), which is possibly due to a competition of Cu with Zn for complexation with the organic ligands. The highest Zn concentrations, up to 0.3 μM, were observed for Experiment 3, which was likely related to the smaller volume of solution used in this experiment (0.25 L instead of 0.9 L). At smaller volume, the same rate of root exudation results in higher concentrations of organic ligands, and consequently higher metal concentrations.

At the end of Experiment 2, DGT measurements were made on selected nutrient solutions. The DGT-measured concentration gives an indication of the lability of the metal complexes in solution. If all metal complexes are fully labile (i.e. dissociate within the experimental time scale, which depends on the thickness of the diffusion gel layer), c_{DGT} equals the total metal concentration in solution. The R value, the ratio of DGT-measured to total solution concentration, was around 0.4 for Cu and >0.4 for Zn in the nutrient solutions (Table 3). These large values of R denote a high degree of lability of the Cu and Zn complexes present in the nutrient solutions at the end of the experiment.

Release of organic compounds.

Exudation of organic compounds was assessed by measuring the concentration of dissolved organic carbon and of carboxylates in the solutions on which the plants were transferred for about 5 hours before harvest. The exudation of carboxylic acids was mostly between 10 and 100 nmol per g root fresh weight per hour, and showed considerable variation between treatments and replicates, without any consistent effect of treatment on composition or rate of organic acid exudation. The release of DOC in these solutions was, on average, 14 µg DOC per g root fresh weight per hour (range: 4–40 µg g^{-1} RFW h^{-1}). The specific UV absorbance (SUVA), i.e. the UV absorbance divided by the DOC concentration, of these root exudate solutions was mostly between 10 and 15 L g^{-1} cm^{-1}. Root exudates of plants that had suffered from Zn deficiency, showed however higher SUVA (Table 2). The SUVA is a measure of aromaticity of organic C, and a relation between SUVA and metal affinity of DOC has been demonstrated (Amery et al., 2007).

The 4-hour root exudate collection at the end of the experiment gives only a measure of the exudation rate at time of harvest. To evaluate the exudation over the whole course of the plant growth experiment, DOC concentrations were also measured in the nutrient solutions of Experiment 3, where no MES was used. The DOC concentrations were in the order of 23 mg L^{-1} at 15 DAT and 33 mg L^{-1} at 20 DAT (Figure 3), with no significant differences between treatments. The SUVA of these nutrient solutions was also not affected by treatment, and was, on average, 9.6 L g^{-1} cm^{-1}. Theoretically, the amount of DOC released in the nutrient solution during the plant growth (Q_{DOC}, mg) can be calculated from the DOC exudation rate (J_{DOC}, mg g^{-1} RFW day^{-1}) as follows:

$$Q_{DOC} = \int_{t_1}^{t_2} J_{DOC} . W_{root}(t) . dt \qquad (1)$$

where t_1 is the time of transfer and t_2 the time of harvest. If the temporal change in root fresh weight is described with exponential growth ($W_{root}=W_0.e^{RGR.t}$), integration of Eq. (1) yields following equation:

$$Q_{DOC} = \frac{J_{DOC} \cdot W_0}{RGR} \cdot (e^{RGR.t_2} - e^{RGR.t_1}) \qquad (2)$$

where RGR is the relative growth rate and W_0 the initial root weight. The growth of plants on the non-deficient nutrient solutions was well described assuming values of 0.03 g (per 2 plants) for W_0 and of 0.2 day^{-1} for RGR. Using these values and the average DOC release rate during the root exudates collection at harvest (14 µg g^{-1} RFW h^{-1} or 0.34 mg DOC g^{-1} RFW day^{-1}), Equation (2) predicts that 9 mg DOC is released in to solution at 20 DAT, which corresponds to a concentration of 10 mg DOC L^{-1} in the 0.9-L solutions (Exp. 1 and 2) and 36 mg DOC L^{-1} in the 0.25-L solutions (Exp. 3). The DOC concentrations in the nutrient solutions were only measured for Exp. 3 (where no MES was used), and the measured concentrations agreed well with the predicted values (Figure 3), suggesting that the DOC release rate as measured during the root exudate collection (at harvest) gives a value comparable to the net release rate during the whole plant growth experiment. These calculations do not take into account microbial decomposition of the root exudates. The gross exudation rate is therefore likely underestimated. The decomposition rate of root exudates depends on the compound. Decomposition of organic acids such as malate and citrate is usually fast, with half-lifes in the order of hours (Jones, 1998), while other root exudates components are more recalcitrant (Uselman et al., 2000).

The amount of metal mobilized by the root exudates (Q_M, µmol) during the plant growth can be related to the 'metal mobilization rate' (J_M, µmol g^{-1} RFW day^{-1}) in the same way as for the DOC release:

$$Q_M = \frac{J_M \cdot W_0}{RGR} \cdot (e^{RGR.t_2} - e^{RGR.t_1}) \qquad (3)$$

Figure 3 shows the evolution of soluble Zn in solutions buffered at pZn 8.8 and pCu 11.8, for a solution volume of either 0.9 L (Exp. 2) or 0.25 L (Exp. 3). For both experiments, the results were well described assuming a Zn mobilization rate of 1.6 nmol g^{-1} RFW day^{-1}. Over all treatments, the estimated metal mobilization rates ranged from 0.3 to 10 nmol g^{-1} RFW d^{-1} for Zn and 6 to 170 nmol g^{-1} RFW d^{-1} for Cu.

Plant concentrations.

Median shoot concentrations were 71 mg Fe kg^{-1}, 62 mg Mn kg^{-1}, 5.7 g Mg kg^{-1}, 33 g K kg^{-1}, 30.5 g Ca kg^{-1} and 4.7 g P kg^{-1} for tomato, and 52 mg Fe kg^{-1}, 63 mg Mn kg^{-1}, 4.7 g Mg kg^{-1}, 46 g K kg^{-1}, 16.3 g Ca kg^{-1} and 3.0 g P kg^{-1} for spinach. Concentrations of P were higher for Zn-deficient plants, a symptom frequently observed in Zn-deficient plants grown in solution culture (e.g. Pedler et al., 2000). The P concentrations showed an inversely proportional relation to the shoot yield, probably due to a growth dilution effect at the high biomass (Figure 4). A similar inverse relation between yield and plant concentration was also observed for Mg and, less pronounced, for Ca.

Zinc concentrations in the plant decreased with decreasing Zn^{2+} activity in the solution (Figure 5). The critical Zn concentration was around 6 mg kg^{-1} in the shoot and 20 mg kg^{-1} in the root. Plant concentrations did not decrease below these values but yield was affected instead at low Zn supply (cf. Table 2). Critical Zn concentrations in shoot are usually in the order of 10–20 mg kg^{-1}, but lower values have been reported (e.g. Ozkutlu et al., 2006). Copper concentrations in the root increased with increasing Cu^{2+} activity, but shoot Cu concentrations, known to be strongly regulated (e.g. Song et al., 2004), were only marginally affected in tomato (Figure 5). The shoot Cu concentrations of spinach showed somewhat more variation (3–17 mg kg^{-1}).

The variation in plant metal concentrations among replicates was sometimes large. This variation could partly be explained by variation in total metal concentration in solution

among replicates (not shown). We hypothesize that this variation in total metal solution concentrations is due to the variable degree of root exudation among replicates of the same treatment.

Soil experiment

Plant growth and deficiency symptoms.

Plants grown on the soil without added Zn showed deficiency symptoms (chlorosis) at time of harvest and also reduced yield, though this yield reduction was only significant for the treatments with added Cu (Table 4). There were no yield differences between the treatments with or without FeHBED, but the plants on the control treatments were less chlorotic when FeHBED was added.

