Availability of Zinc and the Ligands Citrate and Histidine to Wheat: Does Uptake of Entire Complexes Play a Role?

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S *Supporting Information*

ABSTRACT: Organic ligands in soils affect the availability of trace metals such as Zn to plants. This study investigated the effects of two of these ligands, citrate and histidine, on Zn uptake by wheat under hydroponic conditions. Uptake of ^{65}Zn in the presence of these ligands was compared to uptake in the presence of EDTA at the same free Zn concentration ($Zn^{2+} \sim 50$ nM). In the presence of citrate Zn root uptake was enhanced $\sim$3.5 times and in the presence of histidine, by a factor of $\sim$9, compared to the EDTA treatments. Citrate uptake was slightly reduced in the treatment containing ligands and Zn compared to the treatment containing the same ligand concentration but no Zn. In addition, a higher uptake of Zn than of citrate was observed. This suggests that the enhanced Zn uptake was primarily due to increased supply of Zn^{2+} by diffusion and dissociation of Zn−citrate complexes at the root surface. Histidine uptake was much higher than citrate uptake and not influenced by the presence of Zn. As histidine forms stronger complexes with Zn than citrate, the results suggest that the enhancement of Zn uptake in the presence of histidine was in part due to the uptake of undissociated Zn−histidine complexes.

KEYWORDS: *organic ligands, wheat, Zn bioavailability, Zn−citrate, Zn−EDTA, Zn−organo complexes, Zn−histidine*

INTRODUCTION

Zinc (Zn) is often a limiting micronutrient in crop production. Low concentrations of plant-available soil Zn are also a frequent cause of low Zn contents in the edible parts of crops, for example, in cereal grains.[1] In many soils worldwide Zn deficiency in crops is not caused by a low total Zn status but by the insufficient availability of Zn for uptake by the plants, which depends largely on its speciation, for example, strong sorption to soil particles.[2] Organic ligands occurring in soils[3] can mobilize trace metals into soil solutions.[4−6] On the other hand, they may also limit metal uptake by organisms, by forming complexes that are less bioavailable than the free ions. According to the concept underlying the free ion activity model (FIAM) and the biotic ligand model (BLM), cells in general take up metals such as Zn from the soil solution only if they are present as free ions and not in the form of metal−organic complexes.[7] These models are based on the assumption that the free ions are taken up through ion channels or transporter enzymes in the cell membranes and that transmembrane transport is the rate-limiting step. However, under conditions of deficiency, the transfer of Zn from the bulk soil to the root surfaces may become limiting, and in this case Zn acquisition by plants could be enhanced through the delivery of metals in the form of labile metal−organic complexes dissociating at the root surface.[8−11] The binding strength of the respective metal−ligand complexes is a crucial factor in this process.[8,10] Organic ligands can also increase metal acquisition if there is uptake of undissociated complexes. This has been found, for example, in the case of lipophilic metal−rhamnolipid complexes, which can cross root cell membranes simply via diffusion.[12] Such transmembrane transfer may also be possible for neutral hydrophilic complexes. Bell et al.[13] found higher uptake of EDTA by Swiss chard from solutions in which it was present primarily in the form of neutral metal−EDTA complexes than from solutions in which it was mainly present in the form of negatively charged complexes. It is possible that the neutral complexes passed unselectively through the cell membranes via aquaporins.[14,15] Overall, complexes with no physiological role are expected to be taken up only to a small extent. The main causes for it are the incomplete formation of the Casparian strip at root tips and at places where lateral roots develop or possibly membrane disruption of endodermal cells.[16−18] Selective uptake of metal−organo complexes also contributes to metal internalization by plant cells. Uptake of metal−siderophore complexes are central for iron (Fe) acquisition by grasses and has also been found to contribute to Zn uptake.[19,20]

Here, our question was, do organic ligands naturally present in soil solutions, other than phytosiderophores, play a role in Zn uptake by wheat? Our focus was on citrate and histidine, because both ligands are involved in metal transport and storage in plants.[20] They are not only exuded[21,22] but also taken up by plant roots, probably through active uptake mechanisms.[13,23] Citrate is a common exudate of wheat roots, whereas histidine exudation by wheat has been detected only under aluminum stress.[3,24] In addition, there are other important sources of organic ligands in soil such as bacterial exudates[25] and decomposition of organic matter. Citrate is a major ligand involved in storage of Zn in plant cell vacuoles,[20,26] whereas histidine is one of the most important ligands for Zn xylem transport and cytosol storage.[20] The knowledge of whether organic ligands play a role in Zn uptake by plants could be

Table 1. Treatments for Experiment 2 on the Influence of Organic Ligands on Zn Uptake[a]

	Zn 20 μM + citrate	Zn 20 μM + histidine	Zn 20 μM + EDTA	Zn 2 μM + EDTA	Zn 20 μM
total Zn (μM)	20	20	20	2	20
total ligand (μM)	1450	1300	20.2	2	
Zn^{2+} modeled (μM) (% of $[Zn]_{total}$)	0.05 (0.25%)	0.05 (0.25%)	0.044 (0.22%)	0.032 (1.6%)	19.6 (98%)

[a]Potassium citrate, L-histidine, and H_4EDTA were used. Background solution contained in all treatments: 500 μM KNO_3, 400 μM $Ca(NO_3)_2$, and 2.5 mM MOPS at a pH of 7.2. Zn^{2+} concentrations were calculated using MINEQL.

instrumental in improving Zn nutrition of crop plants growing on soils with low Zn availability, for example, through addition of organic ligands to soil via incorporation of crop residues or organic fertilizers or by means of intercropping, as suggested by Zuo and Zhang.[27]

To investigate the potential influence of citrate and histidine on the transfer of Zn from soil solution into plants, we performed two experiments with radiolabeling of Zn and ligands under hydroponic conditions, because only such well-controlled systems allow specific investigation of the influence of particular ligands on metal uptake.[9] In a first step, we compared Zn uptake and translocation into shoots from solutions with citrate or histidine to uptake from solutions with the same total Zn and the same Zn^{2+} concentrations buffered with EDTA. In a second step, we investigated the influence of Zn on citrate and histidine uptake, using double-labeling of Zn and ligands.

