JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Uptake of Zn and Fe by Wheat (*Triticum aestivum* var. Greina) and Transfer to the Grains in the Presence of Chelating Agents (Ethylenediaminedisuccinic Acid and Ethylenediaminetetraacetic Acid)

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A way to decrease iron and zinc deficiency in humans is to biofortify foods by increasing the bioavailable contents in these elements. The aim of this work was to study if chelating agents could be used to increase the capture of Fe and Zn by wheat grains. Zn and/or Fe in combination with the chelating agents ethylenediaminedisuccinic acid (EDDS) or ethylenediaminetetraacetic acid (EDTA) were added at various times (i.e., at flower head formation, anthesis, and postanthesis) to spring wheat (*Triticum aestivum* var. Greina) grown in nutrient solution. Treatments lasted for 2 weeks, and the plants were harvested at grain maturity. The shoots of treated plants accumulated higher Zn and/or Fe concentrations than untreated plants, depending on the treatment. The plants also accumulated significant concentrations of EDDS or EDTA in their shoots. Elevated Zn and Fe concentrations in the shoots did in most cases not lead to significantly higher Zn and Fe concentrations in the grains. The grains of plants treated with EDDS during flower head formation accumulated elevated Fe and Zn concentrations but at the cost of a reduction in yield. The control plants transferred higher percentages of Fe and Zn from the shoot into the grain than the treated plants. This indicates that EDTA and EDDS inhibited in most cases the translocation of Fe and Zn from the shoots into the grains. The amounts of EDDS and EDTA found in the grains of treated plants were very small. This indicates that there was little transfer of the chelates into the symplast and that the apoplastic pathway, which is important for the transport of chelants into the shoots, is efficiently blocked between shoots and seeds.

INTRODUCTION

Iron and zinc are essential elements for human nutrition (*1*). Worldwide, cereals are a main staple for humans, but unfortunately, the concentrations of bioavailable Zn and Fe in grains are rather low and antinutrients such as phytic acid reduce the absorption of Fe and Zn into the body. The nutritional value of grains may be enhanced by increasing accumulation without reducing the availability of the metals or by increasing their bioavailability (*2*).

One possible way to increase the uptake of metals by plants is the addition of chelating agents to soil or nutrient solutions (*3*). Whereas low concentrations of chelating agents were found to produce Zn deficiency in corn and barley (*4, 5*) and to reduce biomass and Zn uptake of wheat (*6*), high concentrations ($\geq$50−100 μM) have often been shown to enhance metal uptake. Increases in shoot Pb concentration in the range from 2 to 10 times the control have been observed with maximum values of up to 400 times (*7–9*). The effect of chelating agents on shoot uptake differs among plant species (*10*). The uptake of Cu and Zn in the presence of chelants often remained unaffected or even decreased. In sunflower, for example, the uptake of the essential metals Cu and Zn decreased in shoots in the presence of EDDS (ethylenediaminedisuccinic acid), whereas uptake of the nonessential Pb was enhanced (*11*). On the other hand, wheat showed a significant increase in shoot Zn, Cd, and Pb concentration after the addition of chelating agents, in both hydroponics and pot experiments (*12–14*).

Metal−chelant complexes are taken up by plants through the apoplast (*15*). This means that the complexes pass through the free space of the roots, which is made up of root cell walls and water-filled intercellular spaces in the root cortex and which is continuous with the surrounding soil solution (*16*). The Casparian strip is a barrier for the apoplastic pathway. It forces solutes to cross the cell membranes of the endodermis to reach the root stele and aboveground plant parts via the xylem. As most chelates are electrically charged, large in size, and have

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10.1021/jf800041b CCC: $40.75 © 2008 American Chemical Society
Published on Web 05/31/2008

Table 1. Composition of the Nutrient and the Experimental Solution

nutrients	nutrient solution (μM)	experimental solution (μM)
$Ca(NO_3)_2 \cdot 4H_2O$	400	400
$MgSO_4 \cdot 7H_2O$	200	200
KH_2PO_4	100	
KNO_3	500	500
EDTA/EDDS	10	500[a]
Fe	10	0/125[a]
H_3BO_3	10	
$MnSO_4 \cdot H_2O$	2	
$ZnSO_4 \cdot 7H_2O$	0.2	0/125[a]
$CuSO_4 \cdot 5H_2O$	0.2	
$Na_2MoO_4 \cdot 2H_2O$	0.1	
NaCl	20	
MES		2000
pH	6.0	6.0

[a] See **Table 2** for chelating agent, Fe, and Zn concentrations.

no known specific transporters, it is unlikely that they can pass through the cell membrane. However, the Casparian strip is not a perfect barrier. At the root tips, it is not yet formed (*15, 17*), and where lateral roots branch off, the Casparian strip can be disrupted. Through such leaks, the surrounding solution may enter the stele without passing through a cell membrane (*17, 18*). Several reports have shown that chelating agents are indeed taken up into the shoots and that they can facilitate the uptake of metals that are not normally taken up to a larger extent such as Pb at concentrations equal to their own accumulation (*7, 19–24*).