Metal concentrations in soil and soil solution composition.

Most of the added metals were still EDTA-extractable at the end of the experiment: the EDTA-extractable concentration was ca. 50 mg Cu kg^{-1} in Cu amended soils and ca. 85 mg Zn kg^{-1} in the Zn-amended soils. In contrast, the soil-borne Cu and Zn was for a large part not EDTA-extractable (Table 1).

Soil solution was isolated and analyzed at the start of the plant experiment, 5 days after addition of the metal salts, and at the end of the plant experiment (Table 5). The measurement of the pH was problematic as the solution pH increased with time after isolation, which can be attributed to CO_2 volatilization. Nevertheless, the results indicated that the pH of the soil solution was around 7.6 with no effect of treatments. The solution composition was similar for the treatments with or without FeHBED, except for the Fe concentration. The Fe concentration in the soil solution was ca. 0.5 μM when no Fe was added to the soil, and initially around 100 μM for the FeHBED amended soils. At the end of

the experiment, however, the concentration in the FeHBED treated soils had generally decreased to between 5 and 20 μM, most likely because of degradation of HBED.

The Cu concentrations had decreased during the experiment in the Cu amended soils on which no plants had grown, suggesting slow reaction of the added Cu with the solid phase or a decrease in DOC concentrations during the experiment. Initial DOC concentrations were not measured, but DOC concentrations of the unplanted soils were low at the end of the experiment, in the order of 5 mg DOC L^{-1}. At the end of the experiment, differences in Zn concentrations between treatments for the unplanted soils were surprisingly small. In the planted soils, concentrations of Cu and Zn in soil solution were generally larger than in the corresponding unplanted treatments. These larger metal concentrations in planted soils were related to the higher DOC concentrations (Table 6). Also the UV absorbance indicated higher concentrations of organic components in the soil solutions of the planted soils. The SUVA of the soil solutions was higher for the Zn-deficient treatments, which agreed with the observations in the nutrient solutions.

The DGT-measurements confirmed the mobilization of metals by presence of the plants, as DGT-measured concentrations were larger in planted soils than in the corresponding unplanted treatment (Table 3). Not surprisingly, the ratio of DGT-measured to total solution concentration was generally smaller than in the nutrient solutions. The R values are usually smaller than 0.5 upon a 4-days deployment in soils, because there is a depletion of the solution concentration near the DGT interface (Ernstberger et al., 2002).

Plant concentrations.

Median concentrations in the shoot were 33 mg Mn kg^{-1}, 6.7 g Mg kg^{-1}, 34 g K kg^{-1}, 55.9 g Ca kg^{-1} and 3.3 g P kg^{-1}. Concentrations of P were higher for Zn-deficient plants (up to 7800 mg kg^{-1}), and inversely related to shoot yield, as was also observed in the solution experiment (Figure 4).

Zinc concentrations in the deficient plants were around 6 mg kg^{-1} in the shoot and 20 mg kg^{-1} in the root (Table 4), similarly as for the solution experiment. The plants on the FeHBED amended soils had higher Fe concentrations than those on the corresponding treatments without Fe amendment, but showed otherwise a similar composition. Root Fe concentrations are not reported in Table 4, because these were very high (around 5000 mg kg^{-1}) compared to Fe concentrations in the roots of the solution grown tomato plants, which were in general between 50 and 150 mg kg^{-1}. Most likely, these elevated Fe concentrations in the roots of the soil grown plants were due to soil contamination (Strasser et al., 1999).

Discussion

Metal mobilization by root exudates: chemical considerations

Our results show that root exudates of dicotyledonous plants are able to mobilize metals at relevant conditions. Analysis of the root exudate solutions indicated the exudation of carboxylates and DOC by the plants. Concentrations of carboxylates were not measured in the nutrient solutions because there was too much interference of the sample matrix for the chromatographic analysis. Based on the carboxylate exudation rate measured at harvest in deionized water (10–100 nmol g^{-1} RFW h^{-1}), concentrations of carboxylates in the nutrient solutions at the end of the experiment are expected to be 70 μM at most. Calculations with GEOCHEM-PC indicated that the exudation of carboxylates could not explain the large increase in metal concentrations. Even assuming that there is 70 μM oxalate, the carboxylate with the highest metal mobilization potential of the ones detected, the calculated degree of complexation (concentration ratio of complexed to free metal) is only 9 for Cu and 0.3 for Zn under the experimental conditions. The degree of complexation in the nutrient solutions at the end of the experiment was between 10^4 and 10^7 for Cu and between 1 and 1000 for Zn, indicating that other ligands than carboxylates are responsible for the metal mobilization.

The measured carboxylates accounted only for ca. 10% of the DOC measured in the root exudate solutions. Also the measured UV absorbance of the root exudate solutions could not be explained by the carboxylates, which confirms the presence of other, likely more aromatic, organic components. Several studies have reported the presence of phenolics in root exudates (e.g. Juszczuk et al., 2004), and we hypothesize that the metal mobilization is related to these types of components.

Complexes of metals with root exudates increase metal uptake.

The experimental results not only showed that root exudates mobilize Cu and Zn, but also suggested that these complexes affect metal uptake. For instance, for treatments buffered at same pCu and different pZn, the plant concentrations of Cu showed a significant positive correlation with total Cu concentration in solution. At pZn 9.6, clear Zn deficiency was observed in the 0.9-L solutions but not in solutions of smaller volume (0.25 L), which could be explained by the larger Zn concentrations in the solutions of small volume (cf. Figure 3). These results suggest that the complexes of metals with root exudates contribute to the metal uptake, similarly as has been observed for complexes with synthetic ligands (Degryse et al., 2006a). We hypothesize that the uptake is limited by diffusion of the free ion to the root surface, and that the complexes contribute by enhancing the diffusion flux through dissociation of the complex in the diffusion layer. The DGT measurements indeed showed that the metal complexes with root exudates are partially labile and enhance the diffusion flux under zero sink conditions.

Flux calculations were made to add further evidence to the hypothesis that the complexes contribute to the uptake by enhancing the diffusion flux. The metal uptake flux by plants in a solution without metal complexes can theoretically not exceed the maximal diffusion flux of the free ion, F (nmol g^{-1} RFW d^{-1}), and therefore the maximal plant concentration equals:

$$M_{pl} = \frac{RWR}{RGR} \cdot F = \frac{RWR}{RGR} \cdot SRA \cdot \frac{D}{\delta} \cdot [M^{2+}] \qquad (4)$$

where M_{pl} is the concentration in the plant (nmol per g FW), SRA is the specific root area (assumed to be 0.02 m^2 g^{-1} RFW), RWR is the root weight ratio (g root per g plant) on fresh weight basis, RGR is the relative growth rate (d^{-1}), D is the diffusion coefficient (4.3×10^{-5} m^2 d^{-1}), δ is the thickness of the diffusion layer (4×10^{-4} m) and $[M^{2+}]$ is the free ion concentration (in nmol m^{-3}). For a more detailed explanation and justification of the parameter values, we refer to Degryse et al. (2006a). Figure 6 shows that the observed plant concentrations are smaller than the predicted values for Cu and for Zn (at low Zn^{2+} activity), indicating that the uptake is limited by diffusion of the free ion to the root surface. The observed uptake can therefore only be explained if a contribution of the complexes in solution to the diffusion flux is assumed:

$$F_t = \mathrm{SRA} \cdot \frac{D}{\delta} \cdot ([\mathrm{M}^{2+}] + \xi[\mathrm{ML}]_t) \qquad (5)$$