■ MATERIALS AND METHODS

Cultivation of Plants. Two spring wheat cultivars differing in Zn efficiency (ratio between grain yields obtained under Zn-deficient and Zn-sufficient conditions) were used for the experiments: the Zn-inefficient cultivar 'Kavir' and the Zn-efficient cultivar back cross (BC) 'Rushan'.[28] Both cultivars are widely used by local farmers in central Iran and were provided from the Seed and Plant Improvement Institute (SPII), Karaj, Iran. After germination on filter paper for 5 days, the seedlings were grown in opaque containers in a greenhouse with a 16/8 h day/night cycle with a day temperature of 22 °C and a night temperature of 14 °C, in a 20% Hoagland solution (800 μM $Ca(NO_3)_2$, 1000 μM KNO_3, 400 μM $MgSO_4$, 200 μM KH_2PO_4, 40 μM NaCl, 20 μM $Fe(NO_3)_3$, 20 μM H_3BO_3, 4 μM $MnSO_4$, 0.4 μM $Cu(NO_3)_2$, 0.2 μM Na_2MoO_4, 0.2 μM $ZnSO_4$), buffered with 2.5 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and adjusted using 1 M NaOH to a pH between 6.5 and 6.9. The cultivars were distributed randomly in the greenhouse. During the whole growth period, the solutions were well aerated. In both experiments, the plants were cultivated in the same way for 3 weeks. If not stated otherwise, all treatments had four replicates, with four plants per replicate container.

Experiments. Prior to the experimental treatments, the plants were washed for 10 min in nanopure water and kept for 5 h in a pretreatment solution consisting of 500 μM KNO_3, 400 μM $Ca(NO_3)_2$, and 2.5 mM MOPS at a pH of 7.2 (adjusted with 1 M NaOH). No $Ca(NO_3)_2$ was used in experiment 3. MOPS buffer was used because it does not influence metal speciation in the medium.[29] To apply experimental solutions, the plants were gently transferred to 1 L containers consisting of opaque dark plastic to prevent degradation of the ligands by light.

Experiment 1: Zn Uptake Kinetics. By adding $ZnSO_4$ to a background of 500 μM KNO_3, 400 μM $Ca(NO_3)_2$, and 2.5 mM MOPS (adjusted with 1 M NaOH to a pH of 7.2) experimental solutions with the following six Zn^{2+} concentrations were produced: 0.005, 0.032, 0.5, 2, 10, 20 μM. To obtain the three Zn^{2+} concentrations <2 μM, 2.5, 2, or 1.5 μM EDTA was added to experimental solutions with 2 μM Zn, to buffer the respective concentrations of 0.005, 0.032, and 0.5 μM Zn^{2+} (calculated with MINEQL 4.6). In addition, 66.7 kBq L^{-1} $^{65}ZnCl_2$ were applied (Los Alamos National Laboratory, Los Alamos, NM, USA) to all samples.

Plants were immersed into these experimental solutions for 3 h. They were neither stirred nor aerated during this period.

Experiment 2: Influence of Organic Ligands on Zn Uptake by Wheat. The experimental solutions all consisted of a background of 500 μM KNO_3, 400 μM $Ca(NO_3)_2$, and 2.5 mM MOPS at a pH of 7.2 (adjusted using 1 M NaOH). A total Zn concentration of 20 μM was used in the treatments, and ligand concentrations (citrate, L-histidine, and EDTA) required to reach a Zn^{2+} concentration of ~50 nM were calculated using MINEQL 4.6. In addition, two controls were used, one with a total Zn concentration of 20 μM and no ligands and one containing only 2 μM total Zn and EDTA, also adjusted to a Zn^{2+} concentration of ~50 nM. The respective amounts of ligands added to the experimental solutions and treatment identification are given in Table 1. The conditional stability constants used for the calculations are given in Table 2. Stability constants for the Zn–ligand complexes

Table 2. Conditional Zn−Ligand Stability Constants Calculated for the Experimental Conditions of Experiment 2 Using MINEQL[a]

ligand (charge)	log K ZnL cond	log K ZnL$_2$ cond	log K ZnHL$_2$ cond
EDTA (−IV)	18.0 (99.8%)		
histidine (−I)	6.85 (16.6%)	12.6 (79.7%)	18.4 (3.4%)
citrate (−III)	5.6 (96.3%)	7.18 (3.4%)	

[a]In parentheses: percentage of the total Zn complexed by the respective ligand. Log K values (I = 0, 25°C) were taken from NIST database.[30].

and ligand deprotonation constants were taken from the NIST database[30] and adjusted to ionic strength I = 0 using the Davies equation.[31] After pretreatment, plants were exposed to the experimental solutions containing 277.5 kBq L^{-1} $^{65}ZnCl_2$ for 6 h. The solutions were neither stirred nor aerated during this time.

