DOI:10.1021/jf101197h

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Biofortification and Localization of Zinc in Wheat Grain

I. Cakmak,*,† M. Kalayci,‡ Y. Kaya,§ A. A. Torun,∥ N. Aydin,⊥ Y. Wang,# Z. Arisoy,§ H. Erdem,∥ A. Yazici,† O. Gokmen,† L. Ozturk,† and W. J. Horst#

†Faculty of Engineering and Natural Sciences, Sabanci University, 34956 Istanbul, Turkey, ‡Anatolian Agricultural Research Institute, Eskisehir, Turkey, §BD International Agricultural Research Institute, P.O. Box 125, Konya, Turkey, ∥Department of Soil Science, Faculty of Agriculture, Cukurova University, Adana, Turkey, ⊥Karamanoglu Mehmetbey University, 70100, Karaman, Turkey, and #Institute for Plant Nutrition, Leibniz University Hannover, 30419 Hannover, Germany

Zinc (Zn) deficiency associated with low dietary intake is a well-documented public health problem, resulting in serious health and socioeconomic problems. Field experiments were conducted with wheat to test the role of both soil and foliar application of $ZnSO_4$ in Zn concentration of whole grain and grain fractions (e.g., bran, embryo and endosperm) in 3 locations. Foliar application of $ZnSO_4$ was realized at different growth stages (e.g., stem elongation, boot, milk, dough stages) to study the effect of timing of foliar Zn application on grain Zn concentration. The rate of foliar Zn application at each growth stage was 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1}. Laser ablation (LA)-ICP-MS was used to follow the localization of Zn within grain. Soil Zn application at a rate of 50 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} was effective in increasing grain Zn concentration in the Zn-deficient location, but not in the locations without soil Zn deficiency. In all locations, foliar application of Zn significantly increased Zn concentration in whole grain and in each grain fraction, particularly in the case of high soil N fertilization. In Zn-deficient location, grain Zn concentration increased from 11 mg kg^{-1} to 22 mg kg^{-1} with foliar Zn application and to 27 mg kg^{-1} with a combined application of $ZnSO_4$ to soil and foliar. In locations without soil Zn deficiency, combination of high N application with two times foliar Zn application (e.g., at the booting and milk stages) increased grain Zn concentration, on average, from 28 mg kg^{-1} to 58 mg kg^{-1}. Both ICP-OES and LA-ICP-MS data showed that the increase in Zn concentration of whole grain and grain fractions was pronounced when Zn was sprayed at the late growth stage (e.g., milk and dough). LA-ICP-MS data also indicated that Zn was transported into endosperm through the crease phloem. To our knowledge, this is the first study to show that the timing of foliar Zn application is of great importance in increasing grain Zn in wheat, especially in the endosperm part that is the predominant grain fraction consumed in many countries. Providing a large pool of Zn in vegetative tissues during the grain filling (e.g., via foliar Zn spray) is an important practice to increase grain Zn and contribute to human nutrition.

KEYWORDS: Biofortification; crease phloem; wheat grain; zinc fertilization; zinc deficiency

INTRODUCTION

Zinc (Zn) deficiency is a well-documented public health problem in the developing world, resulting in severe health and socioeconomic problems (*1*, *2*). Impairments in brain function, immune system and physical growth are the major consequences of Zn deficiency in the human body. Based on diet and bioavailability data, it is estimated that at least 1/3 of the world population is affected by the Zn deficiency problem, particularly children (*1*, *3*). Nearly 450 000 children under the age of five die annually because of Zn deficiency (*4*). Together with vitamin A deficiency, Zn deficiency has been identified as the top priority area to be addressed in order to achieve a very rapid and high return for humanity and global stability. This conclusion was made in 2008 by a panel of eight economists including five Nobel Laureates (www.copenhagenconsensus.com).

Low dietary intake of Zn and very little dietary diversity appear to be the major reasons for the widespread occurrence of Zn deficiency in human populations (*4*−*6*). Diets consumed predominantly in the developing world are based on cereals which are poor in the amount and bioavailability of Zn. Enrichment of cereal crops with Zn is, therefore, an important global challenge and a high-priority research area (*7*). Plant breeding (e.g., genetic biofortification) and application of Zn fertilizers (e.g., agronomic biofortification) are two important agricultural tools to improve grain concentration of Zn (*8*−*10*). The plant breeding approach offers the most sustainable solution to enrichment of cereal grains with Zn; it is, however, a long-term process. In addition, the genetic capacity of the newly developed high-Zn genotypes to

*To whom correspondence should be addressed. Phone: +90-216-483-9524. Fax: +90-216-483-9550. E-mail: cakmak@sabanciuniv.edu.

 Published on Web 07/27/2010

accumulate a sufficient amount of Zn in grain may depend on the pool of plant-available Zn in soil solution. As reviewed by Cakmak (*7*), application of Zn fertilizers represents a promising solution to the problem in the short term and a complementary approach to the plant breeding strategy.

Convincing evidence is available about the effectiveness of soil and foliarly applied Zn fertilizers in improving grain Zn concentrations in Zn-deficient wheat growing regions. Strong increases in grain Zn were obtained in the case of combined soil and foliar Zn applications of $ZnSO_4$, raising grain Zn by 3- or 4-fold (*11*). Application of Zn fertilizers not only improved nutritional quality but also contributed significantly to grain production in Zn-deficient soils (*7*, *11*−*13*). Increases in grain Zn concentration by soil and/or foliar application of Zn fertilizers were also shown in pot trials under greenhouse conditions (*14*, *15*). According to Kutman et al. (*15*), the N nutritional status of plants has a critical role in maximizing grain Zn concentration. When Zn nutrition of plants was optimal, improving N nutrition of plants substantially enhanced grain Zn concentrations. There was a close positive correlation between tissue N and Zn concentrations in plants treated sufficiently with Zn and N fertilizers (*15*). These results indicate that a desirable biofortification of wheat grain with Zn depends greatly on adequate supply of N.

Remobilization of Zn from vegetative tissues into grain appears to be an important pathway for accumulation of Zn in grain. More than 70% of the Zn reserves in the vegetative parts of wheat plants was remobilized (*16*), associated with the senescence of leaf tissues and/or grain filling (*17*). There is convincing molecular evidence for the role of leaf senescence in accumulation of micronutrients in wheat grain. Expression of the NAM-B1 gene was shown to be responsible for remobilization of Zn, Fe and also N from leaf tissue into grain during the leaf senescence, and delaying leaf senescence markedly reduced grain concentrations of Zn and Fe (*18*, *19*). The results of Pearson and Rengel (*20*) in wheat showed clearly that Zn reserves in vegetative parts are quickly depleted during the grain development period, indicating that grains are an important sink for Zn. Zinc has relatively high phloem mobility, and foliarly applied Zn is, therefore, easily translocated into grain (*14*, *17*). Based on these results it can be suggested that timing of foliar Zn application could be an important issue in enrichment of wheat grain with Zn. Ozturk et al. (*21*) suggested that foliar application of Zn at an early stage of seed development could be a useful practice to produce a significant increase in grain Zn concentration in wheat because the highest Zn accumulation in grain took place around early milk stage. Despite the existence of many studies related to foliar application of Zn fertilizers, there is, however, no published report about the effect of the timing of foliar Zn application on grain Zn concentration. The importance of the late foliar application of Zn fertilizers has been suggested as an effective way to optimize grain Zn concentration (*7*).