where F_t is the metal flux to the root surface (nmol g^{-1} RFW d^{-1}). The subscript t is added because the concentration of complex, and therefore also the flux, is not constant in time (cf. Figure 3). The parameter ξ is the relative contribution or so-called lability of the complex and has a value of 0 when the complexes are inert and a value of 1 when the complex contributes as much as the free ion. The amount of metal taken up by the plant can be calculated based on equation (5), by integration of $\int F_t.W_{\mathrm{root},t}.dt$. Assuming exponential growth and an exponential increase in the metal complex concentration (cf. Eq. 3) and, for ease of calculation, also assuming that the plants were transferred to the solution at t=0, following equation is obtained for the metal concentration in the plant:

$$\mathrm{M_{pl}} = \mathrm{SRA}.\frac{\mathrm{RWR}}{\mathrm{RGR}} \cdot \frac{D}{\delta} \cdot ([\mathrm{M}^{2+}] + \frac{\xi[\mathrm{ML}]}{2}) \qquad (6)$$

where [ML] denotes the concentration of complex at the end of the experiment. The 2 in the denominator provides a time-averaged value of the complex concentration. The observed plant concentrations could be well described if it was assumed that Zn complexes contribute

for 20% (ξ=0.2) and Cu complexes for 0.4% (ξ=0.004) (Figure 6). Although the relative contribution of the Cu complexes is small, the predicted uptake is 100 to 10^4 times larger than when only the free ion is considered (Eq. 4), because of the very high degree of complexation for Cu. For Zn, the estimated contribution of the complexes is much larger. The DGT analyses indicated a high degree of lability for both the Cu and Zn complexes (Table 3). Under zero-sink conditions, the lability of complexes for plant uptake correlates well with the DGT-measured degree of lability, though the latter is usually higher (Degryse et al., 2006b). The much higher degree of lability for the Zn complexes than for the Cu complexes might therefore indicate that the plant almost acts as a zero sink for Zn at the low Zn^{2+} activities. For Cu, on the other hand, it appears that the plant demand is already satisfied without the plant acting as zero sink, and therefore, the contribution of the complexes is not as large as they possibly could be. This hypothesis is further supported by experimental observations. At low Zn supply, the plants seemed to respond to the sub-optimal supply by exudation of organic components with higher SUVA (Table 2) and higher metal affinity (Figure 1), although this could not prevent that plants started to suffer from Zn deficiency and showed reduced growth. No such observations were made at low Cu supply, suggesting that the 'basal root exudation' resulted in Cu concentrations that were large enough to sustain the plant's demand.

Figure 7 illustrates that the contribution of complexes to plant uptake can indeed explain the different plant response at same pZn between the solutions with small and large volume. The higher total solution concentrations of Zn explain why no yield reduction is observed in the small-volume solutions at the low Zn^{2+} activity (pZn 9.6). Both systems fall on the same response curve when yield is plotted against the 'labile' metal concentration, calculated as $[M^{2+}]+ \xi [ML]/2$ (Eq. 6) using a value of 0.2 for ξ. A similar effect of plant density on Zn deficiency response has been observed in the field, where biomass of rice increased at higher plant density in a low-Zn soil (Hoffland et al., 2006).

Solution versus soil system

Nutrient solutions provide a convenient means to study the interaction between plants and the surrounding solution, as the solution composition can be controlled and the solution can be easily sampled. Notwithstanding the many experimental advantages, there are reservations to the use of nutrient solutions and the general applicability of the results obtained from solution cultures, since plants grown on solutions may be morphologically and physiologically different from those growing in real soil (Pedler et al., 2000; Jones, 1998). Moreover, metals in nutrient solutions are often buffered using soluble ligands such as EDTA or HEDTA to control the metal activities at low, relevant levels. Complexes of Cu and Zn with these ligands have been shown to contribute to the plant uptake (e.g. Degryse et al., 2006a). Resin buffering avoids the need of using soluble ligands to control metal activities and more closely mimics the soil system, where metals are also buffered by a solid phase. Our results suggest that these resin-buffered solutions are a relatively good model to study metal uptake in soil. The Cu and Zn concentrations in soil-grown plants agreed reasonably well with those in plants grown on solutions with similar metal activities. In both systems, zinc deficiency was observed at low Zn^{2+} activities (pZn>9) and root exudates of Zn deficient plants had higher SUVA.

The relative growth rate was smaller for the soil-grown plants than for those grown on solution. The estimated value for RGR ranged between 0.09 and 0.13 d^{-1}. Assuming the same net exudation rate for the soil as for solution experiment (0.34 mg DOC g^{-1} RFW day^{-1}) and taking into account the smaller volume of solution in the soil experiment (ca. 10 mL), Equation (3) predicts DOC concentrations in the range of 80 to 200 mg L^{-1} in the soil solution. The observed DOC concentrations agree reasonably well with these predicted values (Table 6).

Notwithstanding the agreement between the solution and the soil experiment, also resin-buffered solutions suffer from the drawbacks inherent to solution culture and are not a perfect model of the soil system. Ultimately, soil processes must be studied in soil, but the complexity of the soil system often makes it hard to draw clear-cut conclusions from soil experiments, as also discussed below.

Possible artifacts and confounding factors in the soil experiment

Potted soil experiments are obviously more relevant to the 'real world' than nutrient solution experiments, but pose several experimental challenges. Soil solution must be isolated to analyze the solution composition, and the question always remains whether the isolated solution is representative for the solution that is actually accessed by the plant roots. To avoid this problem, we used small volumes of soil so that all soil could be considered to be rhizosphere. As a result, frequent application of fertilizer solution was needed. We tried to tune the fertilization to the plant's need, but this was not completely successful. Because of overdosing of Ca, the Ca concentrations had increased in the planted soils to ca. 15 mM compared to 2 mM in the control soils. The Ca concentration may affect the partitioning of metals, and the observed difference in metal concentrations between planted and unplanted soils could therefore be partly due to this difference in Ca concentration. To further explore this, we assessed the effect of Ca concentration in a batch experiment. Soil was suspended in solutions of varying Ca concentration in a 1:2 solid:liquid ratio and equilibrated for 3 days. The effect of Ca concentration on metal solution concentrations was found to be limited. The metal concentrations increased with 8 % (Cu) or 50 % (Zn) and in the metal spiked soil and decreased with 18 % (Cu) or 30 % (Zn) in the control soil (not spiked with metals) when the Ca concentration increased from 2 to 20 mM. Therefore, it seems not likely that the larger metal solution concentrations observed for the planted soils (Table 5) are solely due to the larger Ca concentrations.

Another problem with the soil experiment is that no distinction can be made between soil-borne and plant-borne (root exudates) DOC. In the analysis of our results, we assumed that the DOC was plant-borne, since DOC concentrations in solutions of plant-free controls were very small. However, it is possible that the presence of the plant changed the solubility of the soil organic C. For instance, the presence of plants may stimulate the microbial activity, resulting in enhanced mineralization of soil organic matter and the release of soluble products (priming effect; e.g. Cheng et al., 2003).

