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Metal complexes increase uptake of Zn and Cu by plants:

Implications for uptake and deficiency studies in chelator-buffered solutions

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Abstract

The uptake of trace metals by plants is commonly assumed to depend on the free metal-ion activity, rather than on the total concentration of dissolved metal. Although this free-ion hypothesis has proved to be useful for the interpretation and prediction of metal uptake, several exceptions have been reported where metal complexes also affected metal uptake by plants. In this study, we measured uptake of Zn and Cu by spinach (*Spinacia oleracea* L.) and tomato (*Lycopersicon esculentum* L.) in chelator-buffered or resin (Chelex)-buffered solutions, under Zn-deficient and non-deficient conditions. Several ligands, with differing dissociation rates, were used in the chelator-buffered solutions. At the same free-ion activity, Cu and Zn uptake was less in Chelex-buffered than in chelator-buffered solutions. In the chelator-buffered solution, uptake of Cu and Zn at same free-ion activity and same total concentration followed the order: NTA > HEDTA > EDTA > CDTA, i.e. the same order as the dissociation rate. These differences in metal uptake were also reflected in the deficiency symptoms and plant yield in the experiments where Zn deficiency was imposed. The critical Zn^{2+} activity for Zn deficiency varied by one order of magnitude depending on the buffer, and followed the order HEDTA < CDTA < resin-buffered (no soluble ligand). These results suggest that, when present, aqueous complexes can increase metal uptake by plants because uptake is rate-limited by diffusion of the free ion to the root or cell surface. Thus, the critical free-ion activity in chelator-buffered solutions depends on the type and concentration of the ligand employed.

Introduction

Studies of plant mineral nutrition have long relied on solution culture methods, as solutions are better-defined chemically and a more homogenous medium than soils. In recent decades, trace metal uptake and nutrition has frequently been investigated using chelator-buffered solutions, which can maintain trace-metal activities at low, relevant levels.

Moreover, deficiencies of trace elements such as Zn can be quite difficult to induce in hydroponically grown plants when using unbuffered solutions (Parker et al., 1995a).

‘Conventional’ solutions are therefore not well suited to the study of Zn deficiency.

Chelator-buffered solutions, where metals are buffered using synthetic metal chelators such as EDTA or HEDTA, have been successfully used to induce Zn deficiency in higher plants, and have proved useful in the derivation of critical levels and in ranking genotypes for sensitivity to Zn deficiency (e.g., Cakmak et al., 1996). Based on studies using chelator-buffered solutions, critical values between 10^{-11} and 10^{-10} M have been reported for the free Zn^{2+} activity below which growth of soybean (Chaney et al., 1989), tomato (Parker et al., 1992), wheat, maize (Parker, 1993), barley (Norvell and Welch, 1993), and rice (Yang et al., 1994) is affected.

The use of chelator-buffering in nutrient solutions relies on the assumption that metal uptake is not affected by the presence of complexes, but is solely controlled by the free-ion activity. However, it has been shown on several occasions that this assumption does not always hold. Uptake of Cu, Zn, and Mn by barley (Bell et al., 1991) and Cd and Zn by lettuce (McLaughlin et al., 1997) was not only controlled by the free-metal activity, but also depended on the concentration of metal-ligand complexes. It has been suggested that the contribution of metal complexes to metal uptake by plants is caused by direct uptake of the complexes, or by alleviation of diffusional constraints for uptake of the free ion, through dissociation of labile metal complexes in the diffusive boundary layer (Berkelaar and Hale,

2003). To test the latter hypothesis, Degryse et al. (2006) measured Cd uptake by spinach in nutrient solutions at same free-ion concentration, in presence of complexes with different dissociation kinetics. Uptake was greatest in the presence of rapidly dissociating (labile) complexes. The observed effect of complexes on Cd uptake was in good agreement with predictions based on the assumption that uptake is limited by diffusion of the free ion through an unstirred layer to the root surface. Although these observations were for a non-essential metal, the same processes may also occur with essential metals, suggesting that the critical free Zn activity will differ between chelator-buffered solutions and solutions without Zn complexes, and will depend on the type of ligand and concentration of complexes in chelator-buffered systems.

In this study, we measured uptake of Zn and Cu by spinach and tomato in both chelator-buffered and resin-buffered solutions. Different ligands, with varying dissociation rate, were used in the chelator-buffered solutions. In the resin-buffered solutions, metals were buffered with a Chelex resin (Checkai et al., 1987). Since the solid phase resin is physically separated from the root system, there is little possibility that this type of buffering increases metal uptake, whether it is by enhancing the diffusion flux through dissociation of the complex near the root surface or by direct uptake of the complex.

Materials and methods

Zn deficiency experiments (Experiments 1 and 2)

Treatments for the Zn deficiency experiments are summarized in Table 1. In the first experiment, Zn deficiency in two dicotyledonous species was assessed in solutions buffered with the ligands HEDTA (N-(2-hydroxyethyl)-ethylene-diaminetriacetate) or CDTA (trans-1,2-cyclohexylenediaminetetraacetate), or with a chelating resin (Chelex-100, 100–200 mesh), with three zinc levels for each type of buffering. The nutrient solutions had a basal