Little is also known about how Zn is distributed within grain of plants treated with foliar Zn fertilizers at various vegetative and reproductive growth stages. In most countries, wheat is eaten after milling. The milling process removes the bran and embryo parts of seeds that are highly rich in Zn, whereas the endosperm (e.g., the part consumed in human diet) is low in Zn (*21*, *22*). Therefore, biofortification of the endosperm with Zn is an important topic in terms of human nutrition. Currently, most molecular genetic approaches aimed at improving Zn and Fe concentrations in seeds focus on endosperm expression of proteins with high Fe- and/or Zn-binding capacity, like ferritin (*9*, *23*, *24*). Lack of information about the role of foliar Zn fertilization in Zn enrichment of endosperm makes this issue an interesting research topic. According to Pearson et al (*25*), transport of Zn into the endosperm increased as the grain matured, leading to a suggestion that late-season foliar application of Zn may maximize Zn accumulation in the endosperm. The crease phloem appears to be important in releasing Zn into endosperm. Destroying the crease phloem in wheat grain reduced substantially Zn transport into the endosperm (*25*).

Table 1. Concentration of DTPA-Extractable Zn, Fe, Mn and Cu, Mineral N (Nmin), Organic Matter (OM), pH and Texture of Soils in the Experimental Sites in Samsun, Konya and Adana

	concn (mg kg^{-1})							
location	Zn	Fe	Mn	Cu	Nmin (kg ha^{-1})	OM (%)	pH	texture
Samsun	1.59	18.6	3.48	6.96	57	2.7	7.75	clay
Konya	0.18	8.3	5.13	1.97	110	1.5	8.31	clay
Adana	0.49	5.8	6.64	1.24	22	1.0	7.51	clay

In the present study, field experiments were conducted in 3 locations with wheat to study the role of timing of the foliar Zn application on Zn concentrations of the whole grain and grain fractions (e.g., bran, embryo and endosperm) at different soil Zn and N applications. The locations selected were different in levels of plant-available Zn concentration in soil. Laser ablation (LA)-ICP-MS analytical technique has been used to monitor the localization of Zn in the grains of the plants treated by foliar Zn application at various growth stages. Currently, LA-ICP-MS technology is being increasingly used in localization of metals such as Zn and Fe in plant samples (*24*, *26*−*28*).

MATERIALS AND METHODS

Field Experiments. Field experiments were conducted at 3 locations as follows: Bahri Dagdas International Agricultural Research Institute in Konya (32° 33′ E, 37° 51′ N, 1009 m above sea level), Cukurova University Research Farm in Adana (35°19′ E, 37°02′ N; 59 m above sea level) and Black Sea Agricultural Research Institute in Samsun (36° 20′ E, 41°17′ N, 4 m above sea level). The experiments in Konya were carried out in 2007 and 2008 using durum wheat (*Triticum durum*, cv. Selcuklu-97), while the experiments in Samsun and Adana were conducted only in 2007 using bread wheat (*Triticum aestivum*). The bread wheat cultivars used were Sagittario in Adana and Özcan in Samsun. The experiments in the Konya location were not conducted on the same field so that there was not any concern with the residual Zn fertilizer effect. The total precipitation was 242 mm in 2007 and 291 mm in Konya in 2008, 582 mm in Samsun in 2007 and 354 mm in Adana in 2007.

Soil properties at the experimental sites were different, especially regarding the plant-available (DTPA-extractable) micronutrient concentrations that were measured as described by Lindsay and Norvell (*29*). The soils at the Konya location are known to be severely deficient in Zn (*30*) and had lower levels of DTPA-extractable Zn than the Samsun and Adana locations (**Table 1**). The soils of all three locations had adequate Fe, Mn and Cu concentrations. Mineral N concentrations measured according to Fabig et al. (*31*) were also different in all locations, being much higher at the Konya location than the Samsun and Adana locations. The Adana location had the lowest organic matter content. All soils were clay soils with high pH values (**Table 1**).

The experimental design consisted of split-plots in a randomized complete block design with five replications in each of 3 locations. Plot size was 6 m^2 (1.2 × 5 m^2). In Konya (e.g., Zn-deficient location), plants were grown with (50 kg of $ZnSO_4 \cdot 7H_2O$/ha) and without soil Zn application over 2 years. An aqueous solution of $ZnSO_4$ was sprayed on the soil surface just before the planting and then incorporated into soil (approximately 15−20 cm) by a disk-plowing. The basal fertilizer treatments in Konya were 70 kg of P_2O_5/ha applied as triple-superphosphate and 80 kg N/ha applied as ammonium nitrate. In the Samsun and Adana locations, plants were grown with low (80 kg N/ha) and high (240 kg N/ha) soil N applications which were applied as ammonium nitrate. The rate of the P application was 80 kg of P_2O_5 ha^{-1} in each Adana and Samsun locations applied during the planting as triple superphosphate. Due to

existence of an adequate amount of plant-available Zn in soil, Zn was not applied into soil in the Samsun and Adana locations.

In Konya, foliar Zn applications were applied twice at different growth stages as follows: (i) stem elongation + booting, (ii) booting + early milk and (iii) early milk + early dough, whereas in the Samsun and Adana locations five different foliar Zn spraying times were applied as follows: (i) stem elongation + booting, (ii) booting + early milk, (iii) early milk + early dough, (iv) booting + anthesis + early milk, and (v) stem elongation + booting + early milk + early dough. The control plots (no foliar Zn application) were treated with a corresponding amount of water. At each foliar Zn application, approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} was applied by spraying 0.5% (w/v) $ZnSO_4 \cdot 7H_2O$ solution. Foliar spray of Zn was performed either on cloudy days or in the very late afternoon to avoid possible leaf damage caused by salts on sunny day and at high day temperature. Under given conditions, there was no visible leaf damage associated with foliar applications of 0.5% (w/v) $ZnSO_4 \cdot 7H_2O$.