The soil solution was isolated by centrifugation, which raises the concern that damage of the plant roots may have caused a partial release of the root content into the solution. If that is the case, the higher DOC concentrations in the planted soils (~100 mg DOC L^{-1}) compared with unplanted controls may (partly) be an artifact. However, the metal (Cu/Zn) to organic C ratio was much higher in the isolated soil solution than in the plant roots. Therefore, the higher concentrations of metals in the solution of planted soil cannot be explained by possible root damage. We can, however, not exclude the possibility that the elevated DOC concentrations in planted soils were partly due to root damage during centrifugation. The effect of centrifugation on the release of organic carbon from roots was studied by Nambu et al. (2005). They found that the amount of DOC released by roots of *Pinus sylvestris* was higher if roots were packed in quartz sand and centrifuged (0.56 mg DOC g^{-1} root dry weight), than when the roots were dipped for 40 min in distilled water (0.3 mg g^{-1} RDW). Since root dry weight was around 0.05 g in our soil experiment, a release of 0.56 mg g^{-1} RDW would correspond to a release of 0.03 mg DOC from the roots, or only ~3 mg L^{-1} (since the soil solution volume was around 10 mL), suggesting that the problem of DOC contamination due to root damage is maybe not that important after all. Experiments by Yu et al. (1999) also suggested that release of symplastic components by plant roots during centrifugation is negligible at relative centrifugal forces up to 3000 g.

Some other studies have also shown an increase in DOC and metal concentrations in the rhizosphere. For instance, Cattani et al. (2006) found that DOC concentrations were 3-fold higher and soluble Cu concentrations 6-fold higher in the rhizosphere of a polluted calcareous soil than in the bulk soil. In contrast, Cornu et al. (2007) found lower Cu concentrations in the rhizosphere of tomato than in the bulk soil for 2 Cu-contaminated soils.

Implications

Only few studies have shown that root exudates of dicotyledonous plant species can mobilize Cu and Zn (e.g. Mench and Martin, 1991). Most of these studies used concentrated root exudates. The resin-buffering method used in this study allowed assessing metal mobilization by the root exudates under realistic conditions and at low metal activities. The results clearly show that root exudates mobilize metal at relevant metal activities and also strongly suggest that these complexes contribute to the metal uptake.

Other studies have reported increased root exudation under Zn-deficient conditions (e.g. Cakmak and Marschner, 1988). Our results show that the Zn-deficiency induced root exudation also has an impact on metal mobilization. At low Zn supply, the root exudates had higher SUVA values and showed higher metal affinity than at adequate Zn supply, as evident from the stronger Cu mobilization in solutions with same Cu^{2+} activity.

Also in the soil experiment, solution concentrations of Cu, Zn and DOC were higher for planted soils than for unplanted controls, and the SUVA of the soil solution after plant growth was higher under Zn deficient conditions. Our findings indicate that root exudation plays a role in increasing Zn availability to dicotyledonous plants under zinc deficient conditions, but more research is needed to assess the importance of this process.

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References

Amery F, Degryse F, Degeling W, Smolders E, Merckx R (2007) The copper-mobilizing-potential of dissolved organic matter in soils varies 10-fold depending on soil incubation and extraction procedures. Environ Sci Technol 41: 2277-2281.

Cakmak I, Marschner H (1988) Increase in membrane permeability and exudation in roots of zinc deficient plants. J Plant Physiol 132: 356-361.

Cakmak I, Sari N, Marschner H, Ekiz H, Kalayci M, Yilmaz A, Braun H J (1996) Phytosiderophore release in bread and durum wheat genotypes differing in zinc efficiency. Plant Soil 180: 183-189.

Cattani I, Fragoulis G, Boccelli R, Capri E (2006) Copper bioavailability in the rhizosphere of maize (*Zea mays* L.) grown in two Italian soils. Chemosphere 64: 1972-1979.

Chaignon V, Di Malta D, Hinsinger P (2002) Fe-deficiency increases Cu acquisition by wheat cropped in a Cu-contaminated vineyard soil. New Phytologist 154: 121-130.

Chaney R L (1988) Plant can utilze iron from Fe-N, N'-Di-(2-Hydroxybenzoyl)-ethylenediamine-N,N'-diacetic acid, a ferric chelate with 10^6 greater formation constant than Fe-EDDHA. J Plant Nutr 11: 1033-1050.

Checkai R T, Hendrickson L L, Corey R B, Helmke P A 1987 A method for controlling the activities of free metal, hydrogen and phosphate ions in hydroponic solutions using ion exchange and chelating resins. Plant Soil 99, 321-334.

Cheng W X, Johnson D W, Fu S L (2003) Rhizosphere effects on decomposition: controls of plant species, phenology, and fertilization. Soil Sci Soc Am J 67: 1418-1427.

Cornu J Y, Staunton S, Hinsinger P (2007) Copper concentration in plants and in the rhizosphere as influenced by the iron status of tomato (*Lycopersicon esculentum* L.). Plant Soil 292: 63-77.

Degryse F, Smolders E, Parker D R (2006a) Metal complexes increase uptake of Zn and Cu by plants: implications for uptake and deficiency studies in chelator-buffered solutions. Plant Soil 289: 171-185.

Degryse F, Smolders E, Merckx R (2006b) Labile Cd complexes increase Cd availability to plants. Environ Sci Technol 40: 830-836.

Ernstberger H, Davison W, Zhang H, Tye A, Young S (2002) Measurement and dynamic modeling of trace metal mobilization in soils using DGT and DIFS. Environ Sci Technol 36: 349-354.

Gries D, Klatt S, Runge M (1998) Copper-deficiency-induced phytosiderophore release in the calcicole grass *Hordelymus europaeus*. New Phytologist 140: 95-101.

Hoffland E, Wei C Z, Wissuwa M (2006) Organic anion exudation by lowland rice (*Oryza sativa* L.) at zinc and phosphorus deficiency. Plant Soil: 283, 155-162.

Ingestad T (1982). Relative addition rate and external concentration; driving variables used in plant nutrition research. Plant Cell Environ: 5, 443-53.

Jones D L (1998) Organic acids in the rhizosphere - a critical review. Plant Soil 205, 25-44.

Juszczuk I M, Wiktorowska A, Malusa E, Rychter A M (2004) Changes in the concentration of phenolic compounds and exudation induced by phosphate deficiency in bean plants (*Phaseolus vulgaris* L.). Plant Soil 267: 41-49.

Keller H, Römer W (2001) Cu, Zn, and Cd acquisition by two spinach cultivars depending on P nutrition and root exudation. J Plant Nutr Soil Sci 164: 335-342.

Ma J F, Nomoto K (1996) Effective regulation of iron acquisition in graminaceous plants. The role of mugineic acids as phytosiderophores. Physiol Plant 97: 609-617.

Mench M, Martin E (1991) Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. Plant Soil 132: 187-196.

Nambu K, van Hees P A W, Essén S A, Lundström U S (2005) Assessing centrifugation technique for obtaining soil solution with respect to leaching of low molecular mass organic acids from pine roots. Geoderma 127: 263-269.

Ozkutlu F, Torun B, Cakmak I (2006) Effect of zinc humate on growth of soybean and wheat in zinc-deficient calcareous soil. Commun Soil Sci Plant Anal 37: 2769-2778.

Parker D R, Reichman S M, Crowley D E 2005 Metal chelation in the rhizosphere. In: Wright S F and Zabeb R W (eds) Root and soil management: interactions between roots and the soil. American Society of Agronomy Monograph, Soil Sci Soc Am, Madison, WI, pp 57-93.

Pedler J F, Parker D R, Crowley D E (2000) Zinc deficiency-induced phytosiderophore release by the triticaceae is not consistently expressed in solution culture. Planta 211: 120-126.

Song J, Zhao F J, Luo Y M, Mcgrath S P, Zhang H (2004) Copper uptake by *Elsholtzia splendens* and *Silene vulgaris* and assessment of copper phytoavailability in contaminated soils. Environ Pollut 128: 307-315.