Experiment 3: Effect of Zn on Ligand Uptake by Wheat. Only the Zn-efficient cultivar BC Rushan was used for this experiment. No $Ca(NO_3)_2$ was added to the background solution (consisting of 500 μM KNO_3 and 2.5 mM MOPS, adjusted to a pH of 7.2 with 1 M NaOH) to achieve a higher degree of complexation. Each ligand was applied in combination with three different total Zn concentrations: 0, 20, and 100 μM Zn. In the treatments with 20 and 100 μM Zn >99% of the Zn was complexed with the ligands according to the speciation calculations (Table 3). Control treatments without the experimental ligands were established by adding EDTA to experimental solutions with 2 μM total Zn at concentrations of 1.9 μM (control Zn low) and 1.45 μM (control Zn high) to obtain the same Zn^{2+} concentrations as in the respective treatments with citrate and histidine. The solutions were labeled with 162.8 kBq L^{-1} $^{65}ZnCl_2$ and 888 kBq L^{-1} ^{3}H-histidine [ring-2,5-^{3}H] (Hartmann Analytic GmbH, Braunschweig, Germany) or 162.8 kBq L^{-1} ^{14}C-citrate [1,5-^{14}C] (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA), respectively. The citrate treatments had three replicates; the other treatments had four replicates. After pretreatment, plants were exposed to the experimental solutions for 6 h without stirring and aeration.

Plant Processing after Exposure to Radiolabeled Experimental Solutions. After the experimental treatments, plants were washed for 30 s in ice-cold nanopure water and then immediately transferred into a desorption bath to replace ^{65}Zn adsorbed at the root surface and also potentially root-bound ligands in experiment 3. The desorption bath contained ice-cold background solution plus 100 μM $ZnSO_4$, and in

Table 3. Zn and Ligand Concentrations Used in Three Treatments of Experiment 3 (Excluding Controls)[a]

	citrate	citrate + Zn low	citrate + Zn high
citrate total (μM)	450	370	450
citrate free (92% in the form of Cit^{3-}) (μM)	440	342.5	341.9
Zn tot (μM)		20	100
Zn free (μM)		0.11	0.54
Zn–Cit complexes (μM)		19.9	99.4
$ZnCit^-$ (μM)		19.7	98.4
$Zn(Cit)_2^{4+}$ (μM)		0.19	0.97

	histidine	histidine + Zn low	histidine + Zn high
histidine total (μM)	980	850	980
histidine free (92% in the form of $HHis^0$) (μM)	979.7	804.6	805
Zn total (μM)		20	100
Zn free (μM)		0.11	0.54
Zn–His complexes (μM)		19.8	99.3
$ZnHis^+$ (μM)		4.6	23.4
$Zn(His)_2^0$ (μM)		14.6	72.9
$ZnH(His)_2^+$ (μM)		0.61	3.0

[a]Background solution consisting of 500 μM KNO_3, 2.5 mM MOPS at a pH of 7.2 (adjusted with 1 M NaOH).

addition in experiment 3, 5 mM $CaCl_2$ (adapted from Hart et al.[32] and Panfili et al.[9]). After 15 min in the desorption bath, the plants were washed once more for 30 s in ice-cold nanopure water, cut into pieces, and dried at 60 °C for 5 days. It can be assumed that no additional Zn uptake occurred in the post-treatments. Hacisalihoglou et al.[33] found only negligible Zn uptake by wheat from solution at 2 °C as compared to 23 °C.

Ligand Degradation in Nutrient Solution. Ligand degradation was tested under the same experimental conditions as applied in experiment 2, but without applying labeled Zn. The composition of the experimental solutions is given in Table 1. Total ligand concentrations were measured before the plants were immersed in the nutrient solution, after 3 h of incubation, and after 6 h. To prevent biological degradation after sampling, NaN_3 was applied at a final concentration of 0.02%, and the samples were stored at 4 °C.[34] Citrate and histidine measurements were carried out within 1 day after incubation.

Chemical Analysis. *Total Zn Analysis.* After weighing, samples of the dried roots and shoots were digested in 15 mL of 69% (15.6 M) HNO_3 in a heating block at 120 °C. Total Zn concentration in digests

and experimental solutions without radiolabel was measured using ICP-OES (Vista-MPX, Varian).

Citrate Analysis. Citrate was analyzed by ion chromatography (Advanced Compact IC, Metrohm) using a Metrosep A Supp 5 150/4 mm column, 13 mM Na_2CO_3 as eluent, and a flow rate of 0.8 mL min^{-1}.

Histidine Analysis. Histidine concentrations were determined by ultrapressure liquid chromatography (UPLC) (Waters ACQUITY UPLC, equipped with fluorescence (FLR) and photodiode array detectors (PDA)). All samples were analyzed on an ACQUITY UPLC BEH130 C_{18} column (2.1 × 150 mm, 1.7 μm particle size) at 35 °C. The mobile phases were composed of solutions of 15.7 mM sodium acetate (pH 5.9) in nanopure water (solution A) and acetonitrile (solution B) and applied according to the following 7.0 min gradient at a flow rate of 0.35 mL min^{-1}: 0–0.5 min, 90% A; 0.5–2 min, 80% A; 2.0–5.5 min, 65% A; 5.5–6.0 min, 90% A. Sample preparation: 40 μL of the sample was dissolved in 80 μL of borate buffer (0.1 M, pH 8.8) containing 20 μM phenylalanine and, after the addition of 20 μL of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), an amino acid derivatizing agent, immediately capped, vortexed for 5 s, and stored at 4–10 °C until UPLC analysis. AQC was synthesized according to the methods of Cohen et al.[35] and Remucal and McNeill.[36]

EDTA Analysis. EDTA in the nutrient solution was measured by HPLC (Jasco PU-980) with UV detection at 258 nm (UV 970), a Dionex Ion Pac AS11 column (4 × 250 mm), injection volume of 50 μL, and a flow rate of 1 mL min^{-1}. Analyses were carried out with gradient elution, using deionized water as eluent A and a 5 mM $(NH_4)_2SO_4$ (pH 5.3) solution as eluent B. Eluent B was applied in a gradient, increasing from 10 to 75% in 6 min. For the analysis of EDTA, samples were acidified to pH 3 by adding 1 M HCl; then $FeCl_3$ was added at a final concentration of 25 μM.