Measurement of N, Zn and Fe in Whole Grain and Grain Fractions. At full maturity, grains were harvested to determine grain yield and the grain concentrations of N, Zn and Fe. Iron and Zn were measured by an inductively coupled plasma optical emission spectrometer (ICP-OES; Vista-Pro Axial; Varian Pty Ltd., Mulgrave, Australia) after digesting the grain samples in a closed microwave digestion system (MarsExpress CEM Corp., Matthews, NC) in the presence of concentrated HNO_3 and H_2O_2. The grain N concentration was measured by a Leco TruSpec CN analyzer (Leco Corp., St. Joseph, MI) using a 0.2 g ground grain sample. Grain samples were also analyzed for aluminum (Al) concentrations to estimate possible contamination of grain samples by soil, and the results showed that Al concentrations were low in grain (< 3 mg kg^{-1}) indicating no contamination of grain samples with soil. Analytical data were checked against certified values of a standard reference material (SRM 8436 Durum Wheat Flour, National Institute of Standards and Technology, Gaithersburg, MD).

By using the same methods as described above, the grain fractions (e.g., embryo, bran and endosperm) were also analyzed for Zn, Fe and N. Fractionation of grain parts was performed by using the method of Hidalgo and Brandolini (*32*) with some modifications. Initially, whole embryos (i.e., the germ, including the scutellum) of about 100 seeds were severed using a surgical blade. To separate the endosperm, de-embryonated seeds were milled with a vibrating agate cup mill (Pulverisette 9, Fritsch GmbH, Idar-Oberstein, Germany) for 20 s for bread wheat grains and 50 s for durum wheat grains at 700 rpm, and the resulting flour was sieved with a 100 μm mesh plastic sieve. The sieved particles were treated as the endosperm, whereas the fraction remaining on the sieve (bran and shorts) was sieved again with a 1000 μm mesh plastic sieve in order to separate the bran from the shorts.

Zinc Staining of Grains. Zinc staining of grains was conducted using dithizone reagent as described by Ozturk et al. (*21*), by incubating seeds with 500 mg L^{-1} dithizone (1,5-diphenyl thiocarbazone) at room temperature for 30 min. The stained seeds were then rinsed with water and analyzed qualitatively by using a reflectance light microscope (Nikon SMZ1500, Melville, NY) with a high-resolution digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Localization of Zn in Grains Using LA-ICP-MS. In preparation of grain samples for LA-ICP-MS analyses, wheat grains were first soaked in deionized water overnight. Cross sections of the softened grains were obtained by using a microtome (MT.5530, Euromex Microscopen B.V., Arnhem, Netherlands). The cross sections were fixed on glass slides using double face photostrip (Tesa AG, Hamburg, Germany) and subsequently air-dried for at least 24 h before analysis.

The matrix-matched calibration standards were prepared for the calibration of the grain Zn concentration. Wheat flour (10 g) was spiked with 10 mL of Zn standard solution (ranging from 0.05 to 1000 Zn mg L^{-1}) prepared from a 1000 mg L^{-1} ICP SPEX standard (Spex Industries Inc., Edison, NJ) and dried at 60 °C for 48 h. The Zn-spiked flour was homogenized using a vibrating cup mill (Puverisette, Fritsch GmbH, Idar-Oberstein, Germany) for 10 min. The flour was then pressed to pellets of 5 mm diameter. The Zn concentration of the flour pellets was determined by digesting in concentrated nitric acid and measured by an ICP-EOS (Spectroflame EOS, Spectro Analytical Instruments GmbH, Kleve, Germany) and ICP-MS (7500cx, Agilent Technology, (Santa Clara, California, USA). A quadrupole ICP-MS coupled to a laser ablation system UP193SS (New Wave Research, Fremont, CA) was used for determination of the Zn concentration and its distribution in wheat grains. Samples and matrix-matched calibration standards were arranged in a laser ablation chamber (Supercell, New Wave Research, Fremont, CA) and ablated under identical experimental conditions, in order to allow the calculation of Zn concentrations. The ablated material was transported by argon as a carrier gas to the plasma. The ICP-MS instrument tuning was optimized with respect to the maximum ion intensity of low masses. The carrier gas flow rate was adjusted to optimum output. ^{13}C was used as an internal standard to normalize for different quantities of seed tissue ablated. The parameters for the LA-ICP-MS operation are summarized below:

ICP-MS:	Agilent 7500 cx, Quadropol
Carrier gas flow:	Ar 0.25 L min^{-1}
Make up gas flow:	Ar 1.25 L min^{-1}
Reaction mode:	off
RF power:	1300 W
Laser:	UP193SS, 193 nm
Output energy:	2 J/cm^2
Repetition rate:	10 Hz
Crater size:	25 μm
Scan speed:	10 μm s^{-1}
Measured elements:	^{64}Zn, ^{13}C

For calculation of grain Zn concentration, the Zn/C ratio (^{13}C as internal standard) of the calibration standards and the grains was used. Four cross sections from four grains of each treatment were analyzed. The images shown in this paper were representative of four independent measurements for each treatment.

Statistical Analysis. JMP statistical software (SAS Institute, USA) was used in analysis of the experimental data. Comparison of means was performed by Student's *t* test. Data was analyzed by ANOVA using the general linear model (GLM). JMP Statistical software (SAS Institute) was used in performing the statistical analyses. Student's *t* test was used to compare means whenever ANOVA indicated significant differences among treatments.

Table 2. Grain Yield of Bread Wheat Grown under Field Conditions with Low (N 80: 80 kg N ha^{-1}) and High (N 240: 240 kg N ha^{-1}) N Applications and Treated by Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] at the Adana and Samsun locations in 2007[b]

	grain yield (tons ha^{-1})			
	Adana		Samsun	
foliar Zn application stages	N 80	N 240	N 80	N 240
control	3.33	3.44	6.50	6.48
stem + booting	3.63	3.66	5.61	6.00
booting + milk	3.83	4.03	5.51	6.42
milk + dough	3.61	3.74	6.95	6.42
booting + anthesis + milk	3.95	3.65	6.01	5.56
stem + booting + milk + dough	4.01	3.66	5.20	5.89
mean	3.70	3.72	5.96	6.17
$LSD_{0.05}$ for Zn applications	ns[c]	ns	ns	ns
$LSD_{0.05}$ for N rates	ns		ns	

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The bread wheat cultivars used were Sagittario and Ozcan at Adana and Samsun locations, respectively. The values shown are means of 5 independent replications. [c] Not significant.

RESULTS

As presented in **Table 2**, neither foliar Zn applications nor an increase in N rate from 80 to 240 kg N ha^{-1} affected grain yields at the Adana and Samsun locations. At the Konya location, with very low available soil Zn, applying Zn to the soil increased the grain yield by 23% and 21% in the 2007 and 2008 trials, respectively (**Table 3**). In the case of foliar Zn

application, wheat grain yield was not statistically affected in either of the two years.