Sterckeman T, Perriguey J, Cael M, Schwartz C, Morel J L (2004) Applying a mechanistic model to cadmium uptake by *Zea mays* and *Thlaspi caerulescens*: Consequences for the assessment of the soil quantity and capacity factors. Plant Soil 262: 289-302.

Strasser O, Köhl K, Römheld V (1999) Overestimation of apoplastic Fe in roots of soil grown plants. Plant Soil 210: 179-187.

Tipping E (1998) Humic ion-binding model VI: an improved description of the interactions of protons and metal ions with humic substances. Aquat Geochem 4: 3-48.

Tipping E, Rieuwerts J, Pan G, Ashmore M R, Lofts S, Hill M T R, Farago M E, Thornton I (2003) The solid–solution partitioning of heavy metals (Cu, Zn, Cd, Pb) in upland soils of England and Wales. Environ Pollut 125: 213-225.

Uselman S M, Qualls R G; Thomas R B (2000) Effects of increased atmospheric CO2, temperature, and soil N availability on root exudation of dissolved organic carbon by a N-fixing tree (*Robinia pseudoacacia* L.) Plant Soil 222: 191-202.

von Wíren N, Marschner H, Römheld V (1996) Roots of iron-efficient maize also absorb phytosiderophore-chelated zinc. Plant Physiol 111: 1119-1125.

Yu Q, Tang C, Chen Z, Kuo J (1999) Extraction of apoplastic sap from plant roots by centrifugation. New Phytol 143: 299-304.

Zhang F, Römheld V, Marschner H (1989) Effect of zinc deficiency in wheat on the release of zinc and iron mobilizing root exudates. Z Pflanzenernähr Bodenk 152: 205-210.

Zhang F S, Römheld V, Marschner H (1991) Release of zinc mobilizing root exudates in different plant-species as affected by zinc nutritional status. J Plant Nutr 14: 657-686.

Zhang H, Davison W (1995) Performance characteristics of diffusion gradients in thin films for the in situ measurement of trace metals in aqueous solution. Anal Chem 67: 3391-3400.

Figure Captions

Figure 1. Temporal evolution of the Cu concentrations in resin-buffered nutrient solutions on which spinach was grown (2 plants per 0.9-L solution). Metal activities in the solutions were buffered at pCu~11.6 and at pZn 10.1 or 8.0. The error bars denote standard errors of 3 replicates (Exp. 1).

Figure 2. The concentration ratio of complexed to free Cu in the resin-buffered nutrient solutions on which tomato or spinach plants had grown for 20 days (2 plants per 0.9-L solution) as a function of Cu^{2+} activity at which the solution was buffered (Results from Experiments 1 and 2) .

Figure 3. Measured (symbols) and modeled (lines) concentrations of Zn and of dissolved organic C (DOC) in resin-buffered nutrient solutions buffered at pZn 8.8 and pCu 11.8. Two tomato plants were grown on a solution of 0.9 L (Exp. 2) or 0.25 L (Exp. 3). Zinc concentrations were modeled with Eq. (3) assuming a Zn mobilization rate of 1.6 nmol g^{-1} RFW day^{-1}. The DOC concentrations were predicted with Eq. (2) using the average DOC release rate as measured during root exudates collection at harvest (0.34 mg DOC g^{-1} RFW day^{-1}).

Figure 4. The P concentration in the plant shoot as a function of the relative shoot yield, i.e. the ratio of the observed shoot fresh weight and the shoot fresh weight of treatments without growth reduction, for solution and soil grown plants. The line shows an inverse relation between P concentration and yield.

Figure 5. Concentrations of Zn and Cu in shoots and roots of tomato plants grown on resin-buffered nutrient solutions as function of the free metal activity (Exp. 2). The error bars show standard errors for 2 or 3 replicates.

Figure 6. Observed and predicted Cu and Zn concentrations in tomato grown on resin-buffered solutions, as function of the free metal activity. The full line shows the predicted values assuming that the diffusive transport is limiting and only the free ion contributes to the diffusion flux (Eq. 4). The dotted line shows the prediction if a contribution of the complexes (as denoted by ξ) is assumed (Eq. 6). Vertical bars represent the standard error.

Figure 7. Relative shoot yield as a function of free Zn^{2+} activity, labile Zn concentration or total solution concentration, for tomato plants grown on resin-buffered solutions at different plant to volume ratios (Exp. 2: two plants per 0.9-L solution; Exp. 3: two plants per 0.25-L solution). All solutions were buffered at pCu~11.7. The labile Zn concentrations were calculated assuming that complexes contribute for 20% (or $[Zn_{lab}] = [Zn^{2+}] + 0.1\ [ZnL]$, cf. Eq. 6 with ξ=0.2).

Table 1. Selected soil properties of the soil used in the pot experiment

pH ($CaCl_2$)	7.7
Organic C (%)	0.3
CaCO3 (%)	29.0
clay (%)	17
Fe_{dith} (g kg^{-1})	11
Fe_{ox} (g kg^{-1})	0.24
Cu_{tot} (mg kg^{-1})	7
Cu_{EDTA} (mg kg^{-1})	2.0
Zn_{tot} (mg kg^{-1})	28
Zn_{EDTA} (mg kg^{-1}	2.2

Fe_{dith}: dithionite-extractable Fe; Fe_{ox}: oxalate-extractable Fe; Cu_{tot} and Zn_{tot}: aqua regia extractable Cu and Zn; Cu_{EDTA} and Zn_{EDTA}: Cu and Zn extractable with 25 mM EDTA (S:L 1:5 kg L^{-1})

Table 2. Shoot fresh weight (SFW) of tomato plants grown on resin-buffered solutions, metal concentrations in these nutrient solutions at the end of the plant growth (20 DAT), and the specific UV absorbance at 254 nm (SUVA) of the root exudate solutions (5 hours collection before harvest; Exp. 2).

Treatment		SFW	Metal in nutrient solution		SUVA ($L\ g^{-1}\ cm^{-1}$)
		(g per 2 plants)	Cu (μM)	Zn (μM)	of root exudates
pZn	9.6	4.5 b	1.17	0.02	26.6 b
	9.3	7.3 b	2.49	0.02	35.6 a
	8.8	14.4 a	3.07	0.02	12.6 c
	7.6	13.0 a	2.36	0.07	12.5 c
	6.6	11.4 a	1.32	0.27	14.4 c
pCu	13.0	14.2 a	1.03	0.07	12.1 c
	12.4	14.0 a	2.02	0.03	8.1 c
	11.8	14.4 a	3.07	0.02	12.6 c
	11.2	11.7 a	3.15	0.03	14.0 c
	10.4	14.4 a	2.50	0.02	10.3 c

Values within the same column with a different letter are significantly different at P=0.05

Table 3. The DGT (diffusive gradients in thin films) measured concentration (c_{DGT}) of Cu and Zn, and the ratio of c_{DGT} to the solution concentration (R) for selected treatments of the solution (Exp. 2) and soil experiment.