composition of (in mM) 2 Ca(NO₃)₂, 0.5 MgSO₄, 1.2 KNO₃, 0.1 KH₂PO₄, and (in μM) 25 NaCl, 15 H₃BO₃, 0.07 (NH₄)₆Mo₇O₂₄, 25 FeHBED. The solutions were buffered with 2 mM MES at pH 6. The FeHBED (Fe-N,N'-bis (2-hydroxybenzyl)-ethylenediamine-N,N'-diacetate) stock solution was prepared according to Chaney (1988) from HBED (acid form) and FeCl₃ in a 30% molar excess over HBED to ensure that no free HBED was left in solution. The chelator-buffered solutions contained 5 μM MnSO₄, 2 μM CuSO₄ and 1, 10 or 100 μM ZnCl₂. Solution speciation was calculated using the GEOCHEM-PC program (Parker et al., 1995b). The solutions were buffered at a total Zn concentration that was 10⁵ times larger than the free Zn²⁺ concentration (10⁻¹¹, 10⁻¹⁰ or 10⁻⁹ M Zn²⁺) using a ligand excess (above the sum of Mn, Cu, and Zn concentrations, since Fe was buffered with HBED) of 75 μM for HEDTA and 121 μM for CDTA. The Chelex-buffered solutions were prepared by mixing Zn-, Cu-, Mn- and Ca-chelex in ratios of 0.11:0.10:0.01:0.78 (low-Zn), 0.28:0.10:0.01:0.61 (medium-Zn), and 0.50:0.10:0.01:0.39 (high-Zn), respectively. The Chelex was loaded with the metals by equilibrating the resin with a concentrated solution of the metal salt, and rinsing several times with deionized water and finally with a 2 mM MES (pH 6.0) solution, to remove excess metal. Two g of moist Chelex mixture was added to each 3-L solution, and the solutions were equilibrated for three days. All treatment solutions were prepared in duplicate. Seeds of spinach, *Spinacia oleracea* L. ('Correnta'), and tomato, *Lycopersicon esculentum* L. ('Brandywine'), were surface sterilized for 15 minutes in 0.04 M NaOCl, rinsed with deionized water, and germinated for six days at 25 °C between sheets of germination paper continuously wetted with metal-free nutrient solution. Two seedlings of each species were transferred to each aerated 3-L pot. The plants were grown in a growth chamber with a 12-12 h day-night cycle, day-night temperatures of 20-16 °C, and photosynthetic photon flux density (PPFD) of 400 μmol m⁻² s⁻¹. The pH was measured daily and adjusted to 6.0 ± 0.1 when necessary. The Chelex-buffered solutions were stirred every

day for 5 minutes with a magnetic stirrer, to ensure proper buffering of the solution. At 12 days after transplanting (DAT), KH_2PO_4 was added to each container in a concentration of $30\ \mu\text{M}$. The plants were taken from the nutrient solution at 18 DAT. The roots were rinsed and blotted, and individual root and shoot fresh weight was recorded. The shoots and roots within each container were pooled and dried (2 days at $65\ ^\circ\text{C}$). Dried samples were weighed, and ground to pass a 0.43-mm sieve. Samples (0.25 g) were then digested by microwave digestion in a mixture of 2 mL HNO_3 (70% w:w), 2 mL H_2O_2 (30%) and 1 mL H_2O , and brought to a final volume of 25 mL. Because root dry-weights were small, the root samples of duplicate containers were pooled before digestion. The digests were analyzed using inductively coupled plasma – optical emission spectrometry (ICP-OES; Perkin Elmer 3300 DV). A blank sample and a sample of certified plant material issued from the National Institute of Standards and Technology (NIST 1515, apple leaves, or NIST 1573a, tomato leaves) was analyzed with each batch of digestions (14 samples). Observed metal (Zn, Cu, Mn, Fe) concentrations were almost always within 15% (and for Zn within 10%) of the certified values.

Because Zn deficiency was not obtained with any of the Chelex-buffered treatments, a second experiment (Exp. 2) was carried out with tomato (same variety), where Zn was either buffered with HEDTA or with Chelex at lower Zn loadings than those used in the first experiment; there were four zinc levels for each type of buffering. The solutions with HEDTA were buffered at a total Zn concentration that was 3.3×10^4 times larger than the free Zn^{2+} concentration (7.4, 18, 46 and $120\ \text{pM}\ \text{Zn}^{2+}$) using an HEDTA excess (above the sum of Mn, Cu, and Zn concentrations) of $25\ \mu\text{M}$. The Chelex-buffered solutions were prepared by mixing Zn-chelex, Cu-chelex, Mn-chelex and Ca-chelex in ratios of 0.01:0.10:0.01:0.88, 0.02:0.10:0.01:0.87, 0.04:0.10:0.01:0.85 and 0.08:0.10:0.01:0.81 respectively. One gram of Chelex was added to each 1-L solution. All treatment solutions

were prepared in duplicate. The basal composition of the solutions was the same as for the first experiment. Tomato seeds were germinated for seven days at 25 °C between sheets of germination paper continuously wetted with metal-free nutrient solution. Two seedlings were transferred to each aerated 1-L pot. The plants were grown in a growth chamber with a 12-12 h day-night cycle, day-night temperatures of 20-16 °C, and a PPFD of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The Chelex-buffered solutions were stirred daily. To avoid depletion of P in solution, KH_2PO_4 was added at a concentration of 30 μM to each solution at 9 DAT, and again at 14 DAT to the Chelex-buffered solutions. The HEDTA-buffered solutions were completely renewed at 14 DAT, since mass-balance calculations indicated that depletion of Zn in the solution could occur if solutions were not renewed. The plants were harvested at 17 DAT, and analyzed as described above.

The solution Zn concentration was below detection limit for most of the Chelex-buffered treatments. To determine the free Zn concentration for these treatments, the Zn concentration was measured after equilibration with a ligand (NTA) at the end of the plant experiment. The Chelex was suspended in 100 mL of the nutrient solution and NTA was added at a total concentration of 0.2 mM. The solutions were equilibrated for three days and total metal concentrations were measured by ICP-OES after filtration ($<0.45 \mu\text{m}$) of the solution. The free metal concentrations and activities were then calculated with GEOCHEM-PC, using the basal solution composition, the added NTA concentration (0.2 mM), and the measured metal concentrations as input. Metal concentrations in the Chelex-buffered solutions with Zn concentrations above the detection limit (i.e., the treatments with the two highest Zn loadings in Experiment 1) were measured regularly during the plant-growth phase of the experiment.

Zn and Cu uptake by spinach (Experiment 3)

The effect of ligand type on Cu and Zn uptake in buffered solutions was assessed by measuring uptake of Cu and Zn by spinach in solutions with same free-ion and total metal concentration using different ligands (Table 2). Two series of four solutions were buffered with the ligands NTA, HEDTA, EDTA or CDTA. Free-ion concentrations of Cu, Mn and Zn were the same for all chelator-buffered treatments: 10^{-12} M for Cu^{2+} , 10^{-9} M for Zn^{2+} , and 10^{-7} M for Mn^{2+} . In the first series of solutions, total Zn was $10\ \mu\text{M}$, and in the second series total Cu was $5\ \mu\text{M}$. Two treatments were also included in which the metals were buffered with Chelex. The Chelex-resin was prepared by mixing Zn-, Cu-, Mn- and Ca-Chelex in ratios of 0.03:0.03:0.01:0.93 (low Zn and Cu) and 0.16:0.16:0.01:0.67 (high Zn and Cu), respectively. One g of (moist) Chelex was added to each (1-L) solution. All treatment solutions were prepared in duplicate.