^{65}Zn, ^3H, and ^{14}C Analyses. ^{65}Zn was analyzed in samples of roots, shoots, experimental solutions (taken immediately before and after plant incubation) and desorption solution samples using γ-spectrometry (high-purity germanium detectors, ORTEC, USA). ^3H and ^{14}C contents in plant and solution samples were analyzed by liquid scintillation counting (TRI-CARB 2200, Perkin-Elmer SA). The counting efficiency was 0.25 for ^3H and 0.91 for ^{14}C. Plant samples were prepared for scintillation counting by combustion in a split tube furnace (Carbolite) at temperatures increasing gradually from 400 to 800 °C over 1.25 h for root and over 3 h for shoot samples. Organic carbon was completely oxidized to CO_2 using a copper oxide catalyst. The oven was attached to a vacuum pump, and the radiolabeled gas was trapped in three sequential 300 mL bottles containing nanopure water for ^3H and 0.1 M NaOH for ^{14}C samples (i.e., to convert CO_2 into aqueous carbonate, CO_3^{2-} ion). For analysis of ^3H and ^{14}C in all samples, 10 mL of sample and 10 mL of Ultima Gold LSC-cocktail (Perkin-Elmer) were used.
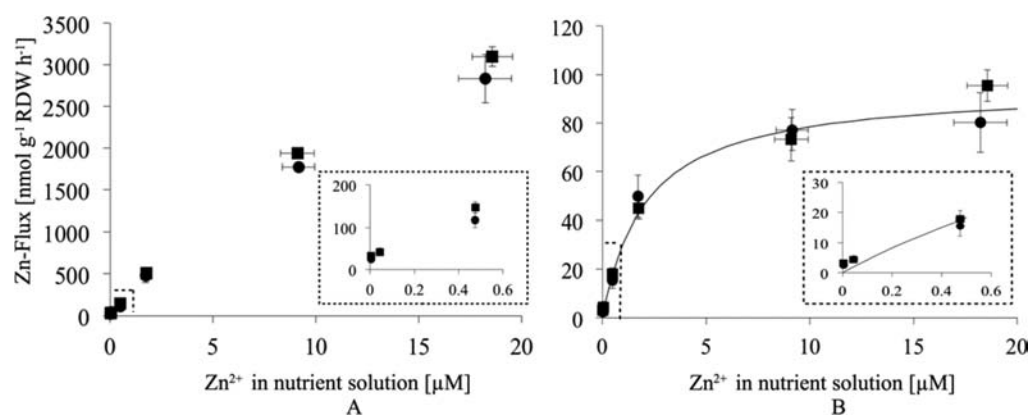

Figure 1. Zn influxes to roots (A) and translocation into shoots (B) of the Zn efficient cultivar 'BC Rushan' (■) and the Zn inefficient cultivar 'Kavir' (●) at various Zn^{2+} concentrations. Lines represent best-fit Michaelis–Menten kinetics (average of the two cultivars). Vertical bars represent SE; horizontal bars represent maximum differences in nutrient solution Zn^{2+} concentrations between the beginning and end of the experiment.
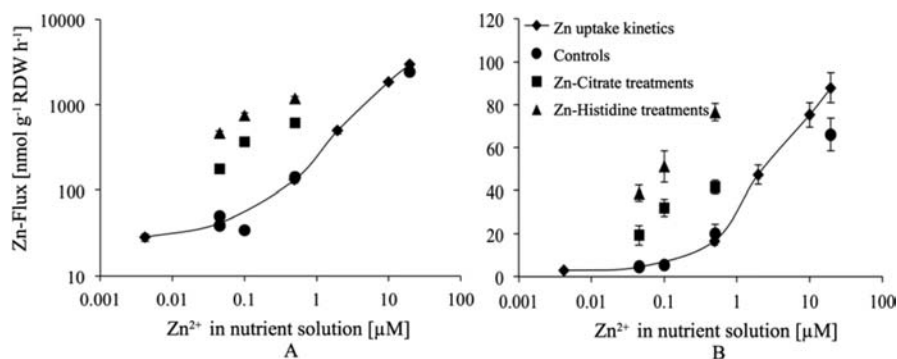
Figure 2. Zn fluxes into wheat roots (A) and translocation into shoots (B) at different Zn^{2+} concentrations in the absence and presence of organic ligands. Data points connected with the line represent the Zn uptake kinetics experiment (Zn^{2+} concentrations < 2 μM in the nutrient solution were buffered with EDTA); data points at ~0.05 and 20 μM Zn^{2+} concentration are from experiment 2; data points at the 0.1 μM and 0.5 μM Zn^{2+} concentrations are from experiment 3. Except for the 20 μM Zn^{2+} treatment, the controls are buffered with EDTA. Vertical bars represent SE.