An increase in the N application rate significantly increased grain N concentration at Adana and Samsun, while foliar Zn applications did not affect grain N (**Table 4**). All combinations of foliar Zn treatments significantly increased grain Zn concentrations at both locations, when compared to the control plots where plants were not treated with foliar Zn. The increase in grain Zn by foliar Zn applications was more pronounced at Samsun (up to 2.4-fold increase) where grain Zn values were lower in the control plots than at Adana. At both locations, the highest grain Zn concentrations were obtained when Zn was applied 4 times. The differences among the foliar Zn applications were relatively small compared to the difference between any foliar Zn application and the control treatment. However, there was a clear trend for later applications (especially for the booting + milk stages) to result in higher grain Zn concentrations than the earlier applications, particularly at Samsun. Increasing the N rate from 80 to 240 kg N ha^{-1} enhanced grain Zn concentrations at Samsun, but the magnitude of increase was not as high as in foliar Zn treatments. A similar trend was also observed in Adana, although not statistically significant, possibly resulting from relatively large field variations. Nitrogen application also increased grain Fe concentrations, although the effect was not statistically significant at Adana. Foliar Zn applications did not influence grain Fe concentrations at Adana, but resulted in a significant increase at Samsun (**Table 4**).

At the Konya location, soil and foliar Zn applications did not influence grain N concentrations in either year (**Table 5**). Soil Zn application increased grain Zn concentration nearly 2-fold in 2007 and 2008. Relative increases in grain Zn concentrations following foliar Zn application were much greater when soils were not treated with Zn. Compared with soil Zn application, enhancement in grain Zn concentrations was greater in foliar Zn treatments. A combined application of Zn to soil and foliage at the booting and milk stages increased grain Zn concentration almost 2.5-fold in 2007 and 2.6-fold in 2008. Iron concentrations were not affected by the foliar Zn applications, but were decreased by soil Zn application.

Among the grain fractions, the endosperm had the lowest Zn concentration (**Table 6**). In the case of the control plants (without foliar Zn application), the embryo had either greater (in Adana) or similar (in Samsun) Zn concentrations compared to the bran fraction. Foliar Zn treatments significantly increased Zn concentrations in all three grain fractions. Although the endosperm had the lowest Zn concentrations, generally the greatest relative increases in Zn concentrations were obtained in this fraction. Regarding the timing of foliar Zn applications, earlier application (at stem and booting stages) had less effect on grain Zn than the later applications. A combination of booting and milk stage applications resulted in the highest Zn concentration in all grain fractions at both locations. Foliar Zn treatment at booting + milk stages increased Zn concentrations in the endosperm from 11.5 to 18.5 mg kg^{-1} in Adana and from 6 to 13.5 mg kg^{-1} in Samsun as an average of the two N rates. Embryos were the least affected, and the bran fractions were intermediate in terms of increase in Zn concentration as a result of foliar Zn application. Increases in grain Zn concentrations by the foliar Zn application has been also demonstrated by using the staining method for the grains from the Samsun location (**Figure 1**). Zinc was particularly concentrated in the aleurone and embryo fractions as shown by the intensity of red color (**Figure 1**).

Increased N rates showed a trend to increase grain Zn, although the effect was not as consistent as that of foliar Zn treatment (**Table 6**). A combination of Zn and N treatments caused the greatest increase in endosperm Zn concentration, especially when foliar Zn was applied at booting + milk stages (e.g, from 11 to 20 mg of Zn kg^{-1} at Adana and from 5 to 15 mg

Table 3. Effect of Soil and Foliar-Applied $ZnSO_4 \cdot 7H_2O$ on Grain Yield of the Durum Wheat Cultivar Selcuklu-97 Grown in 2007 and 2008 on a Zn-Deficient Calcareous Soil at the Konya Location[a]

soil Zn (kg of $ZnSO_4$ ha^{-1})	foliar Zn application stages	grain yield (ton ha^{-1}) 2007	2008
0	control (no Zn application)	1.45	2.20
	stem + booting	1.49	2.42
	booting + milk	1.38	2.38
	milk + dough	1.41	2.28
50	control (no Zn application)	1.79	2.81
	stem + booting	1.69	2.88
	booting + milk	1.77	2.68
	milk + dough	1.75	2.88
$LSD_{0.05}$ for soil Zn application		0.14	0.20
$LSD_{0.05}$ for foliar Zn application		ns[b]	ns

[a] Foliar Zn application was performed at different growth stages by applying 0.5% $ZnSO_4 \cdot 7H_2O$ (approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage), while soil Zn application was prior to seeding. [b] Not significant.

Table 4. Nitrogen, Zn and Fe Concentrations in Whole Grain of Bread Wheat Grown under Field Conditions with Low (N 80: 80 kg N ha^{-1}) and high (N 240: 240 kg N ha^{-1}) N Applications and Treated by Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] at the Adana and Samsun Locations in 2007[b]

	concn											
	grain N (%)				grain Zn (mg kg^{-1})				grain Fe (mg kg^{-1})			
	ADANA		SAMSUN		ADANA		SAMSUN		ADANA		SAMSUN	
foliar Zn treatment stages	N 80	N 240	N 80	N 240	N 80	N 240	N 80	N 240	N 80	N 240	N 80	N 240
control	1.97	2.32	1.89	2.27	32	37	23	29	36	41	29	32
stem + booting	2.07	2.29	1.95	2.20	51	58	42	42	39	41	35	36
booting + milk	2.12	2.25	1.83	2.21	56	56	49	55	38	40	34	36
milk + dough	2.07	2.39	1.92	2.31	57	64	44	51	35	41	33	34
booting + anthesis + milk	1.97	2.45	2.02	2.37	58	65	53	60	38	41	36	38
stem + booting + milk + dough	2.07	2.37	1.96	2.40	65	70	56	63	36	43	36	39
mean	2.05	2.34	1.93	2.29	53	58	45	50	37	41	34	37
$LSD_{0.05}$ for foliar Zn applications	ns[c]	ns	ns	ns	7.4	7.4	5.1	5.1	ns	ns	3.2	3.2
$LSD_{0.05}$ for soil N applications	0.12		0.27		ns		2.3		ns		0.9	

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The bread wheat cultivars used were Sagittario and Ozcan at Adana and Samsun locations, respectively. The values shown are means of 5 independent replications. [c] Not significant.