Seeds of spinach, *Spinacia oleracea* L. ('Correnta'), were surface sterilized for 15 minutes in $0.04\ \text{M}$ NaOCl, rinsed with deionized water, and germinated for six days at $25\ ^\circ\text{C}$ between sheets of germination paper continuously wetted with metal-free nutrient solution. Two seedlings were transferred to each aerated 1-L pot. The plants were grown in a growth chamber with a 14-10 h day/night cycle, day-night temperatures of $22\text{-}16\ ^\circ\text{C}$, and a PPFD of $400\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. Daily, the pH was checked and adjusted to 6.0 ± 0.1 as necessary, and the Chelex-buffered solutions were stirred. At 12 and 17 DAT, KH_2PO_4 was added to each container at a concentration of $30\ \mu\text{M}$. The plants were taken from the nutrient solution at 19 DAT. Plants were analyzed in the same way as described previously.

Dissociation kinetics

The dissociation constant, k_d , of the metal-ligand complexes was measured with the competitive ligand exchange method, as described by Degryse *et al.* (2006). Chelex-100

resin was converted to the Ca-form and buffered at pH 6.0 (MES). Solutions were prepared containing 2 mM Ca(NO₃)₂, 2 mM MES (pH 6), 0.1 mM ligand, and 10 μM of Cd(II), Cu(II), Pb(II), Ni(II) and Zn(II). The Chelex was added to the stirred solution, in a ratio ranging from 1 g (for the weaker ligands) to 20 g Chelex per 50 mL of solution. Subsamples were removed from the solution at various times (up to four days), filtered through a 0.45-μm membrane, and analysed by ICP-OES. The first-order dissociation rate constant, k_d , was estimated by fitting the data to following equation, using least-squares non-linear regression:

$$c = c_{\text{ini}} \exp(-k_d t) \quad (1)$$

where c_{ini} is the initial metal concentration in solution and c is the concentration measured at time t .

Results

Zinc deficiency in chelator-buffered and resin-buffered solutions (Experiments 1 and 2)

Solution composition. Total metal concentrations in the ligand-buffered solutions were measured at the start and end of the experiment and corresponded well to the nominal concentrations (Table 1). The Zn concentrations in the Chelex-buffered solutions were below the detection limit for most treatments. Therefore, Zn concentrations were measured after equilibration of the resin with 0.2 mM NTA, which allowed estimating the free Zn²⁺ concentration (Table 1). For the Chelex-buffered treatments where the Zn concentration in the nutrient solution was above the detection limit of the ICP (i.e., the treatments with the two highest Zn loadings in Experiment 1), the Zn concentrations were measured regularly during the plant-growth. These measured Zn concentrations were relatively stable throughout the experiment, showed no steady decline or increase, and deviated at most 35%

of the initial values, indicating that the resupply of Zn from the resin into the solution was fast enough to compensate for the Zn uptake of the plant. The concentrations of Mn on the other hand showed a steady, almost 3-fold, decline. Buffering of Mn by the Chelex was inadequate, since the amount of Mn taken up by the plants was ~70% of the total Mn present on the resin. The amount of Zn taken up was for all treatments less than 3% of the total Zn amount on the resin. Checkai and Norvell (1992) developed a recirculating resin-buffered system to control activities of nutrient ions, since they had found that resupply from the resin was inadequate when using a more simple system where the resin was enclosed in membrane filter bags. Our results indicate that, in our simple system where the resin was placed directly in the solution, aeration and daily stirring provided sufficient mixing of the system to overcome kinetic constraints in resupply of Zn from the resin.

Deficiency symptoms and dry weight reduction. Zinc deficiency in spinach became apparent through chlorosis of the leaves in the last several days before harvest. Mild symptoms were observed for the HEDTA treatment with the smallest Zn activity (pZn 11.2), and more severe symptoms for the CDTA treatment with the same activity. Yields were depressed for all treatments with CDTA, suggesting that either the ligand itself affected plant growth or that the growth reduction was caused by non-optimal supply of another element (see below). No symptoms were observed for the Chelex-buffered treatments (Table 3).

Zinc deficiency symptoms of tomato plants first became visible in the CDTA treatment with the lowest Zn activity (pZn 11.2). Necrotic patches appeared 10 DAT, and stunting of the plants was visible by 12 DAT. At harvest, the plants grown at pZn 11.2 were much smaller than those grown with CDTA at pZn 9.2. Plants grown at pZn 10.2 showed mild symptoms. For the HEDTA treatments, deficiency symptoms – overall stunting and

necrosis of the leaf tips – were only observed for the treatment with the lowest Zn concentration (pZn 11.2) in the first experiment (Table 3). No deficiency symptoms were observed for the Chelex-buffered treatment, but the plants grown at the highest Zn level were stunted and yield was significantly reduced, indicating Zn toxicity.

In the second experiment, the Chelex solutions were buffered at lower Zn^{2+} activity (see Table 1), and Zn deficiency symptoms became apparent for the treatment with the smallest Zn activity (pZn 9.5). The tomato plants were stunted and leaves were chlorotic. These symptoms were similar to those of the plants grown with HEDTA at pZn 10.5 (Table 4). No symptoms were observed for the HEDTA treatment with pZn 10.1. These results indicate that the critical Zn^{2+} activity determined in chelator-buffered solutions depends on the type of ligand, as will be discussed in more detail below.

Zinc concentrations in plant tissues and Zn uptake. The amount of Zn taken up by the plant decreases at lower Zn supply, since Zn concentrations in the plant are lower and/or yield is reduced. As long as the concentration in the plant tissue exceeds the critical value, which is mostly around 15–20 mg kg⁻¹ dry weight (DW) for Zn in shoot, lower Zn supply mainly affects the uptake through a decrease in Zn concentration in the plant tissues. Near the critical limit, however, the growth rate and hence biomass yield is mainly reduced as a result of low Zn supply.