Most research on the interaction of chelating agents with plants has been conducted with EDTA (ethylenediaminetetraacetic acid). This compound, however, is not readily biodegradable in the environment and is therefore worldwide under scrutiny (*25*). EDDS (ethylenediaminedisuccinic acid) has the potential to be a substitute for EDTA. It is a strong chelator similar to EDTA, but in contrast, it is easily biodegradable (*26–28*). EDDS can readily solubilize metals from soil. At pH 7, it was shown to solubilize more Cu and Zn than EDTA at equimolar ratios of chelating agent to metal (*29*). Several papers have recently been published on the influence of EDDS on increasing the metal uptake (Pb, Zn, Cu, and Cd) in plants (*30–36*).

It is well-known that the chelating agent EDTA increases the bioavailability of Fe and Zn in humans (*37*). Fe(III)EDTA is used as a food additive and supplement in many countries of the world to ameliorate Fe deficiency (*37, 38*). EDTA can be regarded as safe for such uses (*39*). Also, Zn retention by humans is improved in the presence of EDTA (*40*).

The aim of this study was to test if EDTA or EDDS could increase the uptake of the essential metals Zn and Fe into the grains of wheat in a similar way as they can increase the uptake of metals in the shoots. If chelating agents are taken up into the grain, this would not only result in higher metal contents in the grain but at the same time also increase human bioavailability of Zn and Fe; thus, it is an efficient way of biofortification. The experiments have been carried out in hydroponic solution to allow full control over the composition of the solution, including metal speciation.

MATERIALS AND METHODS

Experimental Setup. Seeds of summer wheat (*Triticum aestivum* var. Greina, UFA Samen, Switzerland) were sterilized in 10% H_2O_2 (v/v) and rinsed with distilled water. They were germinated on vermiculite and watered regularly with distilled water. After 3 weeks, the seedlings were placed in aerated modified Hoagland solution and kept floating by means of perforated styropor plates. **Table 1** gives the composition of the nutrient solution. The solution was exchanged every 2 weeks. Sixty percent of the plants were grown in solution containing 10 μM Fe(III)EDDS; the other 40% were grown in 10 μM Fe(III)EDTA. The plants were grown in a climate chamber with 16 h of daylight, 2 h of twilight (23 °C, 75% relative humidity), and 6 h of night (15 °C, 75% relative humidity).

After 5 weeks, single plants were transferred to 2.5 L brown bottles, replicated four times for each treatment. Eight different treatments were applied by adding Zn, Fe, or Zn + Fe in combination with EDDS or EDDS as specified in **Table 2**. Before and after the treatments, the plants were grown in the nutrient solution. The treatments lasted for 2 weeks during which the solution was exchanged every 4 days. For the EDDS treatments, plants grown in EDDS-containing nutrient solution were taken; for EDTA treatments, the plants were grown in EDTA-containing solution. Two sets of four plants (one set for EDDS and one for EDTA) were not exposed to a treatment solution to serve as controls. The first treatment was started during flower head formation when the plants were 9 weeks old and extended into flowering (denoted with "FH_"). The treatment denoted "A_" started at the end of flowering, and the treatment denoted "PA_" started during the beginning of yellowing of the lower leaves.

All plants were harvested after 15 weeks of growth when the grains were fully mature. The shoots were cut 1 cm above the roots. For each plant, the seeds were removed by hand. All plant parts were dried for 4 days at 60 °C, and the dry weights of shoots and seeds were recorded.

Analyses. Shoots and seeds were milled in a titanium mill. Plant samples of 200 mg weight each were digested in Teflon tubes with 15 mL of HNO_3 (65%). The tubes were placed in a heating block (DigiPREP MES, SCP Sciences) and heated to 150 °C for 15 min. The digested samples were diluted to 15 mL with Millipore water. The digests were analyzed for Zn and Fe by inductively coupled plasma−optical emission spectroscopy. Analytical quality was assured by using reference samples of poplar leaves (NCS DC 73350).

For EDDS and EDTA analysis, the dried plant material was extracted with pure water (10 mL of water and 10 mg of shoots or 100 mg of grains) by heating for 1 h at 100 °C. The samples were then centrifuged and filtered (0.45 μm).

EDDS derivatization and analysis were carried out as described by Tandy et al. (*41*). This method involves the derivatization of EDDS by FMOC (fluorenylmethyl chloroformate) followed by high-performance liquid chromatography (HPLC) (Jasco PU-980) separation using a Phenomenex 5 μm column and fluorescence detection (Jasco 821-FP). Each sample was also spiked with an EDDS standard to help discriminate the EDDS peak at low concentrations from the matrix peaks.