Treatment		Copper		Zinc	
		c_{DGT} (μM)	R	c_{DGT} (μM)	R
Solution					
pZn	8.8	1.00	0.33	0.01	0.44
	7.6	1.01	0.31	0.07	1.03
	6.6	0.47	0.40	0.15	0.47
pCu	13.0	0.42	0.35	0.08	0.99
	10.4	0.42	0.31	0.01	0.45
Soil					
Control	+plant	0.07	0.14	0.05	0.05
	–plant	0.04	0.37	0.02	0.10
+Zn	+plant	0.07	0.18	1.25	0.24
	–plant	0.02	0.18	0.62	0.72
+Cu/Zn	+plant	1.00	0.11	0.82	0.25

Table 4. Shoot fresh weight (SFW) and metal concentrations in shoots and roots of tomato plants grown on a calcareous soil (Table 1) when no metal is added or when Cu and Zn is added to the soil in concentrations of 50 (Cu) or 100 mg kg^{-1}. The FeHBED treated soils were amended with 0.025 mmol FeHBED kg^{-1}. Values between brackets give standard errors of 3 replicates

Treatment	SFW (g/plant)	Cu_{shoot} (mg kg^{-1})	Cu_{root} (mg kg^{-1})	Zn_{shoot} (mg kg^{-1})	Zn_{root} (mg kg^{-1})	Fe_{shoot} (mg kg^{-1})
No Fe added						
Control	1.3 ab	5.1 (0.6)	9.5	5.4 (0.7)	17	45 (7.2)
+Cu	1.1 b	16.2 (1.6)	115	5.9 (0.5)	28	67 (2.8)
+Zn	1.4 ab	3.6 (0.4)	7.7	126 (9.3)	147	45 (0.5)
+Cu/Zn	1.6 a	18.4 (0.8)	40	149 (5.2)	140	48 (3.9)
FeHBED added						
Control	1.5 ab	4.8 (0.2)	13.0	5.0 (0.2)	22	61 (3.4)
+Cu	1.2 b	14.8 (1.5)	91	5.1 (0.2)	31	75 (7.1)
+Zn	1.7 a	4.3 (0.3)	10.4	104 (4.3)	152	64 (1.2)
+Cu/Zn	1.2 ab	13.7 (1.0)	51	84 (2.8)	140	80 (19)

Values within the same column with a different letter are significantly different at P=0.05

Table 5. Copper and zinc concentrations in the soil solution for different metal treatments, 5 days after the metal addition ('initially') or after 4 weeks in a plant growth chamber for soils on which tomato was grown (+ plant) or for plant-free controls. Values between brackets give standard errors of 3 replicates

		Cu in solution (μM)				Zn in solution (μM)		
Treatment	pCu [a]	initially	-plant	+plant	pZn [a]	initially	-plant	+plant
No Fe added								
Control	12.1	0.10	0.14 (0.05)	0.44 (0.09)	9.2	0.16	0.47 (0.19)	1.36 (0.20)
+Cu	10.0	1.66	0.29 (0.02)	3.21 (0.45)	9.0	0.12	0.42 (0.03)	0.36 (0.05)
+Zn	11.9	0.09	0.13 (0.02)	0.13 (0.08)	7.4	0.54	0.48 (0.10)	3.55 (0.54)
+Cu/Zn	9.9	3.08	0.34 (0.10)	4.34 (0.34)	7.2	0.41	0.38 (0.14)	1.89 (0.38)
FeHBED added								
Control	12.1	0.10	0.04 (0.001)	0.39 (0.07)	9.2	0.19	0.37 (0.13)	1.16 (0.32)
+Cu	10.0	1.67	0.74 (0.04)	4.58 (0.21)	9.0	0.02	0.29 (0.06)	0.86 (0.10)
+Zn	11.9	0.10	0.01 (-)	0.52 (0.08)	7.4	0.88	0.87 (-)	5.24 (0.18)
+Cu/Zn	9.9	2.66	0.76 (0.19)	12.5 (1.60)	7.2	0.57	0.32 (0.03)	3.54 (0.25)

[a] Free ion activities were modeled with WHAM/ModelVI

Table 6. The concentration of dissolved organic C (DOC; mg L^{-1}) and the specific UV absorbance (SUVA; L g^{-1} cm^{-1}) of the soil solution for the different metal treatments. The FeHBED treated soils were amended with 0.025 mmol FeHBED kg^{-1}. Tomato plants were grown on the soil for 28 days. The DOC concentration in unplanted soil was ca. 5 mg L^{-1}.

	- FeHBED		+FeHBED	
Treatment	DOC	SUVA	DOC	SUVA
Control	58 b	14.8 a	60 c	13.6 a
+Cu	55 b	15.2 a	68 bc	11.7 ab
+Zn	117 a	9.1 b	119 b	8.8 b
+Cu/Zn	111 a	9.1 b	222 a	7.7 b

Values within the same column with a different letter are significantly different at *P*=0.05

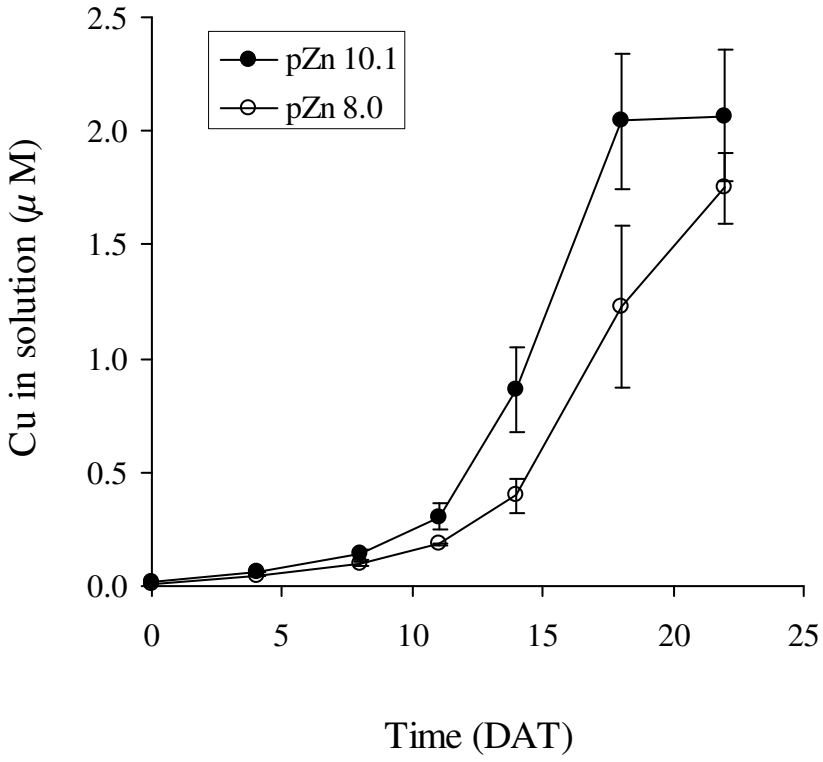
pZn 10.1
pZn 8.0
Cu in solution (μ M)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time (DAT)