In the first experiment, a decrease in Zn^{2+} activity of the solution had little effect on the yield of spinach (Table 3), but strongly reduced the zinc concentrations in root and shoot (Figure 1). With tomato, the yield was affected in the ligand-buffered treatments, but not in the Chelex-buffered treatment (Table 3). For the Chelex-buffered solution with the lowest Zn^{2+} activity (pZn 7.7), zinc concentrations were 260 and 84 mg kg⁻¹ DW in root and shoot, respectively, i.e. well above the critical limits. In Experiment 2, the Zn supply affected both

the Zn concentrations in the plant tissues and the yield of tomato grown with HEDTA. An increase in the Zn^{2+} activity from pZn 11.3 to 10.1 resulted in a ~2-fold increase of the Zn concentration (Figure 2) and a ~3-fold increase in plant yield, resulting in an overall increase of the Zn uptake with a factor 6.6 (Table 4). For the plants grown on the resin-buffered solutions, the plant yield was only reduced for the smallest Zn^{2+} activity, and the effect of varying Zn supply was primarily on the Zn concentrations (Figure 2). In both experiments, the Zn concentrations in the plant were not identical at same activity for the different treatments. The Zn concentrations in spinach plants grown with HEDTA were larger than in those grown with CDTA at the same Zn^{2+} activity. Despite the larger Zn^{2+} activity, Zn concentrations in root and shoot of spinach were smaller for the Chelex-buffered treatment at pZn 7.7 than for the HEDTA-buffered treatment at pZn 9.2 (Figure 1). The uptake by tomato from the resin-buffered solution at pZn 9.5 was smaller than in the HEDTA-buffered solution at pZn 10.1, and similar to that in the HEDTA-treatment at pZn 10.5 (Figure 2, Table 4). These results indicate that, at same Zn^{2+} activity, Zn uptake follows the order: HEDTA > CDTA > no soluble ligand.

As mentioned above, the yield reduction and other deficiency symptoms (e.g. chlorosis) differed, at same Zn^{2+} activity, between treatments with different type of buffering. The difference between the treatments vanished when the response (deficiency symptoms, yield reduction) was related to the internal zinc concentrations. The yield of tomato was similar for different treatments when shoot concentrations or uptake of Zn were similar (Figure 2c). For spinach, there was no clear reduction in yield for the low-Zn treatments (Table 3). However, deficiency symptoms (chlorosis) and reduced shoot-root ratios were observed in treatments where Zn uptake and shoot concentrations were small (Figure 1c). Reduction in shoot-root ratios has been reported to be indicative of Zn deficiency (e.g. Cakmak et al., 1996). Zinc deficient conditions had no clear effect on shoot-root ratio of tomato, as was

also found by Cakmak and Marschner (1988) and Parker (1997). The smaller shoot-root ratio of spinach in the Chelex-buffered treatment with the largest Zn activity is probably related to Zn toxicity (Figure 1c). The Zn concentration in the shoot for this treatment was $768 \text{ mg kg}^{-1} \text{ DW}$, while critical concentrations for Zn toxicity are mostly between 100 and 600 mg kg^{-1} (Macnicol and Beckett, 1985).

Uptake of other micronutrients. In both experiments, the shoot concentrations of Cu, Mn and Fe were in the adequate range for all treatments, except for the CDTA-buffered solutions. Shoot concentrations of spinach grown in these solutions were small for Cu ($2\text{--}4 \text{ mg kg}^{-1} \text{ DW}$) and Mn ($15\text{--}20 \text{ mg kg}^{-1}$), which might have caused the yield reduction of spinach in the CDTA treatments (Table 3). Webb et al. (1993) estimated a critical Mn^{2+} activity of $10^{-8.3} \text{ M}$ required for barley in nutrient solutions buffered with HEDTA at a $25\text{-}\mu\text{M}$ excess. The free Mn^{2+} activity in the CDTA treatments was smaller than this critical value (Table 1), but the use of another plant species and another ligand in different concentration makes comparison difficult. Overall, it is not clear what caused the yield reduction of the spinach plants grown with CDTA.

In Experiment 2, shoot and root concentrations of Cu in tomato remained fairly constant among treatments, and hence, total uptake paralleled the dry-matter production (Table 4). In contrast, Fe and Mn concentrations increased significantly under Zn-deficient conditions. The total uptake, however, was not significantly different for the different Zn levels of the HEDTA- or resin-buffered treatments (Table 4). Increased shoot and root concentrations of micronutrients (Cu, Mn, Fe) has been reported before in Zn- deficient plants (e.g. Parker, 1997). It has been argued that this accumulation of micronutrients is a “concentration effect”, due to reduced biomass production (e.g. Lombnæs and Singh, 2003), while others

found that Fe accumulation in the plant could only in part be attributed to the reduced yield (Cakmak et al., 1996).

Zinc and copper uptake in chelator-buffered and resin-buffered solutions (Exp. 3)

In this experiment, the uptake of Cu and Zn by spinach was measured under non-deficient conditions at constant free-ion activity in solutions buffered with different ligands (Table 2). Plant yields did not differ significantly among treatments, except for the treatment with the greatest NTA concentration (1.9 mM NTA), where shoot and root growth was significantly ($P<0.05$) reduced. Yield of spinach for this treatment was 20 mg root dry-weight and 78 mg shoot dry-weight per plant, compared with, on average, 37 and 131 mg root and shoot dry-weight for the other treatments. The yield reduction for the NTA treatment may be an adverse effect of the ligand itself, but may also result from metal toxicity, because of the larger metal uptake. The shoot Cu concentration (24 mg kg⁻¹ DW) was in the range of toxic levels reported in literature (Macnicol and Beckett, 1985). The yield was also smaller for the other NTA treatment (1.1 mM NTA), where mean root and shoot dry-weight was 27 and 111 mg respectively, but this difference was not significant (at $\alpha=0.05$).