Table 4. Zn Fluxes (with Standard Errors) into Roots and Translocation into Shoots at the Same Zn^{2+} Concentrations in the Presence of Citrate, Histidine, EDTA, or No Ligand in Experiment 2[a]

Zn flux (nmol g^{-1} RDW h^{-1})	Zn 20 μM + citrate	Zn 20 μM + histidine	Zn 20 μM + EDTA	Zn 2 μM + EDTA	Zn 20 μM
absorption by roots	177 (21) c	467 (26) d	50 (3) b	39 (2) a	2449 (204) e
adsorption at root surfaces	59 (4) c	88 (5) d	14 (1) b	10 (1) a	745 (33) e
translocation to shoots	19 (4.6) b	39 (3.7) c	4.7 (0.7) a	4.5 (0.8) a	66 (8) d

[a]For definition of treatments see Table 1. Different letters in a row indicate significant differences between treatments ($p \leq 0.05$). Data are pooled for the two cultivars.

Data Analysis. The fluxes of Zn and ligands into roots were calculated by dividing the respective increases in whole-plant contents of Zn and ligands during exposure to the experimental solutions by dry root biomass and time of incubation. Fluxes into shoots were calculated by dividing the amount of Zn or ligand accumulated in the shoots during exposure by dry root biomass (RDW) and time. Increases in Zn and ligand contents of the respective tissues were calculated on the basis of ^{65}Zn and labeled ligand contents after exposure.

Normal distributions of the sample groups were tested separately using the Shapiro−Wilk normality test. Two-way ANOVA was applied to the (if necessary, log transformed) data to analyze the main effects of cultivars, treatments, and their interactions. Significant differences between treatments were determined by pairwise t tests (p-value adjustment method: holm). Nonlinear regression analysis was used for fitting the Michaelis−Menten equation to the data. All statistical tests were performed using the software package "R", version 2.9.2.[37] The significance level was set at $p < 0.05$, if not otherwise stated.

RESULTS

Root and shoot biomass data are given as Supporting Information in Table S1 along with total Zn concentrations.

Experiment 1: Zn Uptake Kinetics. The average loss of Zn from the experimental solutions was 10.9 ± 8.3% during the 3 h incubation period. The highest losses were found with 23.1 ± 1.5% in the treatment with 2 μM total Zn and no EDTA (Figure 1). Whereas Zn fluxes into the roots did not reach a clear maximum with increasing Zn concentrations (Figure 1A), Zn accumulation in the shoots was close to saturation at 10 μM Zn^{2+} in the nutrient solution (Figure 1B) and well described by Michaelis−Menten kinetics at Zn^{2+} concentrations >0.5 μM:[33,38]

$$I(C) = \frac{I_{max}C}{K_m + C} \tag{1}$$

$I(C)$ is the rate of Zn accumulation in the shoots at a Zn^{2+} concentration C in the nutrient solution, I_{max} is the maximum rate of Zn accumulation, and K_m is the Zn^{2+} concentration at which accumulation reaches half the maximum rate. There was no significant difference between the two cultivars in the transfer of Zn from the solution into roots and shoots. Pooling the data for the two cultivars, the following optimized values (±standard errors) of the Michaelis−Menten parameters for shoot Zn accumulation by curve-fitting were obtained: K_m = 2.1 ± 0.4 μM and I_{max} = 94.9 ± 4.9 nmol g^{-1} RDW h^{-1} (Figure 1B). The amounts of Zn that were recovered from the roots in the desorption baths after incubation were substantial in comparison to the amounts that remained absorbed. The ratio of the Zn adsorbed to the absorbed Zn was on average 0.57 ± 0.02 and not significantly different between treatments. On the basis of the results of this experiment a Zn^{2+} concentration of 0.05 μM was chosen for the next experiments to ensure influx saturation was not reached.

Ligand Degradation in Experimental Nutrient Solutions. At the applied concentrations, none of the ligands used here showed significant degradation during the 6 h of incubation. The concentrations measured after 6 h were 99.4 ± 1.5% of the initial concentration for citrate, 100.4 ± 0.9% for histidine, and 101.7 ± 0.3% for EDTA.

Experiment 2: Influence of Organic Ligands on Zn Uptake by Wheat. A major decrease (13.6%) in the total Zn concentration during incubation was found only in the ligand-free nutrient solutions with 20 μM total Zn. Decreases did not exceed 6% in the other treatments. There were again no significant differences between the two cultivars, and thus the respective flux data were pooled for all further statistical analysis.