Table 5. Nitrogen, Zn and Fe Concentrations in Whole Grain of Durum Wheat Cultivar Selcuklu-97 Grown in 2007 and 2008 on a Zn-Deficient Calcareous Soil with (50 kg of $ZnSO_4 \cdot 7H_2O$) and without Soil Zn Application and Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] at the Konya location[b]

		grain concn					
		2007			2008		
soil Zn application (kg of $ZnSO_4$ ha^{-1})	foliar Zn application stages	N (%)	Zn (mg kg^{-1})	Fe (mg kg^{-1})	N (%)	Zn (mg kg^{-1})	Fe (mg kg^{-1})
0	control (no Zn application)	2.98	11.7	42	3.20	10.4	58
	stem + booting	3.13	18.8	40	3.18	12.7	54
	booting + milk	3.09	26.9	42	3.20	17.9	58
	milk + dough	3.09	25.4	47	3.24	18.8	59
50	control (no Zn application)	3.06	21.7	35	3.32	21.5	46
	stem + booting	2.92	25.5	38	3.34	26.1	46
	booting + milk	3.04	29.0	38	3.32	27.0	45
	milk + dough	2.96	29.3	38	3.32	25.2	44
$LSD_{0.05}$ for soil Zn application		ns[c]	1.8	2.0	ns	2.7	4.0
$LSD_{0.05}$ for foliar Zn application		ns	2.6	3.0	ns	2.8	ns

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The values shown are means of 5 independent replications. [c] Not significant.

Table 6. Zinc Concentrations of the Bran, Embryo and Endosperm Fractions in the Grain of Bread Wheat Grown under Field Conditions with Low (N 80: 80 kg N ha^{-1}) and High (N 240: 240 kg N ha^{-1}) N Applications and Treated by Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] at the Adana and Samsun locations in 2007[b]

		Zn concn (mg kg^{-1})					
		Adana			Samsun		
N application rate (kg ha^{-1})	foliar Zn treatment stages	bran	embryo	endosperm	bran	embryo	endosperm
80	control (no Zn)	42	70	11	64	68	5
	stem + booting	72	96	15	115	102	11
	booting + milk	88	106	17	143	120	12
	milk + dough	88	98	16	121	105	11
240	control (no Zn)	75	75	12	72	74	7
	stem + booting	105	105	18	121	106	12
	booting + milk	104	112	20	156	131	15
	milk + dough	110	112	20	144	109	14
$LSD_{0.05}$ for foliar Zn applications		12	4	1	8	9	2
$LSD_{0.05}$ for N applications		12	n.s	3	11	ns[c]	ns

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The bread wheat cultivars used were Sagittario and Ozcan at Adana and Samsun locations, respectively. The values shown are means of 5 independent replications. [c] Not significant.

of Zn kg^{-1} at Samsun). There was no consistent effect of the N treatments on Fe concentrations in grain fractions, with exception of increased Fe concentration in the bran fraction at the Adana location (data not shown). Increasing the N rate was effective in improving N concentrations in almost all grain fractions at both locations, mainly in the endosperm fraction (**Table 7**). Foliar applied Zn did not influence grain N concentrations in any grain fraction.

Under severe Zn deficiency at the Konya location, soil and foliar Zn applications significantly enhanced Zn concentrations in all grain fractions (**Table 8**). The embryo fraction had more Zn than the bran and endosperm fractions. Endosperm Zn was less affected by soil Zn application than the other grain fractions. However, foliar application of Zn at later growth stages resulted in greater increases (nearly 1.9-fold) in endosperm Zn concentrations than the soil Zn application (0.38-fold). A combination of soil and foliar Zn applied at late growth stages caused more than 2-fold increase in endosperm Zn concentrations (e.g., from 8 mg kg^{-1} to 17 mg kg^{-1}). Similar increases in Zn concentration after foliar application of Zn at late growth stages were also found in the bran and especially in the embryo fractions (**Table 8**). Soil Zn applications resulted in a decrease in Fe concentrations in the all grain fractions, mainly in the bran and embryo fractions (**Table 8**). Iron concentrations in bran and embryo parts tended to increase with foliar Zn applications only when Zn was not applied to the soil. Iron concentration in the embryo also increased with foliar Zn application in the case of soil Zn application, although not as much as with the nil soil Zn treatment. Neither soil nor foliar Zn applications affected N concentrations in any grain fraction (data not shown).

Localization and distribution of Zn within grains were also studied by using LA-ICP-MS (**Figures 2** and **3**) using grain from plants grown with or without foliar application of Zn at Adana and Konya. In all grain samples tested by LA-ICP-MS, Zn was particularly concentrated in the embryo, aleurone layer and crease tissue, whereas the endosperm fraction contained very low Zn (**Figures 2** and **3**). LA-ICP-MS analysis also showed that Zn concentrations greatly increased in the aleurone layer, embryo part and crease tissue after Zn foliar application (**Figures 2** and **3**). In the grain samples without foliar Zn application from both experimental locations, endosperm Zn was uniformly distributed. Foliar application of Zn markedly increased endosperm Zn concentration of the grains. This increase was very distinct at both experimental locations and more pronounced when Zn was sprayed at later growth stages (**Figure 2**). The application of Zn by foliar sprays (particularly at later stages) enhanced endosperm Zn concentrations primarily near the crease, leading to an increasingly steep gradient from the crease tissue to the external border of the endosperm. The greater role of late-season foliar application of Zn in increasing Zn concentrations of the embryo part compared to the foliar Zn application at earlier growth

stage (**Table 8**) was also demonstrated and confirmed by using the LA-ICP-MS analysis (**Figure 3**).

DISCUSSION

Soil and foliar applications of $ZnSO_4$ were highly effective in increasing grain Zn concentration in wheat, confirming earlier results (*11*, *13*, *14*). At the Konya location with low plant-available Zn, soil Zn application also resulted in significant increases in grain yield. Foliar Zn applications were, however, not as effective as the soil Zn application in improving yields, indicating the importance of adequate available soil Zn during the early growth stages. It is known that Zn is particularly important for ensuring good root growth and improving tolerance to various environmental stress factors during the early growth stages (*33*, *34*). It is, therefore, not surprising that seeds with high Zn concentrations contribute greatly to high seedling vigor, good field establishment and large yield under Zn-deficient soil conditions (*35*−*37*).