Although activities of Cu²⁺ and Zn²⁺ were constant over all treatments, uptake of these elements differed substantially. For the same degree of buffering, *i.e.* the same total Zn (10 μ M) and Cu (5 μ M) concentration, uptake of Zn and Cu followed the order: NTA > HEDTA > EDTA > CDTA, which is the same order as for the dissociation rate constant (Table 5). In the resin-buffered (Chelex) treatment with the lowest Cu and Zn activities (pCu 11.9; pZn 8.9), where the metals (except Fe) were not buffered by complexes, the concentrations of Cu and Zn in root and shoot were comparable with those in the CDTA treatments and smaller than those in the other chelator-buffered solutions, although free-ion activities of Cu and Zn were two-fold larger in the resin-buffered than in the ligand-buffered

solutions (Table 2). These results indicate that the complexes contribute to the uptake of Cu and Zn, and this contribution is most pronounced for fast dissociating complexes.

The activity of Mn^{2+} was also constant for all chelator-buffered solutions. Both shoot and root concentrations generally increased with increasing total Mn concentration in solution, indicating that Mn complexes also contributed to Mn uptake by spinach. No treatments were included to specifically test Mn buffering (as was done for Cu and Zn), which makes it more difficult to compare the contribution of complexes with different ligands.

Large differences in translocation occurred between Cu, Mn and Zn. More Zn was translocated to the shoot in treatments where more Zn was taken up (Table 5). In all but one of the treatments, root concentrations of Zn were between 80 and 200 mg Zn kg⁻¹ DW, while shoot concentrations showed more variation. With Cu and Mn on the other hand, translocation decreased as uptake of the element increased. As a result, shoot concentrations of Cu and Mn were more constant than root concentrations (Table 5). Root and shoot concentrations of Fe were about 180 and 80 mg Fe kg⁻¹ DW respectively, and differences among treatments were small and inconsistent.

Discussion

The free-ion activity model (FIAM) postulates that the uptake of metal by an organism is proportional to the free-ion activity of the metal in the surrounding solution. However, several exceptions to the FIAM have been reported (e.g. Bell et al., 1991; McLaughlin et al., 1997). Uptake of intact complexes may explain the larger metal uptake in presence of complexes. Some studies have indeed shown uptake of metal-EDTA complexes (e.g Collins et al., 2002). It has been speculated that these complexes of metals involving hydrophilic ligands enter the xylem vessels through an apoplastic pathway, e.g. at the tips of roots where the Casparian bands are not fully developed or at breaks in the endodermis. Indications have also been found that high concentrations of ligands damage the root

membranes, resulting in leakage of the roots and uptake of intact metal-ligand complexes in high concentrations (Vassil et al., 1998; Collins et al., 2002).

Alternatively, the greater metal uptake in chelator-buffered solutions, compared with unbuffered solutions with same free-ion activity, may be related to diffusive limitations to the transport of the free ion in the unstirred layer near the root surface or in the apoplast. In that case, labile complexes will dissociate within the diffusion layer, thus compensating for the depletion of the free metal and enhancing the diffusive flux (Jansen et al., 2002). We found previously that Cd uptake by spinach, at constant Cd^{2+} activity, was greatest in treatments with fast dissociating (labile) complexes, indicating that Cd uptake is rate-limited by diffusion of the free ion to the root surface, even in well-stirred solutions (Degryse et al., 2006). The results presented above suggest that diffusion limitations also govern uptake of Cu and Zn. At same free-ion activity, uptake was larger in chelator-buffered solutions than in absence of complexes (i.e. in the Chelex-buffered solutions), and at the same degree of buffering, uptake was the greatest in the presence of fast-dissociating complexes.

To analyze these results in a more quantitative manner, observed uptake fluxes were compared with the theoretical uptake flux if diffusion were limiting. The (observed) uptake flux, F_{obs} (in nmol per g root fresh weight (RFW) per day), was calculated as follows:

$$F_{\text{obs}} = M_{\text{pl}} \cdot \frac{RGR}{RWR} \quad (2)$$

where M_{pl} is the concentration in the plant (in nmol per g fresh weight), RWR is the root weight ratio (g root per g plant) on fresh weight basis, and RGR is the relative growth rate (d^{-1}). The relative growth rate was calculated from the fresh weight at the final harvest and the fresh weight recorded at 11 DAT, and was found to be 0.22 d^{-1} . For the resin-buffered (Chelex-buffered) solution, the theoretical uptake flux, $F_{\text{pred,-L}}$ ($\text{nmol g}^{-1} \text{ RFW d}^{-1}$), was assumed to be equal to the limiting diffusion flux (i.e. the diffusion flux if the concentration at the surface is zero), and calculated as:

$$F_{\text{pred},-L} = \frac{D}{\delta} \cdot SRA \cdot [M^{2+}] \quad (3)$$

where D is the diffusion coefficient of the free metal ion ($\text{m}^2 \text{d}^{-1}$), SRA is the specific root area ($\text{m}^2 \text{g}^{-1}$ RFW), δ is the diffusion layer thickness (m), and $[M^{2+}]$ is the free-ion concentration (nmol m^{-3}). A diffusion coefficient of $4.32 \times 10^{-5} \text{ m}^2 \text{d}^{-1}$ (or $5.0 \times 10^{-10} \text{ m}^2 \text{s}^{-1}$) was assumed, and a diffusion layer thickness, δ , of $4 \times 10^{-4} \text{ m}$, since these values well described uptake of Cd by spinach in buffered and unbuffered nutrient solutions (Degryse *et al.*, 2006). A value of $0.02 \text{ m}^2 \text{g}^{-1}$ was used for the specific root area, as was found for spinach plants grown under similar conditions (Degryse *et al.*, 2006). Figure 3a shows the predicted and measured Zn flux as a function of the free Zn^{2+} concentration for all resin-buffered treatments. The observed flux is in reasonable agreement with the limiting diffusion flux, but the (calculated) diffusion flux is proportional to the free-ion concentration, while this is not the case for the observed uptake flux. The observed flux is greater than the calculated diffusion flux at small Zn^{2+} concentrations, while the opposite is true at large Zn^{2+} concentrations. The uptake flux being lower than the limiting diffusion flux for the higher Zn^{2+} concentrations is plausible, since it is likely that diffusion is not limiting at large Zn supply, where the uptake capacity of the root is expected to be saturated (or nearly so). An uptake flux that is greater than the limiting diffusion flux is theoretically impossible. The specific root area (Equation 3) can be somewhat different from the estimated value ($0.02 \text{ m}^2 \text{g}^{-1}$ RFW), but this difference is not likely to explain the discrepancy between uptake flux and diffusion flux. More likely, the limiting diffusion flux is larger than estimated, either because D/δ ($=0.11 \text{ m d}^{-1}$) is larger than assumed, or because complexes in solution contribute to the diffusion flux. The Chelex-buffered solutions (initially) did not contain Zn complexes. However, it is possible that Zn deficiency triggered the root exudation of Zn-complexing organic ligands. Since the free Zn^{2+} concentration was buffered, this would result in an increase of the total Zn concentration, and an enhanced