Figure 1

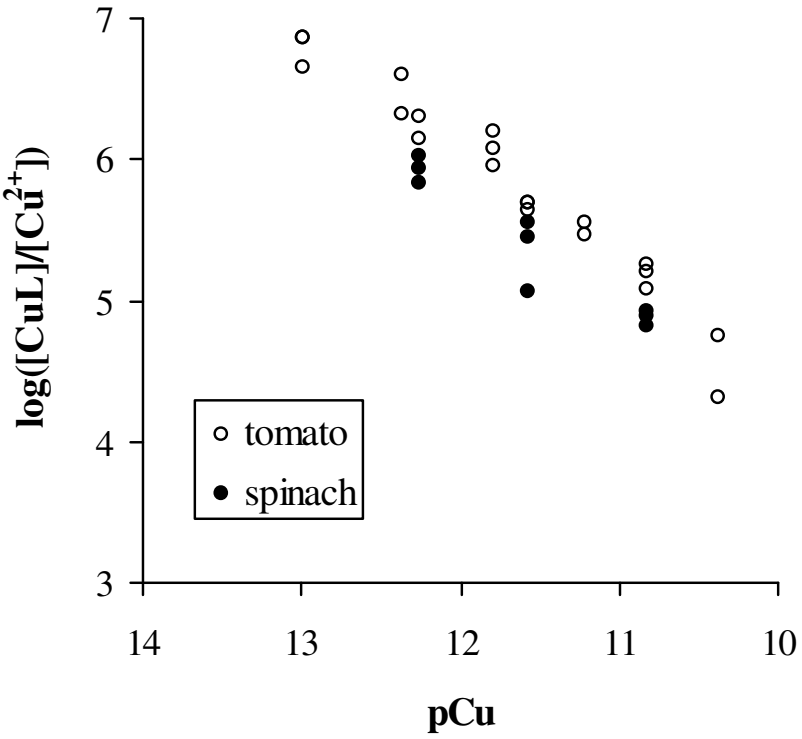

Figure 2

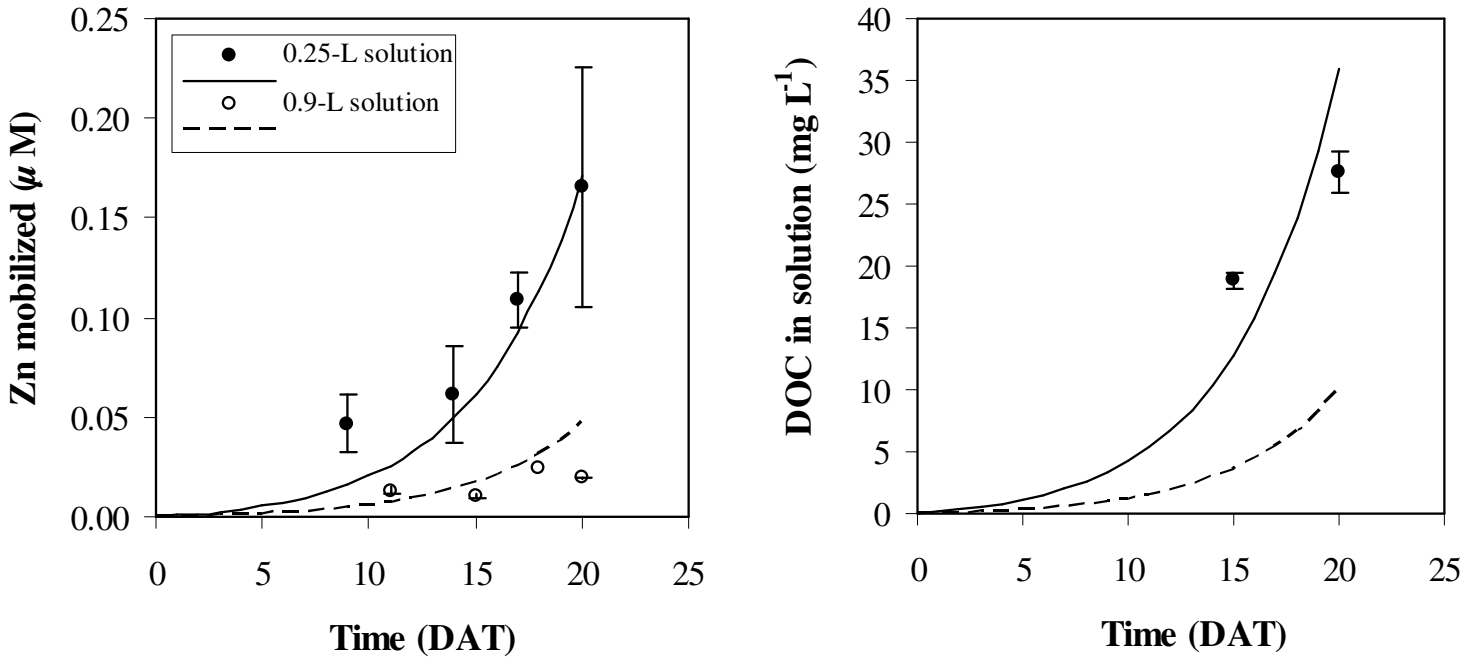
0.25
0.20
0.15
0.10
0.05
0.00
Zn mobilized (μ M)
0.25-L solution
0.9-L solution
0
5
10
15
20
25
Time (DAT)
40
35
30
25
20
15
10
5
0
DOC in solution (mg L-1)
0
5
10
15
20
25
Time (DAT)

Figure 3

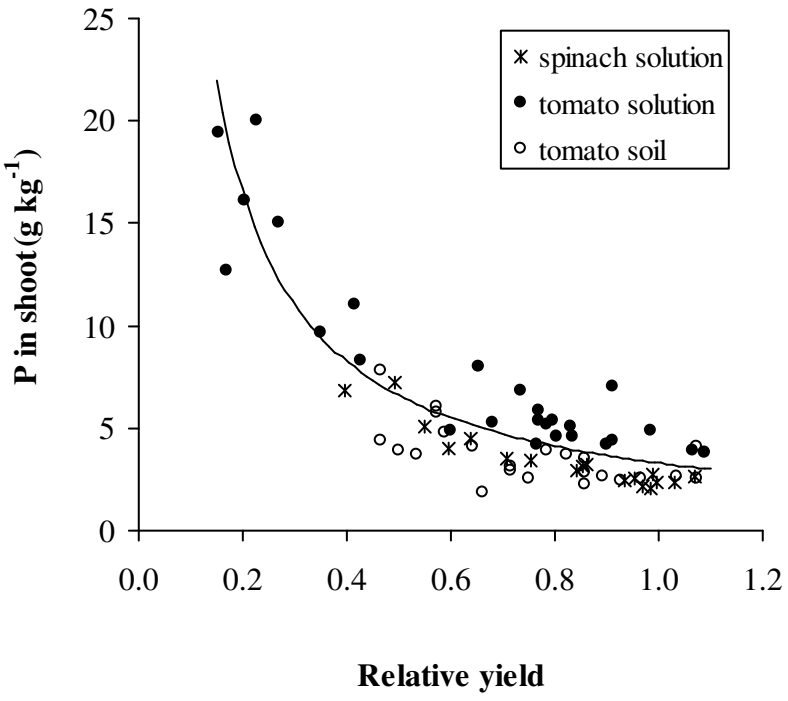
25
20
15
10
5
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
P in shoot(g kg-1)
Relative yield
spinach solution
tomato solution
tomato soil