EDTA analysis was based on the method of Rustandi (*42*) by forming a Tb(III)EDTA complex and fluorescence detection. To 1 mL of the aqueous plant extract, a solution of 60 μL of 10 mM $TbCl_3$ (pH 3) was added and heated to 90 °C for 1 h. HPLC was carried out with the same equipment as the EDDS analysis but using a LiChrosphere 100, RP-18, 5 μm column. Eluent A was 0.01 M Na-formate, 0.45 mM formic acid, and 3 mM tetrabutylammonium bromide (pH 3.5). Eluent B was acetonitrile. A gradient from 0 to 10% B in 6 min and back to 0% in 1 min was used. EDTA was detected by fluorescence (ex, 240 nm; em, 544 nm). Concentrations were quantified by standard addition.

Statistics. All statistical analysis was carried out using SPSS. We tested if treatment means differed from the mean of the controls. Q−Q plots of the residuals were applied to check the distribution of the data. After log-transformation, all of the data were normally distributed. The equality of the variances was tested by scatterplots. In the case of homogeneity of the variance, a one-factorial analysis of variance under application of Bonferroni was conducted. For data with inhomogeneous variances, an independent *t* test assuming no equal variances was made. Differences at the $p < 0.05$ level were considered statistically significant. The error bars shown in the figures represent the standard error of the mean of four replicates.

RESULTS

Biomass and Grain Yield. The biomass of the plants did not vary much between the four replicates of each treatment.

Table 2. Overview of the Experimental Conditions of the Treatments of the Wheat Plants

notation	plant age (weeks)	growth stage	μM			
			Fe	Zn	EDDS	EDTA
FH_Fe/Zn/EDDS	9	before flowering	125	125	500	
A_Fe/Zn/EDDS	11	anthesis	125	125	500	
A_Fe/EDDS	11	anthesis	125		500	
A_Zn/EDDS	11	anthesis		125	500	
A_Fe/Zn/EDTA	11	anthesis	125	125		500
A_Fe/EDTA	11	anthesis	125			500
A_Zn/EDTA	11	anthesis		125		500
PA_Zn/Fe/EDDS	13	post anthesis	125	125	500	

Table 3. Shoot Biomass, Grain Yield, and Grain Weight

phase	treatment	shoot biomass (g/plant)	grain yield (g/plant)	grain weight (g/1000 grains)
flower head formation	FH_Fe/Zn/EDDS	6.35 ± 0.55	2.25 ± 0.34	33.75 ± 2.72
anthesis	A_Fe/Zn/EDDS	4.98 ± 0.37	3.77 ± 0.35	28.76 ± 0.97
	A_Fe/EDDS	6.00 ± 0.45	3.84 ± 0.46	27.61 ± 2.11
	A_Zn/EDDS	5.52 ± 0.29	4.99 ± 0.36	31.60 ± 1.29
	A_Fe/Zn/EDTA	6.54 ± 0.86	3.91 ± 0.63	27.90 ± 0.78
	A_Fe/EDTA	8.03 ± 0.60	4.96 ± 0.69	26.51 ± 0.89
	A_Zn/EDTA	5.40 ± 0.89	4.00 ± 0.37	29.09 ± 1.15
postanthesis	PA_Fe/Zn/EDDS	6.29 ± 0.88	4.75 ± 0.29	28.81 ± 1.18
control	EDDS	5.31 ± 0.27	4.67 ± 0.16	31.85 ± 0.55
	EDTA	4.67 ± 0.29	4.24 ± 0.34	32.35 ± 0.94

In most cases, the relative standard error was less than 10%. The two-week chelant treatments had no detectable toxic effects on the plants throughout the experiment. Only the plants in the Fe/EDTA treatment produced more biomass than the controls ($p = 0.025$); the other treatments showed no significant effect (**Table 3**).

The grain yield was also similar in the various treatments. Only the grain yield of the FH_Fe/Zn/EDDS treatment was significantly lower than for the control ($p = 0.002$) (**Table 3**); the other treatments showed no significant effect on grain biomass.

Fe and Zn Concentration in Shoots. The Fe concentration of the shoots was significantly increased in all treatments receiving Fe ($p \leq 0.024$) (**Figure 1a**). Likewise, the Zn concentration was significantly increased in all treatments receiving Zn ($p \leq 0.004$) (**Figure 1b**). The experimental treatment solution did not contain any micronutrients other than the one for which the treatment was designed. Thus, the plants treated with Zn did not receive Fe for 2 weeks and vice versa. This transient shortage did not lead to detectable effects except for the A_Fe/EDDS treatment, in which a decreased Zn concentration ($p = 0.012$) was observed. In particular, no corresponding effect on the Fe status of plants was observed in the Zn treatments.