diffusion flux. Zinc deficiency has indeed be found to increase root exudation of amino acids, sugars and phenolics (Cakmak and Marschner, 1988; Zhang et al., 1991). Zhang et al. (1991) found that the root exudates of dicotyledonous species did not enhance zinc mobilization from a resin or a calcareous soil, in contrast with those of graminaceous species. On the other hand, Keller and Römer (2001) found that increased Zn uptake by spinach under P-deficient conditions was related to higher exudation rates of oxalate, citrate and malate, and higher Zn concentration in the soil solution. It seems therefore possible that Zn-complexing root exudates enhanced transport of Zn to the root surface for the Zn deficient treatments, but this is speculative and more research is needed to elucidate the possible effects of root exudates on metal speciation.

The discrepancy between uptake flux and (estimated) diffusion flux in the resin-buffered solutions is still larger for Cu than for Zn, which might indicate that the role of root exudates in metal uptake is more important for Cu than for Zn. The free Cu^{2+} concentration is in the order of 10^{-11} M (Table 1), which results in an estimated diffusion flux (Equation 3) of $0.02 \text{ nmol g}^{-1} \text{ RFW d}^{-1}$. The observed uptake flux is on the order of $20 \text{ nmol g}^{-1} \text{ RFW d}^{-1}$, i.e. about 1000-fold larger. Other studies have also pointed out that uptake rates of Cu largely exceed the (estimated) diffusion fluxes to the surface of algae, because of the low environmental Cu^{2+} concentrations (Hudson, 1998; Meylan et al., 2004).

In the chelator-buffered solutions, the uptake flux of Zn was much larger than in ligand-free solutions at similar Zn^{2+} concentration in solution (Figure 3a), suggesting that the diffusion flux was enhanced by the presence of the Zn complexes. The limiting diffusion flux in the presence of complexes, $F_{\text{pred},+\text{L}}$ ($\text{nmol g}^{-1} \text{ RFW d}^{-1}$), can be approximated using the reaction layer concept:

$$F_{\text{pred},+\text{L}} = \frac{D}{\mu} \cdot \text{SRA} \cdot [\text{M}^{2+}] \quad (4)$$

where μ (m) is the thickness of the reaction layer, which can be estimated as

$$\mu = \sqrt{\frac{D}{k_d} \cdot \frac{[M^{2+}]}{[ML]}} \quad (5)$$

The reaction layer is the zone near the surface where the rate of dissociation of the metal complex is not fast enough to keep up with the depletion of the free metal ion. The reaction layer is physically only meaningful if its thickness, μ , is smaller than the diffusion layer thickness, δ , and if the complexes are not fully labile. If these conditions are not fulfilled, Equation (4) cannot be used. The diffusion flux can then be estimated with the following equations:

$$\text{Fully inert } (\mu > \delta, \text{ i.e. } \frac{[M^{2+}]}{[ML]k_d} > \frac{\delta^2}{D}) \Rightarrow F_{\text{pred,+L}} = \frac{D}{\delta} \cdot SRA \cdot [M^{2+}] \quad (6)$$

$$\text{Fully labile } (\frac{[M^{2+}]}{\mu} > \frac{[ML]}{\delta}, \text{ i.e. } \frac{[ML]}{[M^{2+}]k_d} < \frac{\delta^2}{D}) \Rightarrow F_{\text{pred,+L}} = \frac{D}{\delta} \cdot SRA \cdot [M]_{\text{tot}} \quad (7)$$

A more detailed discussion of the reaction layer concept, and assumptions, can be found elsewhere (e.g. van Leeuwen, 2001). Figure 3b shows the observed Zn uptake flux for the treatments of the Cu/Zn experiment that were buffered with different ligands, but had identical free Zn^{2+} concentration ($10^{-9} M$) and total Zn concentration ($10^{-5} M$) (Table 2). The dissociation rate of the complexes was measured with the competitive ligand method, and ranged from $2.7 \times 10^{-5} s^{-1}$ (Zn-CDTA) to $>10^{-2} s^{-1}$ (Zn-NTA). The observed uptake flux is larger for fast dissociating complexes. The theoretical diffusion flux, calculated with Equation (4), is in reasonable agreement with the observed uptake flux, although the uptake flux is again larger than the estimated diffusion flux for the treatments with low Zn supply, i.e. with slowly dissociating complexes.

The uptake flux of Cu also increased with more rapid dissociation of the complexes (Table 4). The observed uptake flux ranged from 6 (buffering with CDTA) to 45 (buffering with NTA) $nmol g^{-1} RFW d^{-1}$ for the treatments with identical Cu^{2+} and total Cu

concentration (Table 2). The theoretical diffusion flux (Eq. 4) ranges from 0.4 (CDTA) to >9 (NTA) $\text{nmol g}^{-1} \text{ RFW d}^{-1}$. The difference between the uptake flux and the estimated (limiting) diffusion flux is smaller than in the unbuffered solutions – about a factor of 10 instead of a factor of 1000. A possible explanation is that the contribution of plant-borne organic complexants to the metal diffusion flux (which is not accounted for in the calculation of the diffusion flux) is more important in unbuffered solutions than in solutions where complexes with synthetic ligands already enhance the diffusion flux.

This simple model, which takes into account the (enhanced) diffusion of the free ion to the site of uptake and assumes that only the free ion reacts with the surface-binding site, is likely an oversimplification of the metal uptake process. For instance, possible binding of the metal-ligand complex at the surface is not considered. The formation of a ternary surface complex could also explain the contribution of metal complexes to metal uptake (Wilkinson and Buffle, 2004), and the observed differences in uptake between the treatments with different ligands, since the rate of break-up of the ternary complex would likely follow the same order as for the metal-ligand complex.