The control treatments (Zn 2 μM + EDTA and Zn 20 μM) gave results that were in very good agreement with the corresponding treatments in the kinetics experiment, demonstrating excellent reproducibility (Figure 2). Zinc fluxes into the roots were slightly higher in the 20 μM + EDTA treatment than in the Zn 2 μM + EDTA treatment (p = 0.01), although the
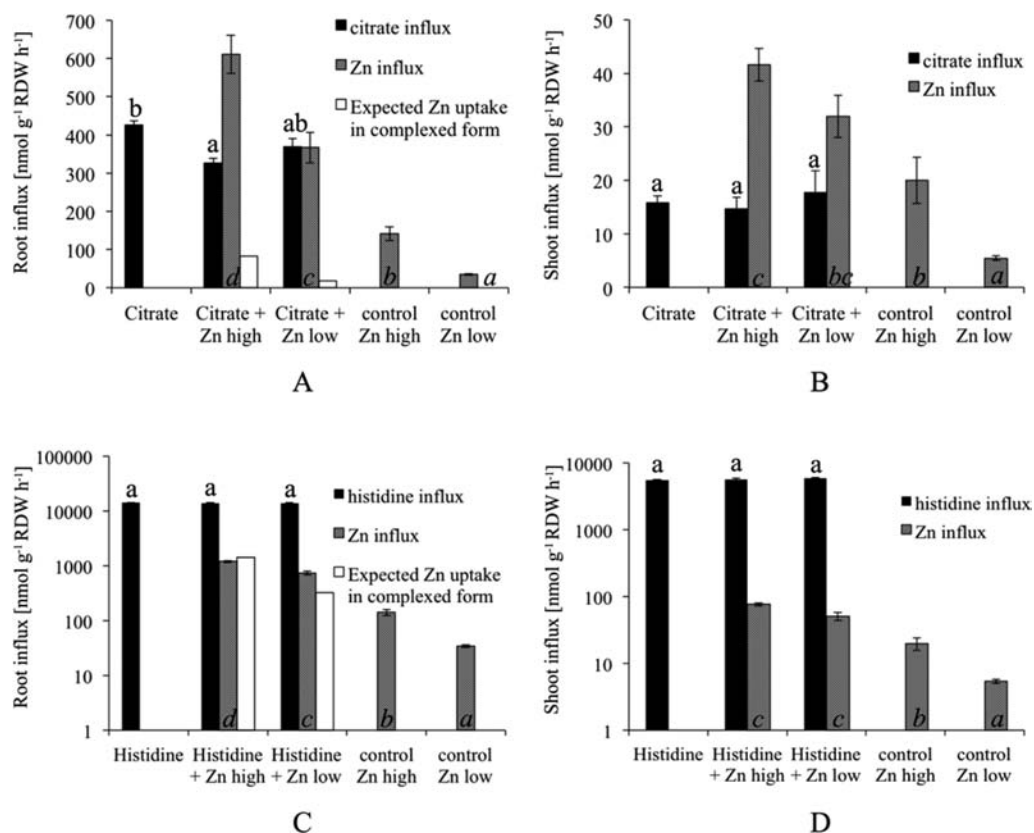
Figure 3. Uptake of citrate (A, B) and histidine (C, D) by wheat in the absence or presence of Zn from simplified nutrient solution in experiment 3. Zn fluxes are also shown for all relevant treatments. Expected Zn fluxes from the complexed fraction are indicated with white bars (Zn flux expected = ligand plant influx × ratio of total complexed Zn in solution to total ligand concentration in solution). Different letters indicate significant differences between treatments ($p \leq 0.05$). Vertical bars represent SE.

Zn^{2+} concentrations in solution were approximately the same, whereas there was no significant difference between these two treatments in shoot Zn accumulation (Table 4). In the presence of citrate or histidine, however, Zn fluxes into roots and shoots were several times larger than in the two Zn + EDTA treatments ($p \leq 0.01$). The absorption of Zn by the roots was increased by a factor of around 3.5 in the citrate treatment (Zn 20 μM + citrate) and by around 9 times in the histidine treatment (Zn 20 μM + histidine). A similar effect was observed on Zn accumulation in the shoots: an approximately 4-fold increase in the Zn 20 μM + citrate treatment and an approximately 8-fold increase in the Zn 20 μM + histidine treatment (Table 4). The translocation index (ratio of ^{65}Zn activity measured in shoots and total activity measured per plant) from roots to shoots was not significantly different between EDTA, citrate, and histidine treatments (0.1 ± 0.01). Only the Zn 20 μM treatment revealed a lower index of 0.03 ± 002, showing the saturation of root to shoot translocation. In parallel with absorption by the roots, the Zn recovered from the roots by the desorption bath solutions increased: Zn 2 μM + EDTA < Zn 20 μM + EDTA < Zn 20 μM + citrate < Zn 20 μM + histidine < Zn 20 μM (Table 4). The adsorption rate of Zn to the roots was related to the Zn uptake by the roots in a linear manner (uptake rate = 3.25 × adsorption rate + 41.8, R^2 = 0.994). However, the ratio of absorption and adsorption rates was higher in the histidine treatment than in all other treatments ($p < 0.05$ in all pairwise comparisons except for the Zn 2 μM + EDTA treatment). By comparison of the Zn fluxes found in the presence of ligands with the results of the

kinetics experiment, Zn uptake in the presence of citrate was equivalent to Zn uptake from a ligand-free solution with 0.55 μM Zn. This is about 10 times more than the Zn^{2+} concentration in the Zn 20 μM + citrate treatment. The effect was even more pronounced with histidine. The equivalent Zn uptake from a ligand-free solution would have required a Zn concentration of 1.55 μM, which is 30 times the Zn^{2+} concentration applied in the Zn 20 μM + histidine treatment (Figure 2).

Experiment 3: Effect of Zn on Citrate or Histidine Uptake by Wheat. *Zn Uptake.* The results of the control treatments agreed again very well with the respective results of the kinetics experiment, and Zn root influxes were again significantly increased in the presence of citrate and even more in the presence of histidine (p value ≤ 0.01), as in experiment 2 (Figures 2 and 3).

Ligand Uptake. In these experiments ^{14}C and ^{3}H from citrate and histidine sources, respectively, were measured. Possible breakdown products within the plants could not be distinguished from the supplied ligands, and when referring to ligand uptake fluxes, we always include possible breakdown products. Citrate uptake by roots was highest (426 ± 11 nmol g^{-1} RDW h^{-1}) in the citrate treatment with no Zn application (Figure 3A). Adding 100 μM Zn (citrate + Zn high) decreased it by 24% ($p = 0.01$). Adding 20 μM Zn (citrate + Zn low) decreased it on average by 13%, but this effect was not significant ($p = 0.1$), nor was the difference between the low and high Zn treatments (Figure 3A). The rate of citrate transfer into the shoots averaged 16.1 ± 1.4 nmol g^{-1} RDW h^{-1} (Figure

3B), and citrate desorption from the roots, 111 ± 17 nmol g^{-1} RDW h^{-1}. Neither differed between treatments.