Fe and Zn in Grains. The observed increase in shoot Fe and Zn in presence of chelating agents did not translate into increased transfer of Fe and Zn into the grains (**Figure 2a**). The only remarkable increase in grain Fe was observed in the FH_Fe/Zn/EDDS treatment ($p = 0.206$), but it was not significant. The Fe-free treatments A_Zn/EDDS and A_Zn/EDTA reduced the Fe concentration in the grains ($p < 0.001$), although they had no effect on shoot Fe concentration (but on shoot growth).

The combined Fe and Zn treatments FH_Fe/Zn/EDDS and A_Fe/Zn/EDDS increased the Zn concentration of the grains ($p < 0.001$) (**Figure 2b**). In one case, the Zn-free treatment did reduce the Zn uptake by the grains significantly (A_Fe/EDTA; $p < 0.001$) and in the other not (A_Fe/EDDS; $p > 0.1$).

Total Fe and Zn amounts taken up by the grains (μmol/plant) were calculated by multiplying grain yields with respective metal concentrations (**Figure 3a**,**b**). The grains from the treatment FH_Fe/Zn/EDDS, which had the highest Fe concentration, had a lower Fe content in the grains than the control plants ($p = 1.0$). The lower amount of Fe in the Fe-free treatments is also visible for A_Zn/EDDS ($p = 0.051$) and for A_Zn/EDTA ($p = 0.008$). The Zn content of the grains was significantly enhanced in the treatments FH_FeZn/EDDS ($p = 0.040$) and A_Fe/Zn/EDDS ($p = 0.014$) (**Figure 3b**) as compared to the controls.

EDDS and EDTA Concentrations in Shoots and Grains. All plants including the controls were exposed for 10 weeks to 10 μM FeEDDS or FeEDTA to ensure a sufficient Fe supply. As a result, small EDDS or EDTA concentrations were found in all shoots and grains, including the control plants. The plants that were exposed additionally for 2 weeks to 500 μM EDDS or EDTA showed a significant enrichment of the respective chelating agent in the shoots (**Figure 4a**). Comparing respective treatments, the enrichment of EDTA was at least twice as high as that of EDDS.

The EDDS and EDTA content in the grains of control plants was about a factor 20 lower than that of the shoots and not significantly increased by treating with experimental solution (**Figure 4b**). Again, the EDTA was at least two times higher than EDDS accumulation in respective treatments (including controls).

Transfer of Zn and Fe to the Grain. We compared the amount of Fe and Zn transferred to the grains with the total amount of these metals in the plants at harvest (shoot + grains). With 72−79%, the control plants transferred the highest percentages of both metals into the grain (**Figure 5**). In the chelant treatments, the percentages of the metals transferred to the grain were reduced to values between 38 (for Fe in A_Fe/EDDS) and 69% (for Zn in A_Fe/EDDS). The transfer of Fe in the Fe-free treatments came closest to the Fe transfer in the control plants (A_Zn/EDDS, 60%; A_Zn/EDTA, 66%). The same effect was observed for the Zn transfer, which was highest in the Zn-free treatment A_Fe/EDDS with 69% and A_Fe/EDTA with 49%. For Fe, the observed translocation efficiencies correspond to values given by Garnett and Graham (*43*).

DISCUSSION

Uptake of Chelated Metals into the Grain. As expected on the basis of previous studies (*11*), the EDTA and EDDS treatments increased the Zn and Fe accumulation in the shoots. This was accompanied by a significant uptake of chelating agents. The uptake of negatively charged metal complexes through the apoplast can be efficient, if the complexes enter the stele through leaks in the root endodermis (*3*).

Low concentrations of chelating agents were measured in the grains. The transfer of chelates from leaves and stems into the developing grain appears to be efficiently controlled. Solute transport to the grains occurs through the phloem and