Our results indicate that metal complexes increase metal uptake by plants, likely because uptake is rate-limited by diffusion of the free ion to the root surface or to the site of uptake into the symplasm. The contribution of complexes to metal uptake implies that uptake is not solely dependent on the free-ion activity. As a result, critical solution activities determined for metal micronutrients are specific for the conditions under which they were determined (e.g. type and concentration of ligand). Reported critical Zn activities are mostly around $10^{-10.5} \text{ M}$ (e.g. Norvell and Welch, 1993; Yang et al., 1994; Parker, 1997). In most of these studies, HEDTA was used to buffer metals in solution. We found a similar critical Zn activity when using HEDTA as ligand. However, the critical Zn activity was larger in solutions buffered with CDTA (between $10^{-10.2}$ and $10^{-9.2} \text{ M}$), and still larger in solutions

where Zn was not buffered by synthetic ligands ($\sim 10^{-9.2}$ M). The degree of Zn complexation in soil solutions is usually low to moderate. Even in soils with high pH (~ 8), the free-ion fraction of Zn is usually 5% or more (Nolan et al., 2003). Since diffusion limitations of Zn^{2+} to the root surface are likely to be more pronounced in soil than in nutrient solutions, the critical Zn^{2+} activity in soil solution is expected to be larger than $10^{-9.2}$ M. There is little information about critical Zn concentrations in soil; based on data from Tiller et al. (1972), we estimate that the critical Zn concentration in soil solution (approximated by the concentration in a 0.05 M CaCl_2 extract) is in the order of 5×10^{-8} to 3×10^{-7} M.

Diffusive limitations in metal uptake explain why metal concentrations measured using DGT (Diffusive Gradients in Thin films) correlate well with concentrations in plant tissues, both for plants grown in soil (Zhang et al., 2001) and in nutrient solutions (Degryse et al., 2006). However, it should be realized that, even when metal uptake is limited by diffusion, the plant has still an active role. Not only is metal translocation to the shoot regulated by the plant, but the plant may also mediate the uptake flux, e.g. through root exudation and acidification of the rhizosphere.

Interestingly, similar concepts are also applicable in other research disciplines. Hudson and Morel (1993) analyzed the kinetics of transport processes for marine microorganisms, and their calculations indicated that diffusion of labile Fe(III) species limits iron uptake for larger cells. It has been speculated that metal (Cu, Zn, Cd) uptake by periphyton (Meylan et al., 2004) and by mussels (Jansen et al., 2002) is limited by diffusion at low concentration of the free ion. In medicinal research, similar reasoning has been used to explain the enhancement of Zn^{2+} transport across the membranes of brain endothelium by histidine (Buxani-Rice et al., 1994), and the albumin-enhanced transport of fatty acids into liver cells (Weisiger et al., 1989).

In conclusion, aqueous metal complexes increase metal uptake by plants and the observed increases can be explained when assuming that uptake is diffusion-limited. Though chelator-buffering has proved to be useful in the study of metal deficiencies, care should be taken in extrapolating results (e.g. critical free-ion activities) to other systems, since the metal uptake at a given free-ion activity depends on the type and concentration of the ligands employed.

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Figure Captions

Figure 1. Zinc concentrations (on dry weight basis) in (a) root and (b) shoot of spinach from Experiment 1 (see Table 1), grown for 18 days in solutions buffered with soluble ligands (HEDTA or CDTA) or with Chelex resin, as a function of the free Zn^{2+} activity in solution, and (c) shoot-root dry weight ratio as a function of the Zn concentration in the shoot. Vertical bars represent the standard error (not shown in panel a, because roots were pooled before digestion).

Figure 2. Zinc concentrations (on dry weight basis) in (a) root and (b) shoot of tomato from Experiment 2 (see Table 1), grown for 17 days in HEDTA- or Chelex-buffered solutions, as a function of the free Zn^{2+} activity in solution, and (c) shoot dry weight as a function of the Zn concentration in the shoot. Vertical bars represent the standard error.

Figure 3. (a) The observed uptake flux of Zn (symbols; Eq. 2) and the calculated limiting diffusion flux (solid line; Eq. 3), as a function of the free Zn^{2+} concentration in resin-buffered or in chelator-buffered (encircled symbols) solutions. The dotted line shows the regression line for all resin-buffered treatments ($F = 66 \times 10^4 \sqrt{[\text{Zn}^{2+}]}$). (b) The observed uptake flux of Zn (symbols; Eq. 2) and the calculated diffusion flux (line; Eq. 4), as a function of the dissociation rate constant of the complex, k_d , in chelator-buffered solutions with identical Zn^{2+} ($10^{-9} M$) and total Zn concentration ($10^{-5} M$). (a-b) Observed and predicted flux increase with increasing concentration of the free ion, and with increasing lability of Zn-complexes in solution (Detailed discussion in text). Symbols: (□) tomato, Exp. 1 (Table 1); (△) tomato, Exp. 2 (Table 1); (■) spinach, Exp. 1 (Table 1); (◆) spinach, Exp. 3 (Table 2). Vertical bars represent the standard error.

Table 1. Summary of treatments for Experiments 1 and 2 where Zn deficiency was assessed in chelator-buffered solutions (HEDTA or CDTA) or in solutions buffered with a chelating resin (Chelex). All solutions contained 25 μM FeHBED, and all chelator-buffered solutions contained 2 μM Cu and 5 μM Mn.

	Ligand	Zn _{tot} ^a	Calculated metal activities ^b			
Buffer	(<i>μM</i>)	(<i>μM</i>)	pZn	pCu	pMn	pFe
Experiment 1						
HEDTA	83	1.0	11.2	13.7	7.3	20.9
	92	10	10.2	13.7	7.3	20.9
	183	100	9.2	13.7	7.3	20.9
CDTA	129	1.0	11.2	13.4	8.6	22.8
	138	10	10.2	13.4	8.6	22.8
	229	100	9.2	13.4	8.6	22.8
Chelex	na	0.03	7.7	11.1	6.3	17.7
	na	0.36	6.6	11.0	6.2	17.7
	na	3.4	5.7	10.8	6.0	17.7
Experiment 2						
HEDTA	32	0.24	11.3	13.2	6.8	20.7
	33	0.60	10.9	13.2	6.8	20.7
	34	1.50	10.5	13.2	6.8	20.7
	36	3.75	10.1	13.2	6.8	20.7
Chelex	na	0.0005	9.5	11.1	6.2	17.7
	na	0.0009	9.2	11.1	6.2	17.7
	na	0.002	8.9	11.1	6.2	17.7
	na	0.006	8.4	11.1	6.2	17.7

^a Zn_{tot} = total Zn concentration in solution; ^b Calculated with GEOCHEM-PC (For the

Chelex-buffered solutions, the solution composition after equilibration with 0.2 mM NTA was used as input for the calculation; details in text.)