With 13.9 ± 0.2 μmol g^{-1} RDW h^{-1} the rate of histidine uptake by the roots was much higher than that of citrate and, in contrast to the latter, did not change with the addition of Zn (Figure 3C). The rates of translocation into the shoots (5.7 ± 0.12 μmol g^{-1} RDW h^{-1}, Figure 3D) and of desorption from the roots (395 ± 21 nmol g^{-1} RDW h^{-1}) also were much higher for histidine than the respective rates for citrate and showed no Zn effect.

■ DISCUSSION

Zn Uptake Kinetics. The rates of Zn uptake into the roots and shoots of the two wheat cultivars found in our study resemble those found in other studies on wheat.[32,33,38] These studies also found no saturation of root Zn uptake kinetics up to concentrations of 20 μM Zn^{2+} in the nutrient solution. Zn translocation into the shoots reached saturation at around ∼10 μM, whereas root Zn concentrations seemed not even close to saturation. The fact that root to shoot translocation reached saturation at lower Zn^{2+} concentrations than root influx suggests that xylem loading of Zn becomes the limiting step at Zn^{2+} concentrations >10 μM. However, it is also possible that there was no saturation reached in root influx because of residual apoplastic Zn^{2+} remaining on the root surfaces after the desorption bath, as also suggested in other studies.[32,38,39] Zn uptakes at the two low Zn^{2+} concentrations of 0.005 and 0.05 μM were similar (Figure 1). This may indicate that Zn uptake occurred there through a high-affinity uptake system and through a low-affinity uptake system at higher Zn^{2+} concentrations, as has been described by Hacisalihoglu et al.[33] However, as our results suggest that diffusion in the rhizosphere and not the internalization rate is the main limiting factor for Zn uptake, it is more likely that these diffusion limitations caused a slight contribution of the strong Zn–EDTA complexes to plant uptake through complex dissociation. Similar effects have already been observed for other synthetic ligands (NTA, HEDTA, EGTA, CDTA), where Degryse et al. found a higher metal uptake in the presence of weaker ligands.[40]

Influence of Wheat Cultivars on Zn Uptake. In this study we found no significant differences in Zn uptake between the two cultivars, although they were found to differ in Zn efficiency in field experiments.[28] This suggests that the differences in Zn efficiency in the field were due to traits other than the efficiency of Zn uptake from soil solution. Such a trait may be the capacity to mobilize Zn in Zn-deficient soils (e.g., exudation of siderophores, organic acid anions, or amino acids). Differences in this capacity have been found among barley cultivars differing in Zn efficiency by Rasouli-Sadaghiani et al.[41] If this was the case, in our short-term hydroponic experiment with a high concentration of an externally applied ligand, the influence of any ligands exuded from the roots during the short duration of the experiments could have had no significant effect on Zn speciation in the nutrient solution. It may also be possible that selective colonization with mycorrhizal fungi may influence the Zn uptake efficiency of crop plants under field conditions[42] or that the cultivars develop different specific root surface areas under field conditions or other traits not relevant under hydroponic conditions.

Effects of Citrate and Histidine on Zn Uptake. Zn uptake was virtually the same for the two EDTA treatments

with a 10-fold difference in the total Zn concentration but the same Zn^{2+} concentration, meaning that Zn uptake was governed by the availability of the Zn^{2+} in the presence of a ligand forming very stable complexes with Zn (Figure 2). Citrate and histidine, however, strongly enhanced Zn uptake into the roots and shoots of the experimental plants. Increased metal uptake in the presence of organic ligands forming rather labile complexes has also been observed by Errecalde and Campbell[43] for cadmium (Cd) and Zn uptake into phytoplankton (*Selenastrum capricornutum*), by Aristilde et al.[44] and Xu et al.[45] for Zn uptake into phytoplankton (*Thalassiosira weissflogii* and *Emiliania huxleyi*), and by Panfili et al.[9] for Cd uptake into wheat roots. Three possible explanations have been suggested: (i) Labile complexes may enhance metal bioavailability indirectly, by contributing to the flux of metal to the root cell membranes, where they dissociate, resupplying free metal ions for uptake.[8–10] (ii) Metals are taken up in the form of undissociated complexes.[12,19,43] (iii) Complexes increase metal bioavailability by forming transient ternary complexes with the biotic ligands catalyzing trans-membrane transfer.[44,46]

The adsorption of Zn at the root surface was also not proportional to the Zn^{2+} concentration in the nutrient solution; it was, rather, correlated with the uptake rate (Table 4). The enhanced ratio of absorption versus adsorption found in the histidine treatment compared to the other treatments may be explained by the fact that surface-bound positively charged Zn–histidine complexes were desorbed with a different efficiency in the desorption bath than Zn ions.

Higher concentrations of organic transporter ligands in plants may also enhance root to shoot translocation of metals. Enhanced translocation of nickel (Ni) has been found in *Alyssum montanum* when histidine was applied externally to the roots, demonstrating the importance of histidine as a transporter ligand within plants.[47] In our experiment 2, however, the Zn translocation index from roots to shoots was not enhanced in the citrate and histidine treatments compared to the EDTA treatments. This suggests that externally applied histidine or citrate has no effect on Zn transport from roots to shoots in wheat.