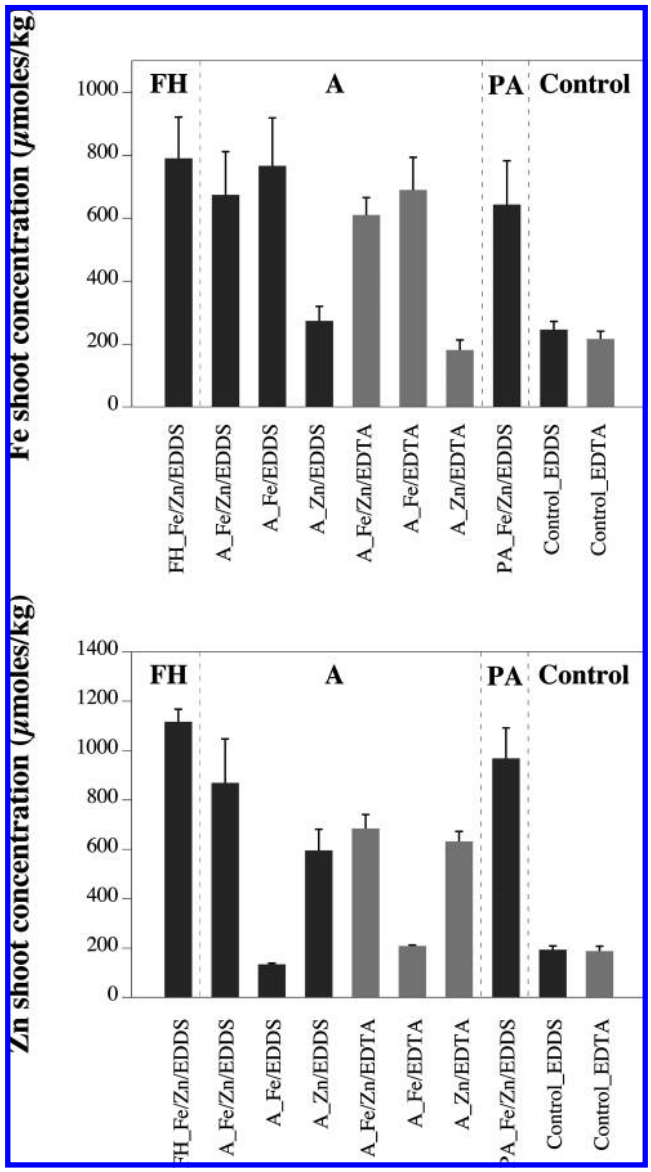

Figure 1. Shoot Fe and Zn concentrations (μmol/kg) in treatments with EDDS (dark gray) or EDTA (light gray). FH, treatment during flower head formation; A, treatment during anthesis; and PA, treatment postanthesis; $n = 4$.

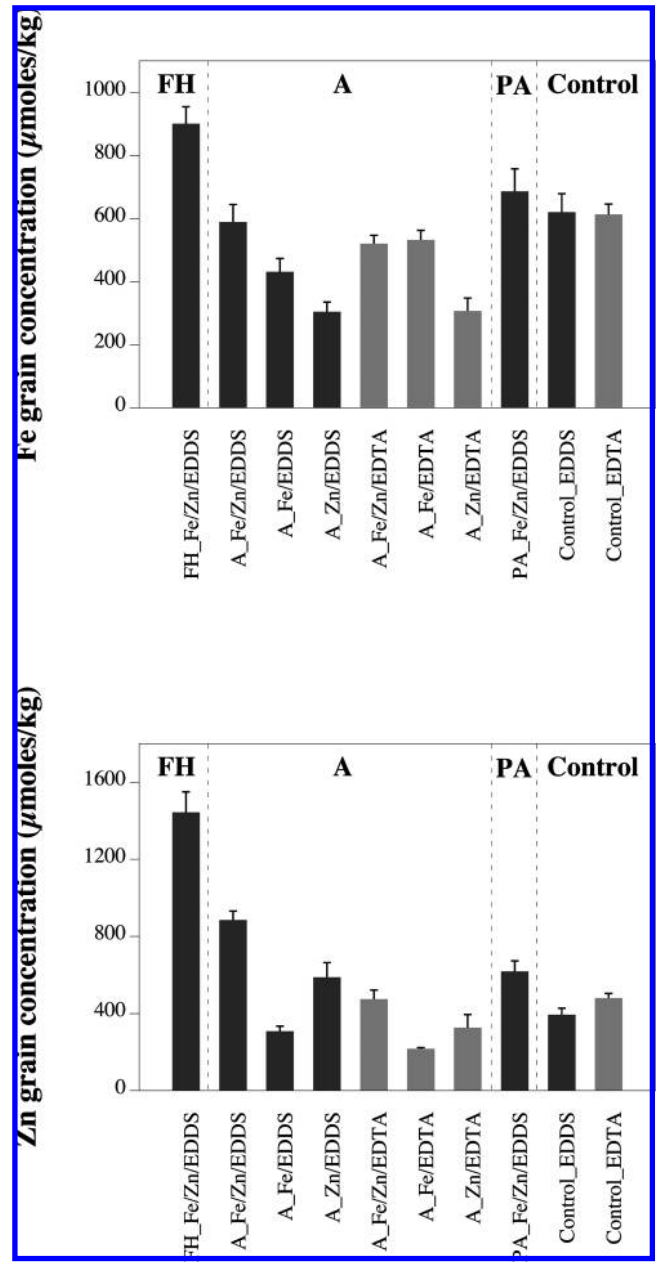

Figure 2. Grain Fe and Zn concentrations (μmol/kg) in treatments with EDDS (dark gray) or EDTA (light gray). FH, treatment during flower head formation; A, treatment during anthesis; and PA, treatment postanthesis; $n = 4$.

xylem (*44, 45*). To reach the endosperm, solutes have to cross two tissues along symplastic pathways (*46*). These barriers obviously are much less leaky for chelating agents than the Casparian strip in the roots. The very low concentrations of chelating agents that were found inside the grains could also have resulted from uptake of chelating agents into the corresponding tissue before it started to differentiate into grain and barriers.