Table 2. Summary of treatments for Experiment 3 where uptake of Cu and Zn was measured in solutions buffered with different ligands or in solutions buffered with a chelating resin (Chelex). All solutions contained 18 μM FeHBED.

	Concentrations in solution (μM)				Calculated metal activities		
	Ligand	Zn	Cu	Mn	pZn	pCu	pMn
Zn_{tot} constant							
NTA	1107	10	2.5	0.7	9.2	12.2	7.2
HEDTA	25	10	6.3	0.7	9.2	12.2	7.2
EDTA	45	10	2.0	2.5	9.2	12.2	7.2
CDTA	40	10	3.5	14.0	9.2	12.2	7.2
Cu_{tot} constant							
NTA	1920	20	5.0	1.4	9.2	12.2	7.2
HEDTA	20	8	5.0	0.6	9.2	12.2	7.2
EDTA	110	25	5.0	6.3	9.2	12.2	7.2
CDTA	59	15	5.0	20.0	9.2	12.2	7.2
Chelex buffered							
none	na	0.002	2.1×10^{-6}	0.8	8.9	11.9	6.3
none	na	0.027	4.3×10^{-5}	1.1	7.8	10.6	6.2

Table 3. Dry weight (DW) and Zn-deficiency symptoms of tomato and spinach at 18 days after transplanting (Experiment 1, see Table 1).

Buffer	pZn	Tomato			Spinach		
		DW (g per 2 plants)		Def ^a	DW (g per 2 plants)		Def ^b
		Root	Shoot		Root	Shoot	
HEDTA	11.2	0.020 bc	0.11 cd	++	0.088 a	0.20 bc	+
	10.2	0.043 a	0.24 a		0.057 bc	0.23 ab	
	9.2	0.051 a	0.25 a		0.055 bc	0.25 ab	
CDTA	11.2	0.013 c	0.05 d	+++	0.045 c	0.11 d	++
	10.2	0.027 b	0.15 bc	+	0.043 c	0.14 cd	
	9.2	0.041 a	0.23 a		0.033 c	0.14 cd	
Chelex	7.7	0.045 a	0.22 ab		0.041 c	0.20 bc	
	6.6	0.047 a	0.21 ab		0.071 ab	0.29 a	
	5.7	0.042 a	0.14 c		0.041 c	0.13 cd	

Means with the same letter are not significant different at $P=0.05$ within the same column (Duncan's multiple range test)

^a Deficiency symptoms: + stunting, downward cupping of older leaves; ++ stunting, necrotic leaf tips, downward cupping; +++ severe stunting, downward cupping, necrotic leaves

^b Deficiency symptoms: + chlorosis; ++ stunting, chlorosis

Table 4. Dry weight (DW), Zn-deficiency symptoms, and metal uptake of tomato at 17 DAT (Experiment 2, see Table 1)

Buffer	pZn	DW (g per 2 plants)		Def ^a	Total uptake (μ g per 2 plants)			
		Root	Shoot		Zn	Cu	Fe	Mn
HEDTA	11.3	0.018 d	0.12 b	+++	2.2 e	2.5 d	62 a	73 ab
	10.9	0.024 d	0.20 b	++	3.8 de	4.7 d	76 a	83 ab
	10.5	0.041 c	0.31 a	+	8.3 c	6.1 cd	68 a	100 a
	10.1	0.058 ab	0.39 a		14.6 b	8.4 bc	53 a	86 ab
Chelex	9.5	0.048 bc	0.30 a	+	6.9 cd	11.1 ab	52 a	50 b
	9.2	0.063 ab	0.36 a		10.7 c	14.1 a	61 a	50 b
	8.9	0.070 a	0.38 a		16.5 ab	13.9 a	72 a	61 ab
	8.4	0.068 a	0.37 a		18.9 a	12.7 a	72 a	60 ab

Means with the same letter are not significant different at $P=0.05$ within the same column

^a Deficiency symptoms: + stunting, downward cupping of older leaves; ++ stunting, necrotic leaf tips, downward cupping; +++ severe stunting, downward cupping, necrotic leaves

Table 5. Metal concentrations in root and shoot of spinach grown for 19 days in resin-buffered (Chelex-buffered) solutions or in solutions buffered with complexes with varying dissociation rate constant, k_d (Experiment 3; see Table 2). Free-ion concentrations of Zn, Cu and Mn were identical in all chelator-buffered solutions.

Ligand	$\log k_d$ (s ⁻¹)	Root concentration (mg kg ⁻¹ DW)			Shoot concentration (mg kg ⁻¹ DW)			Total uptake (μg per 2 plants)		
		Zn	Cu	Mn	Zn	Cu	Mn	Zn	Cu	Mn
Zn_{tot} constant ($\log k_d$ of ZnL given)										
NTA	>-2.0	173	58	7	272	22	82	70	8.0	19
HEDTA	-3.5	159	29	51	124	14	64	45	5.8	21
EDTA	-4.2	131	15	253	95	8.8	149	39	3.9	65
CDTA	-4.6	84	7.8	504	61	4.6	142	20	1.6	68
Cu_{tot} constant ($\log k_d$ of CuL given)										
NTA	>-2.0	575	110	44	295	24	188	68	8.1	31
HEDTA	-2.9	164	28	51	117	13	59	39	5.0	17
EDTA	-3.7	141	22	637	128	11	220	46	4.7	110
CDTA	-4.7	138	14	862	60	6.4	174	31	3.3	132
Chelex Buffered										
none		91	19	512	45	3.2	190	17	2.2	82
none		127	43	577	134	14	274	46	7.2	119

Figure 1

Figure 2

Figure 3