Panfili et al.[9] found that the uptake of Cd by wheat roots was doubled in the presence of citrate compared to ligand-free treatments with the same free Cd concentrations. Considering differences in experimental conditions, this magnitude compares well with the 3-fold citrate-induced increase in Zn uptake found here, although the stability constants for Zn–citrate complexes are slightly higher than for Cd–citrate complexes.[48]

Also, the rates of citrate uptake into roots and shoots observed here were of similar magnitude as in the study of Panfili et al.,[9] who found rates of 710 and 25 nmol g^{-1} RDW h^{-1}, respectively. The fact that Zn reduced citrate uptake into the roots implies that there is no specific uptake mechanism for Zn–citrate complexes that is more efficient than the uptake of the free ligand. Furthermore, comparison of Zn and citrate fluxes show that the Zn flux was by a factor of 1.8 higher than the citrate flux in the citrate treatment with high Zn (Figure 3). If we assume that Zn–citrate complexes are taken up via the same mechanism as free citrate, the ratio of Zn to ligand taken up by the plant should correspond to the fraction of citrate complexed with Zn in the solution (Zn flux expected = ligand plant influx × ratio of Zn complexed to citrate in solution to total ligand concentration in solution). However, the measured

Zn fluxes were much higher than the calculated fluxes, as can be seen in Figure 3. Thus, we conclude that Zn−citrate complexes primarily contributed to Zn uptake by increasing the Zn^{2+} concentration through dissociation at the root cell membranes. Still, some uptake of intact Zn−citrate complexes via citrate transporters cannot be excluded. An analogous uptake of intact Cd−citrate complexes was suggested by the results of Errecalde and Campbell.[43]

If increasing the Zn^{2+} concentration through complex dissociation at sites of uptake would be the dominating mechanism for the enhancement of Zn uptake by histidine also, we would have expected a weaker histidine than citrate effect due to the higher stability of Zn−histidine complexes. However, the opposite was found. One possible explanation for that is that dissociation kinetics are much faster for Zn−histidine complexes than for Zn−citrate complexes. Rapid complex dissociation would also explain why there was no visible influence of Zn on histidine uptake even in the treatment with 100 μM Zn and 980 μM histdine (histidine + Zn high), where 18% of the histidine was complexed by Zn according to the calculations with MINEQL. However, it would seem more plausible that stronger complexes are also more stable kinetically rather than more labile.[49−51] Comparing the influence of NTA, HEDTA, EDTA, and CDTA on Zn uptake into roots of spinach and tomato from nutrient solutions with the same Zn^{2+} concentrations, Degryse et al.[8] found that Zn uptake was inversely related to the stability constants of the respective Zn−complexes as given by Smith and Martell:[52] the highest uptake occurred in the presence of NTA and the lowest in the presence of CDTA. These ligands are rather large molecules compared to citrate and histidine and, being synthetic compounds, not known to be the subject of specific physiological uptake by plant cells. Thus, the dissociation of complexes at the sites of uptake was the most likely mechanism by which they enhanced Zn uptake in the study of Degryse et al.,[8] in line with the assumption of increasing dissociation rate with decreasing complex stability.[53] Our finding that Zn uptake was higher in the presence of histidine than of citrate thus calls for a different explanation.

An important difference between Zn−citrate and Zn−histidine complexes are the complex charges. Whereas Zn−citrate complexes are negatively charged, the complexes formed between Zn and histidine were 80% neutral and 20% positively charged according to our speciation calculations (Table 2). Maurel and Chrispeels[15] found that neutral solutes may be taken up by plants via aquaporins. Thus, it seems possible that also neutral Zn−histidine complexes $(ZnHis_2{}^0)$ are taken up through this pathway. Another possibility is that neutral Zn−histidine complexes are taken up through the same amino acid transporters thought to take up histidine in the neutral form of $HHis^0$.[23,54] Furthermore, histidine may enhance Zn uptake through positively charged Zn−histidine complexes forming transient ternary complexes with biotic ligands at uptake sites on cell membranes. Positively charged Zn−cysteine and Zn−histidine complexes are thought to have enhanced metal uptake by phytoplankton in this way.[44] The hypothesis that the histidine effect on Zn uptake was largely due to direct uptake of Zn−histidine complexes is supported by the finding that total histidine uptake was much higher than total citrate uptake. In addition, the Zn fluxes calculated on the basis of the assumption that complexed histidine is taken up via the same uptake mechanisms as free histidine correspond much closer to

the measured Zn fluxes than in the citrate treatments (Figure 3).

There is evidence suggesting that amino acids can be taken up actively by cells via specific transporters as an additional source of nitrogen.[23] Higher uptake rates for histidine than for citrate were also found in Swiss chard,[13] although the difference was not as large as in our study. Besides species-specific differences, this may also have been due to different nutrient solution pH. Bell et al.[13] carried out experiments at pH 6, whereas the pH was 7.2 in our study. At pH 7.2 about 90% of the total histidine in our solutions is predicted to occur in neutral form, mainly as $HHis^0$, whereas at pH 6 only about 45% is predicted to be in the form of $HHis^0$ (54% as H_2His^+). As histidine is thought to be taken up preferentially in its neutral form,[23,54] this could explain the much larger histidine uptake found in our study than by Bell et al.[13] in relation to citrate uptake.

In conclusion, it is likely that some Zn−histidine complexes entered the roots undissociated contrary to Zn−citrate complexes, where it seems that complex dissociation was responsible for the increased Zn influx. The results of this study indicate that neutral and positively charged complexes in the soil solution may have a significant influence on metal availability to wheat.

■ ASSOCIATED CONTENT

ⓢ Supporting Information
Dry weight and total Zn concentrations of the 3-week-old wheat plants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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