The chelating agents may not even have reached the phloem, if they were already excluded at the site of phloem loading. Results from foliar application of Fe(III)EDTA and other Fe chelates showed translocation of the Fe complex within the plant and therefore the possibility of phloem transport (*47, 48*), although often only a limited mobility was observed within the plant (*49*). However, the fact that glyphosate, a related chelating agent similar to those used here, is very mobile in phloem after foliar application (*50*) suggests that phloem transport is possible and that the chelating agents were probably excluded at the phloem−grain boundary.

EDTA and EDDS are strong chelating agents, especially for Fe(III), and the concomitant presence of metals and chelants in the plant makes it likely that they were present as Fe and Zn complexes. If the chelants are blocked out from the transfer into the grains, the same will apply to the metal chelates as well. This is similar to what was reported for a mutant pea able to overaccumulate Fe (*51*). After fertilization with the complex FeEDDHA, a massive increase in the shoot Fe content was observed but only limited transfer into the beans.

Increased grain Fe and Zn concentrations were observed in the treatment where exposure to EDDS occurred before flowering (treatment FH_). These increased metal concentrations were accompanied, however, by a decrease in grain yield. At the beginning of flowering, the plant determines the number of potential grains that are then reduced until pollination (*52*). This reduction occurred during the exposure to EDDS in the FH_ treatment. The reduced number of seeds in this treatment may

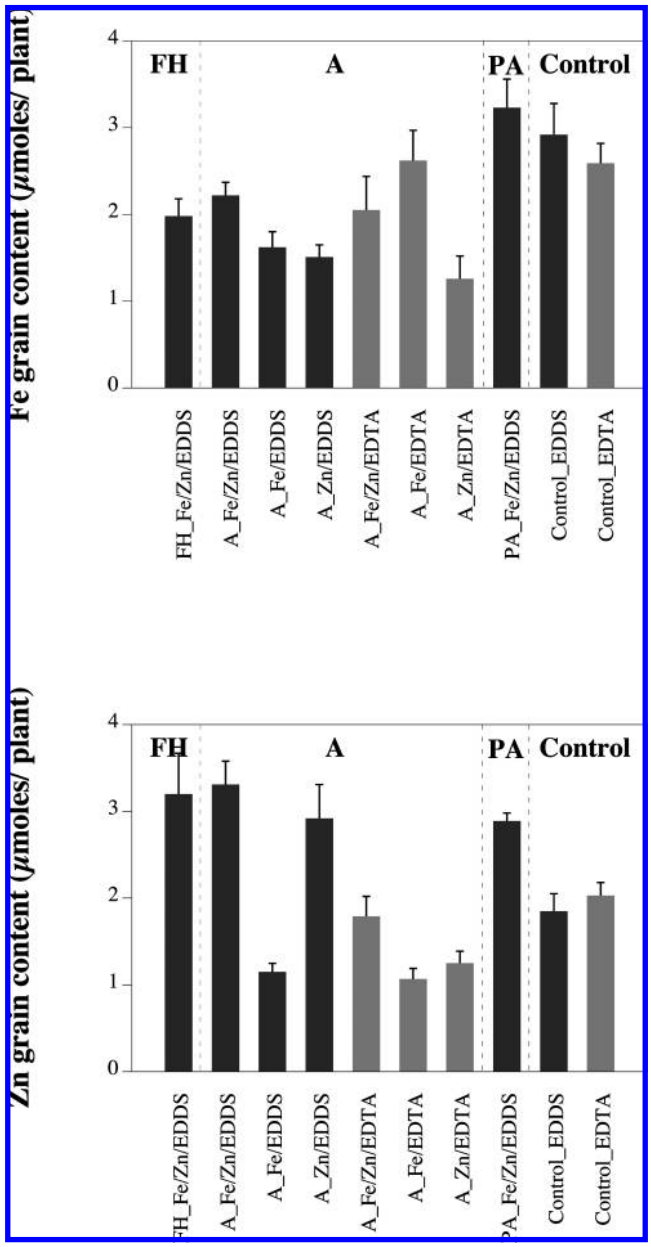

Figure 3. Grain Fe and Zn content (μmol/ plant) in treatments with EDDS (dark gray) or EDTA (light gray). FH, treatment during flower head formation; A, treatment during anthesis; and PA, treatment postanthesis; $n = 4$.