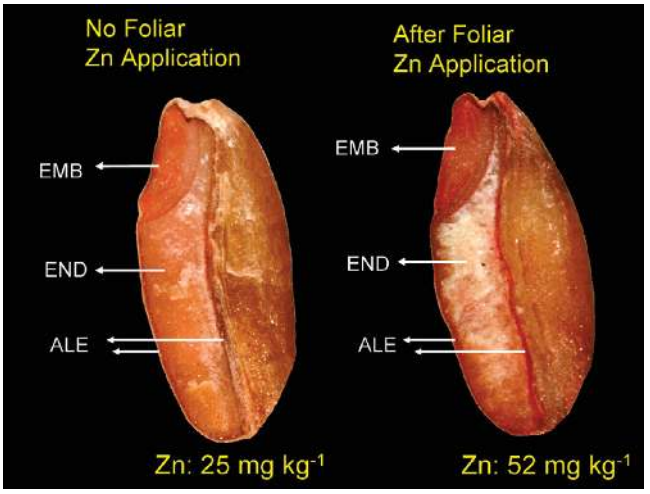

Figure 1. Staining and localization of Zn in a bread wheat grain (cv. Özcan) without (left) and with (right) foliar spray of 0.5% $ZnSO_4 \cdot 7H20$ at booting and milk stages (approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage) at the Samsun location in 2007. Zinc concentrations of the grains were 25 mg kg^{-1} (left) and 52 mg kg^{-1} (right). The longitudinally cut seed surface was stained for Zn by dithizone reagent (500 mg L^{-1} 1,5-diphenyl thiocarbazone dissolved in absolute methanol; incubation at room temperature for 30 min; see Materials and Methods for more detail). Red color formation indicates Zn localization, especially in the embryo and aleuron layer of the grains. (EMB: embryo. ALE: Aleuron. END: Endosperm.)

Foliar Zn applications were more effective at increasing grain Zn concentrations than soil applied Zn. Whereas two foliar applications were effective at achieving high grain Zn concentrations (**Tables 4** and **5**), four foliar applications produced the highest Zn concentrations, but this practice may not be feasible in commercial production. To our knowledge, this is the first study to show that the timing of foliar Zn application is a critical factor in increasing grain Zn concentration. Compared to the early applications (e.g., stem elongation + booting stages), foliar application of Zn later in the growing season (e.g., at booting + milk or milk + dough stages) resulted in greater increases in grain Zn concentration. A combination of late foliar Zn applications with either high soil N or soil Zn application caused further increases in grain Zn (e.g., from 23 to 55 mg kg^{-1} with high N application at Samsun and from 12 to 29 mg kg^{-1} with soil Zn application at the Zn-deficient Konya locations). In order to achieve a measurable biological impact on human health, grain Zn concentrations should be increased by at least 10 mg kg^{-1} in a given region (*7*, *8*). The increases in grain Zn concentrations achieved through foliar Zn applications were around 17 mg kg^{-1} at Konya and more than 30 mg kg^{-1} at the other locations.

High N supply positively affected grain concentration of both Zn and Fe (**Table 4**). Similar results were recently reported by Kutman et al. (*15*) in a greenhouse study with durum wheat. The reasons for the positive impact of N nutrition on Zn and Fe concentrations of plants might be the possible contributions of adequate N nutrition to (i) pool of transporter proteins mediating absorption and transport of Zn and Fe, (ii) release of Zn- and Fe-solubilizing phytosiderophores from roots into the rhizosphere, (iii) facilitation of Zn and Fe translocation from vegetative tissues into grains by nitrogenous compounds such as peptides or nicotianamine (*10*). Understanding the mechanisms contributing to an increase in grain Zn by N fertilization deserves further research.

Increases in the concentration of whole-grain Zn through soil and/or foliar Zn applications were reflected proportionally in all grain fractions analyzed (**Tables 6** and **8**). In the case of the late-season foliar Zn application, increases in Zn concentrations of the grain fractions were more distinct as shown both by the ICP-OES data (**Tables 6** and **8**) and by the LA-ICP-MS data for endosperm and embryo (**Figures 2** and **3**). Here, particular attention should

Table 7. Nitrogen Concentrations of the Bran, Embryo and Endosperm Fractions in the Grain of Bread Wheat Grown under Field Conditions with Low (N 80: 80 kg N ha^{-1}) and High (N 240: 240 kg N ha^{-1}) N Applications and Treated by Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] in the Adana and Samsun locations in 2007[b]

		N concn (%)					
		Adana			Samsun		
N application rate (kg ha^{-1})	foliar Zn treatment	bran	embryo	endosperm	bran	embryo	endosperm
80	control (no Zn)	2.12	2.85	2.15	2.56	2.47	1.57
	stem + booting	2.28	2.80	2.23	2.54	2.54	1.53
	booting + milk	2.30	2.85	2.17	2.50	2.43	1.36
	milk + dough	2.16	2.80	2.09	2.55	2.52	1.46
240	control (no Zn)	2.60	3.11	2.50	2.87	2.68	1.74
	stem + booting	2.50	3.08	2.44	2.77	2.64	1.61
	booting + milk	2.49	3.04	2.46	2.76	2.58	1.71
	milk + dough	2.52	3.02	2.49	2.82	2.64	1.75
$LSD_{0.05}$ for foliar Zn applications		ns[c]	ns	ns	ns	ns	ns
$LSD_{0.05}$ for N applications		0.17	0.15	0.25	0.28	ns	0.17

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The bread wheat cultivars used were Sagittario and Ozcan in Adana and Samsun locations, respectively. The values show the mean of 5 independent replications. [c] Not significant.

Table 8. Zinc and Fe Concentrations of the Bran, Embryo and Endosperm Fractions in the Grain of Durum Wheat Cultivar Selcuklu-97 Grown in 2007 under Field Conditions with (50 kg of $ZnSO_4 \cdot 7H_2O$) and without Soil Zn Application and Foliar Spray of 0.5% $ZnSO_4 \cdot 7H_2O$ at Different Growth Stages[a] in the Konya Location[b]

		concn (mg kg^{-1})					
		Zn			Fe		
soil Zn application (kg of $ZnSO_4$ ha^{-1})	foliar Zn application stages	bran	embryo	endosperm	bran	embryo	endosperm
0	control (no Zn)	20	38	8	58	89	23
	stem + booting	28	47	10	57	89	24
	booting + milk	35	62	15	52	90	27
	milk + dough	41	63	15	79	100	27
50	control (no Zn)	33	52	11	54	85	20
	stem + booting	34	58	13	49	85	23
	booting + milk	44	68	17	52	87	22
	milk + dough	45	69	16	52	90	20
$LSD_{0.05}$ for soil Zn application		3.0	3.4	1.0	5.3	3.8	1.9
$LSD_{0.05}$ for foliar Zn application		4.8	4.2	4.8	5.4	4.2	5.4

[a] Approximately 4 kg of $ZnSO_4 \cdot 7H_2O$ ha^{-1} applied at each growth stage. [b] The values show the mean of 5 independent replications.

be given to the changes in Zn concentration in the endosperm fraction, as this is the predominant fraction consumed in many countries. In addition to the ICP-OES data on Zn concentrations in grain fractions, the LA-ICP-MS data (**Figure 2**) also showed that the application of foliar Zn late in the growing season produced a large increase in the concentration of endosperm and whole-grain Zn. Similarly, the application of foliar N fertilizers after flowering enhanced grain protein concentration more than the N application at earlier growth stages, such as booting (*38*, *39*). During the early stage of seed development, both protein synthesis (*40*, *41*) and Zn accumulation (*21*) are maximized in wheat grain, suggesting that an increase in protein synthesis possibly creates a sink for Zn. Zinc is known to be primarily required for biosynthesis of proteins (*17*, *42*) and affect protein composition of wheat grain (*13*). Up to 10% of proteins in biological systems require Zn for their function and structural integrity (*43*). Accordingly, Zn concentrations are extremely high (e.g., 600 mg kg^{-1}) in the protein bodies of wheat grain (*44*) and correlate positively with the protein concentrations in different wheat genotypes (*45*−*47*). These observations suggest that foliar application of Zn during early stage of seed development may maximize accumulation of Zn in grain due to a possible high sink activity for Zn at this stage of seed development.