Figure 4

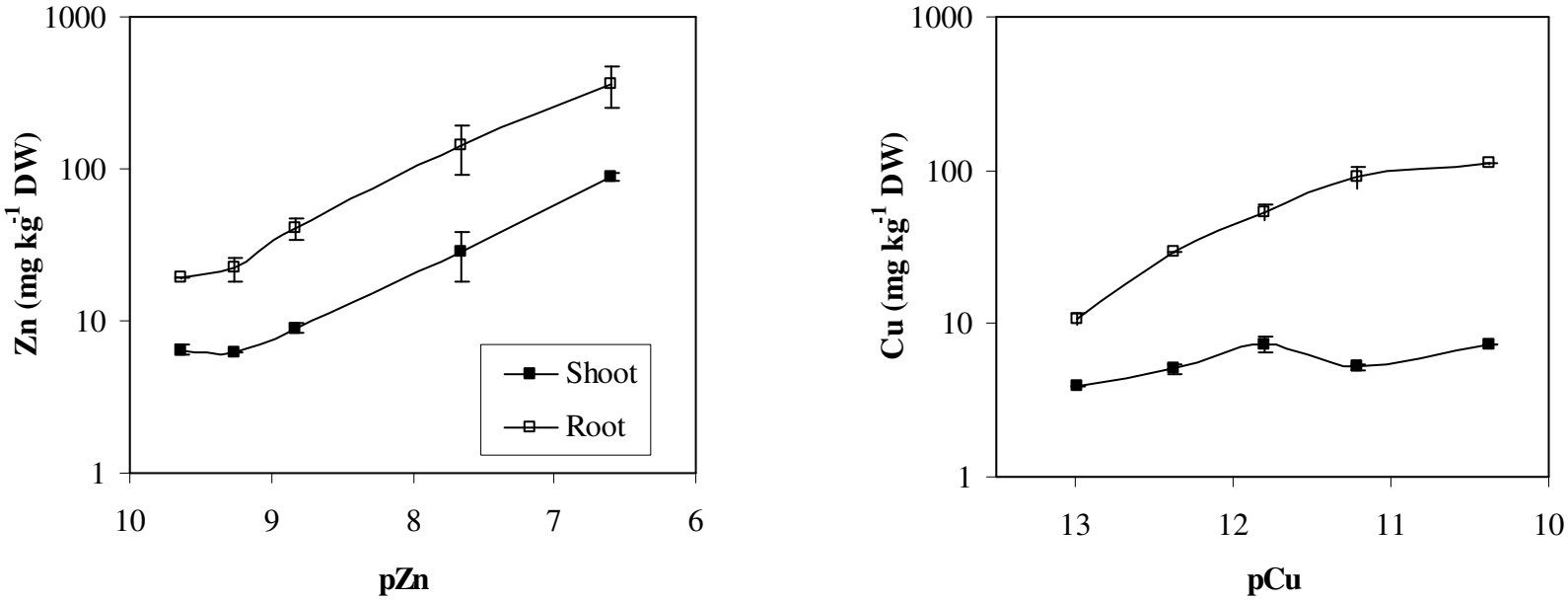
1000
100
10
1
Zn (mg kg-1 DW)
10
9
8
7
6
pZn
Shoot
Root
1000
100
10
1
Cu (mg kg-1 DW)
13
12
11
10
pCu

Figure 5

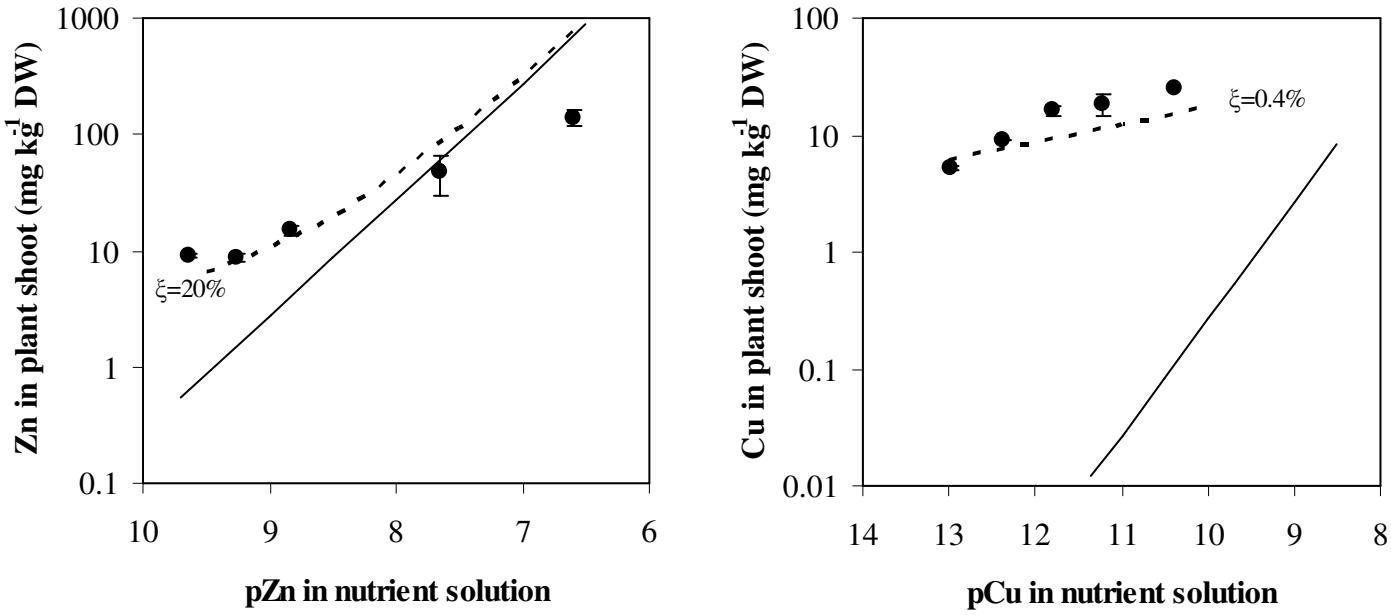
1000
100
10
1
0.1
Zn in plant shoot (mg kg-1 DW)
ξ=20%
10
9
8
7
6
pZn in nutrient solution
100
10
1
0.1
0.01
Cu in plant shoot (mg kg-1 DW)
ξ=0.4%
14
13
12
11
10
9
8
pCu in nutrient solution

Figure 6

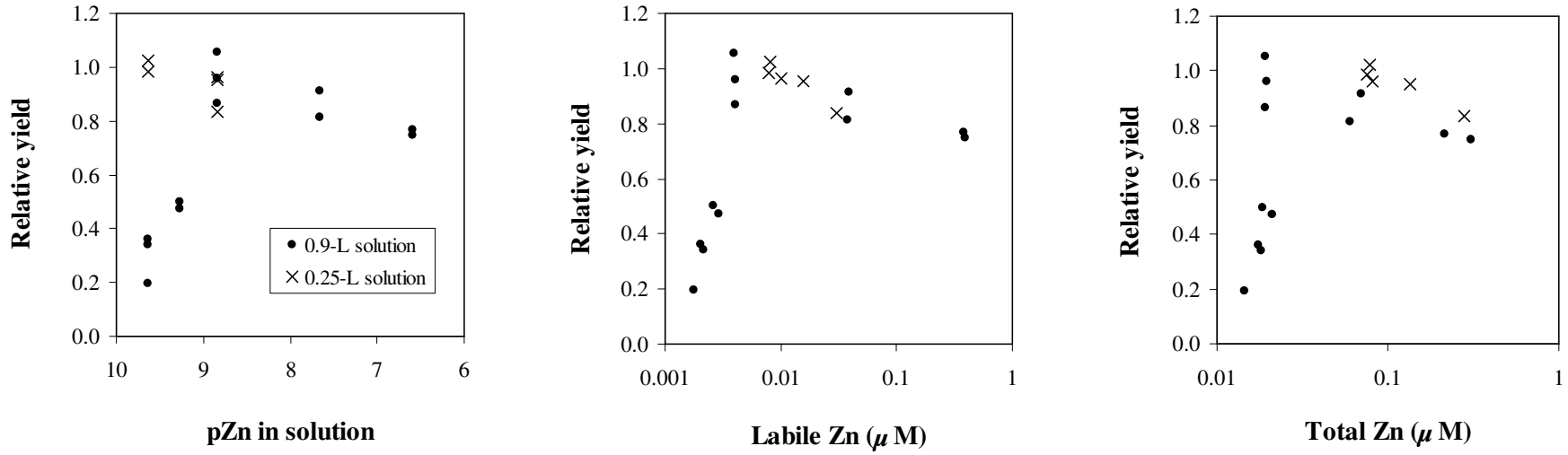
Relative yield
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
9
8
7
6
pZn in solution
0.9-L solution
0.25-L solution
Relative yield
0.001
0.01
0.1
1
Labile Zn (μ M)
Relative yield
0.01
0.1
1
Total Zn (μ M)

Figure 7