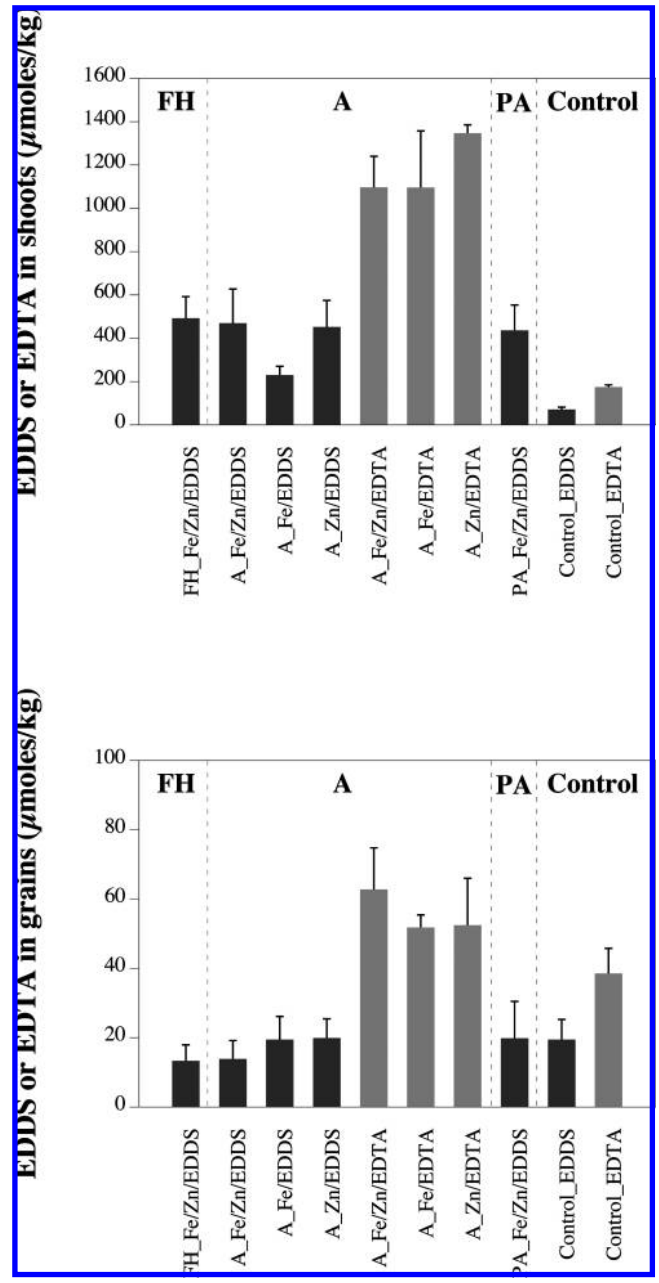

Figure 4. Shoot and grain EDDS and EDTA concentrations (μmol/kg) in treatments with EDDS (dark gray) or EDTA (light gray). FH, treatment during flower head formation; A, treatment during anthesis; and PA, treatment postanthesis; $n = 4$.

thus have resulted from stress caused by the metal + chelant treatment, although no such effect was found in the shoot biomass. While the plants reduced the number of seeds in this treatment, the grains became the largest of all treatments (including control). The fewer seeds later received a larger share of the available Fe and Zn. The absolute amounts of Fe and Zn in the grains were therefore not less at harvest than in the other metal−chelant treatments.

If one compares the fraction of the total Fe and Zn that is transferred to the grains, then the effect of the chelating agents is much more obvious in that in almost all cases the plants exposed to chelating agents transferred less metals into the grains. This means that not only the metal chelates are less likely to be transferred to the grain than noncomplexed metals but also the presence of the chelants inside the plants affected the normal transfer processes into the grain, presumably by changing the speciation of metals and decreasing the amount of metals bound by endogeneous chelators that can pass into the grain.

Differences between EDTA and EDDS. Although EDTA and EDDS were present at the same concentration in the nutrient solutions, their concentrations in the plant were quite different. The EDTA concentration was twice the EDDS concentration or even more. Several explanations for this observation are possible as follows: higher uptake of EDTA, biodegradation of EDDS, and faster photodegradation of EDDS in the plant.

The uptake of metal chelates depends on the type of chelant and its charge, although the dependence is not yet clear (*23, 24*). Large differences in uptake of EDTA and EDDS are unlikely because the two compounds are very similar. According to speciation calculations, the prevalent Fe complexes were monovalent $Fe(III)EDDS^-$ and $Fe(III)EDTA^-$ and the prevalent free forms were the divalent species H_2EDDS^{2-} and H_2EDTA^{2-} at the pH of the nutrient solution. Because of the excess of chelant, the latter dominated. As the charge of the two chelating