The distinct increases in grain Zn concentrations following foliar application (especially at the late growth stage) indicate that Zn is easily translocated via the phloem into grain. Similar conclusions were also reported earlier by Haslett et al. (*48*) and Erenoglu et al. (*49*) in the experiments conducted with wheat under controlled environmental conditions. Based on these results it can be speculated that a major part of the Zn loaded into grains during grain development under field conditions, as described in this study, was most probably due to retranslocation from the vegetative tissues via the phloem. Contrary to this suggestion, some reports for rice and *Arabidopsis* plants show that continued root uptake and translocation into seeds during the seed-filling period appears to be the major way for accumulation of Zn into seed (*50*, *51*). It seems likely that in the case of high availability of Zn in the growth medium, such as in growth chamber or greenhouse pot experiments, continuous root uptake and translocation into grain during the grain-filling period contributes significantly to grain accumulation of Zn (*15*, *50*−*52*). However, when the availability of Zn in the growth medium is limited such as under field conditions in calcareous soils with limited soil moisture, the role of root uptake and translocation into grains during the grain-filling period is possibly minimal. Under field conditions, the remobilization of Zn from the vegetative tissue into developing grain via the phloem may be the major pathway for Zn accumulation in grain. Accordingly, Waters et al. (*52*) showed that withholding supply of Zn in nutrient solution during postanthesis stimulated Zn remobilization from the leaf tissue, while in case of the continuous supply of Zn in the nutrient solution there was no net remobilization from leaf tissue. Based on these results from controlled growth conditions (*15*, *52*) and field trials described in this study, it can be suggested that increasing the pool of Zn in vegetative tissue during the reproductive growth stage (for example through foliar spraying of Zn) is of great importance for maximizing grain Zn accumulation under field conditions with limited soil-Zn supply. In agreement with this suggestion, Pearson and Rengel (*20*) emphasized the importance of the flag leaf and stem as Zn reservoirs in wheat and showed that these plant parts rapidly depleted Zn during the grain filling period. Increasing molecular evidence is also available showing that remobilization of Zn from senescing leaf tissue through the action of the NAM-B1 gene plays a critical role in accumulation of Zn in wheat grains (*18*, *19*, *52*).

Although it was not examined in this study, foliarly applied Zn in the late growth stages might be important for alleviation of subsoil Zn deficiency problems. Previous studies with radiolabeled Zn showed that an important part of foliarly absorbed Zn is translocated to plant roots (*48*, *49*). Under field conditions, transport of Zn into subsoil roots may improve plant growth and yield capacity (*53*). In most cases, concentrations of plant-available Zn in the subsoil is low when compared to the top soil due to the low mobility of Zn in the soil and the impossibility of applying Zn to the subsoil through common fertilizer application methods. In semiarid regions, roots may not be able to absorb sufficient Zn from the topsoil because this part of soil is often dry. Therefore, roots rely on the available Zn in the subsoil (which contains moisture long after the topsoil had dried), especially during the late growth stages. Having adequate amounts of Zn available to subsoil roots is of great importance for the protection of roots from various stress factors such as salinity, B toxicity, and soil-borne diseases, and also for the maintenance of the structural integrity of root cell-membranes (*34*, *54*).

It remains unclear how Zn is transported into the developing grain. The LA-ICP-MS data (**Figure 2**) suggest that Zn is transported into the endosperm through the crease phloem in a similar way to sucrose. It is widely believed that sucrose is transported into the endosperm cavity and then into the endosperm via the crease phloem (*55*). As demonstrated by the LA-ICP-MS measurements, there was a gradient in the endosperm