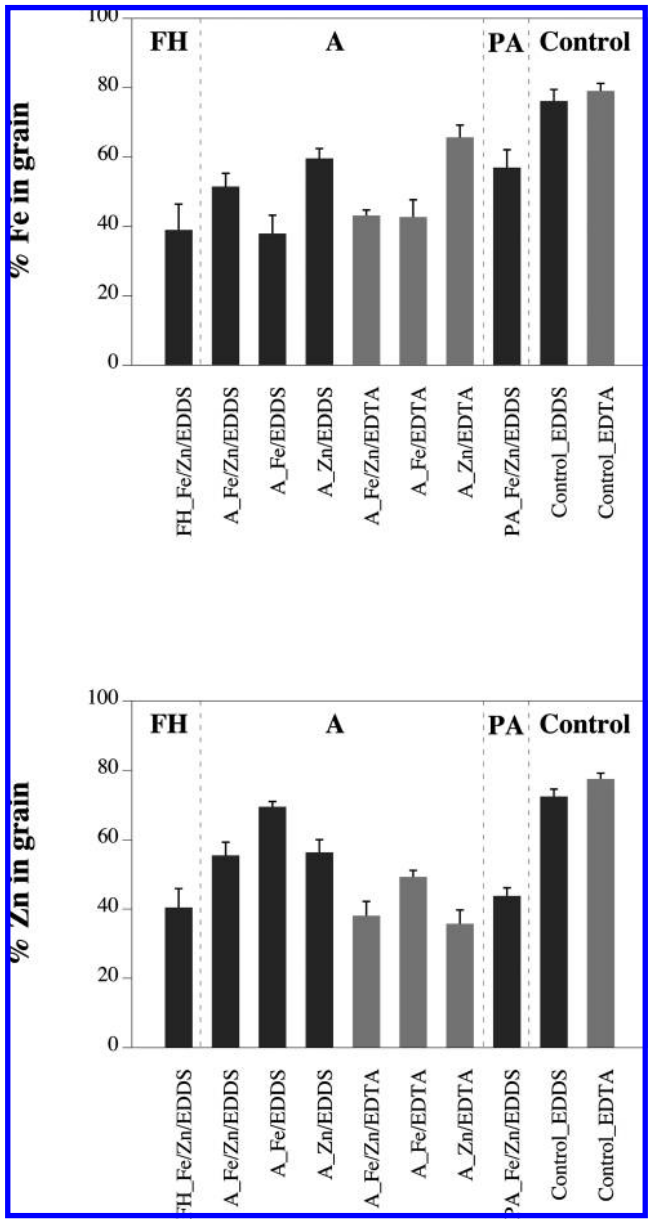

Figure 5. Percentage of total Fe and Zn taken up that is transferred to the grain in treatments with EDDS (dark gray) or EDTA (light gray). FH, treatment during flower head formation; A, treatment during anthesis; and PA, treatment postanthesis; $n = 4$.

agents was the same in both treatments, this does not explain the large difference in uptake.

Being not readily biodegradable (*53*), EDTA is stable in nutrient solution. In contrast, EDDS is rapidly biodegradable (*26, 54*). Although the nutrient solution was exchanged every 4 days, it is possible that the concentration of EDDS in the nutrient solution was thus on average lower than that of EDTA. The reported half-lives for EDDS range from 0.3 days for soil amended with sludge to 6.3 days for river water (*26*). Biodegradation could thus explain a correspondingly lower uptake of EDDS into the plants.

Photodegradation of chelants could not have played a role in the nutrient solutions, as the bottles were not transparent. However, inside the plant photodegradation of Fe complexes is a process that has been shown to occur (*55*). Fe(III)EDDS is faster degraded by light than Fe(III)EDTA (*56*), a process that may also have contributed to the lower EDDS concentration in the shoots and grains. A combination of biodegradation of EDDS and faster photolysis of Fe(III)EDDS in the plant may thus have resulted in the lower EDDS concentrations in the plant.

Effect of Fe-Free and Zn-Free Treatments on Fe and Zn Content in Grains. The Fe-free treatments A_Zn/EDDS and A_Zn/EDTA resulted in plants with significantly reduced grain Fe concentrations, although this treatment lasted for only 2 out of the 15 weeks of growth. In the remaining 13 weeks, the plants received sufficient Fe to compensate for the 2 week shortage. In fact, the Fe concentration in the shoots was not significantly reduced in this treatment. Thus, only the transfer from shoots to grains was affected. Treatment A_ started about 10 days after pollination. Until about 25 days after pollination, the plant fills about 50% of the final grain content (*52*). The Fe shortage thus occurred exactly during the critical grain-filling stage. Only a small fraction of the chelating agent in nutrient solution was complexed with Zn; the major part was in free form. The uptake of free chelating agents can result in significant changes in metal speciation inside the plant and can thus affect retranslocation of Fe by forming a complex that is less likely transported into the developing grain.

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Received for review January 7, 2008. Revised manuscript received March 26, 2008. Accepted April 2, 2008.

JF800041B