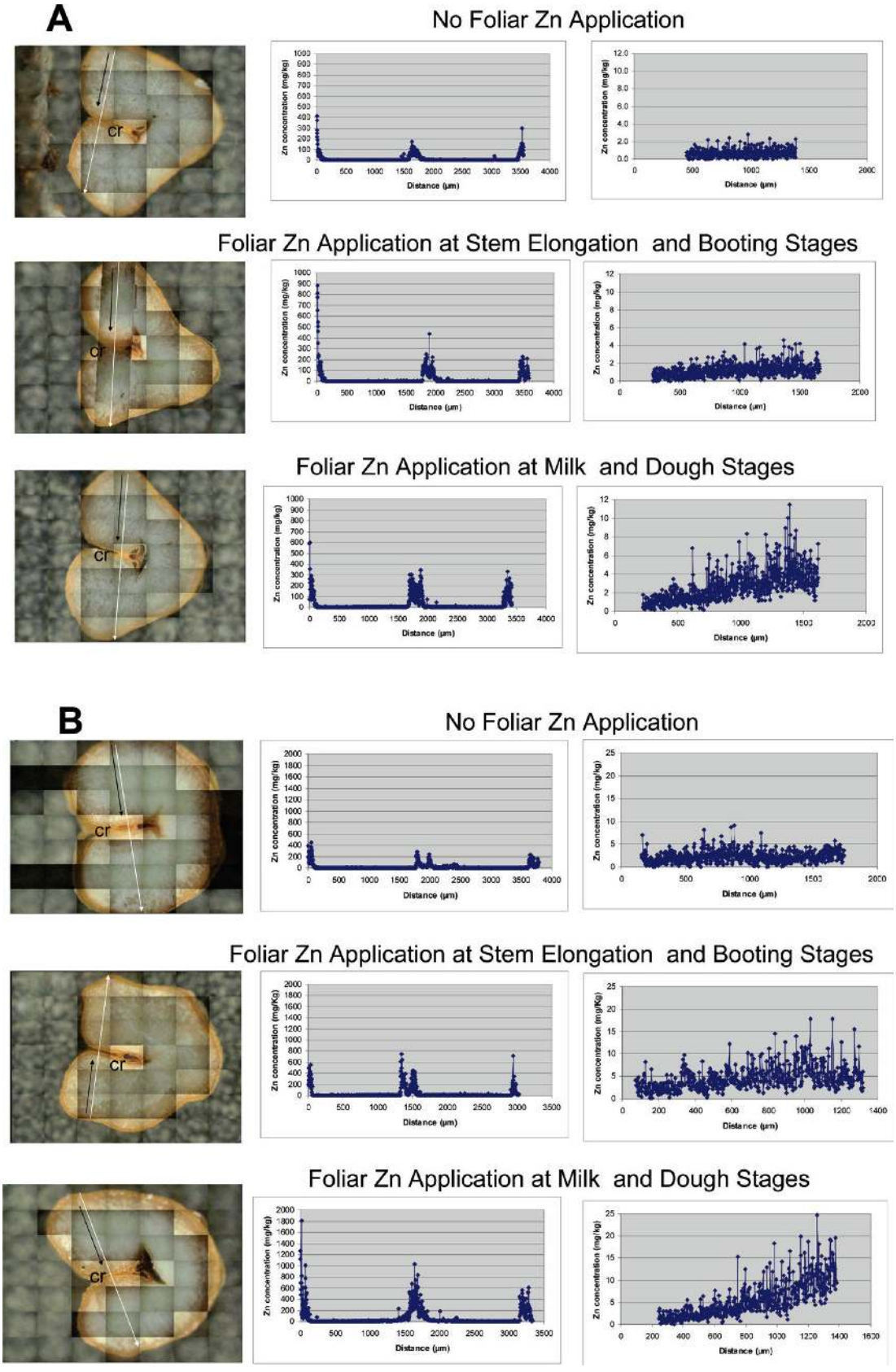

Figure 2. Localization of Zn in cross sections of wheat grains harvested at the Zn-deficient Konya location without soil Zn application (**A**) and Zn-adequate Adana (**B**) locations in 2007. Grains subjected to LA-ICP-MS analysis were from plants which were either not treated (no foliar Zn application) or treated with foliar spray of $ZnSO_4 \cdot 7H_2O$ at the stem elongation and booting or at the milk and dough stages. The laser ablation scanning of the seed cross section started at the seed coat and moved through the cross section passing through the crease (cr) in the direction as shown by the white arrow in the pictures on the left. The diagrams in the middle show the Zn concentrations along the entire cross section (white arrow). The diagrams on the right show the Zn concentrations in the endosperm between the aleurone layers of the seed surface and the crease (black arrow). Four cross sections from four grains of each treatment were analyzed. Thus, the images shown are representative of four independent measurements of each treatment. See Materials and Methods for more details.

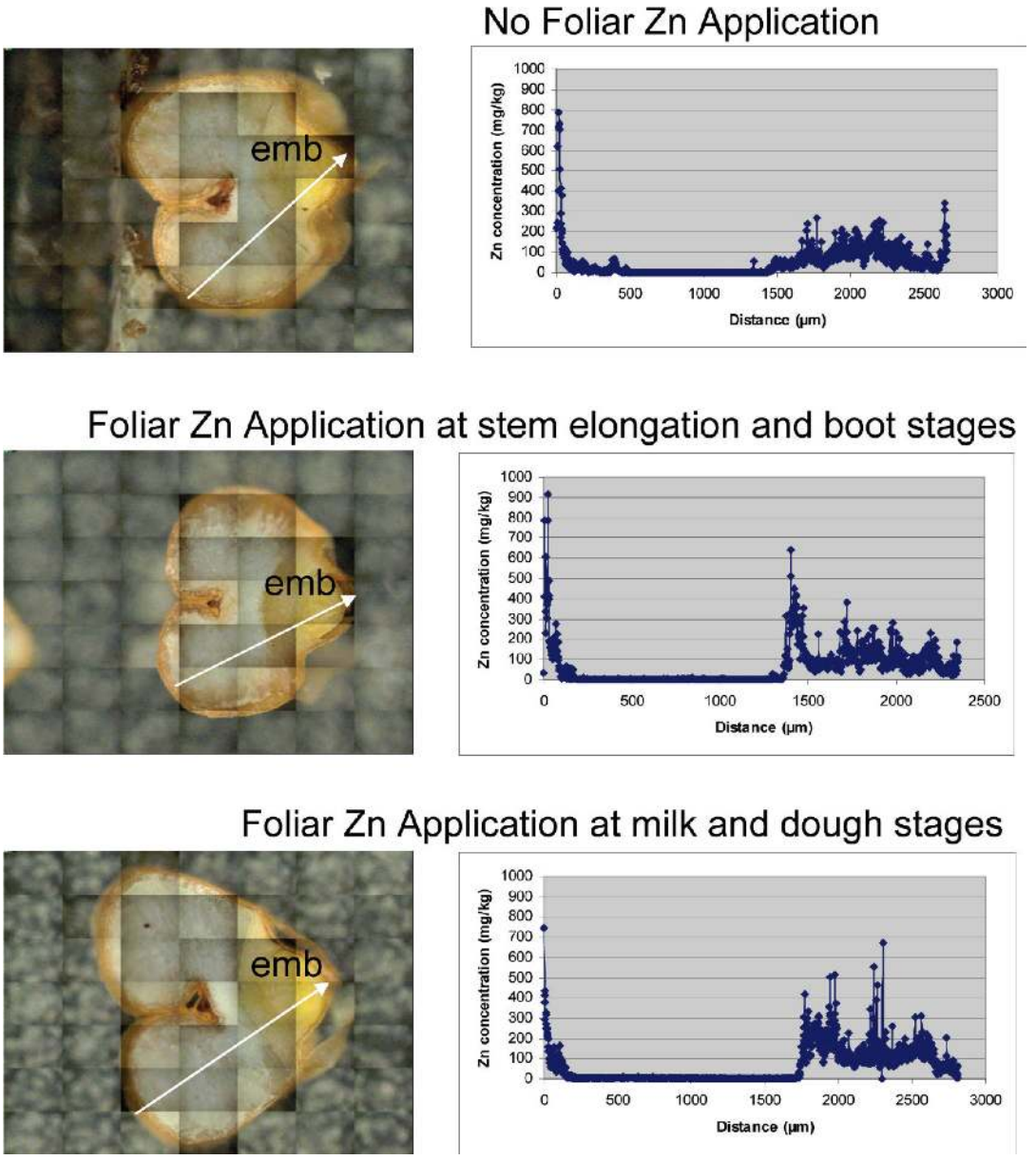

Figure 3. Localization of Zn in cross sections including the embryo (emb) of wheat grains harvested at the Zn-deficient Konya location in 2007. Grains subjected to LA-ICP-MS analysis were from plants which were either not treated (no foliar Zn application) or treated with foliar spray of $ZnSO_4 \cdot 7H_2O$ at the stem elongation and booting or at the milk and dough stages. The laser ablation scanning of the seed cross section started at the seed coat and moved through the cross section passing through the endosperm and the embryo, as shown by the white arrow in the pictures on the left. The diagrams on the right show the Zn concentrations along the entire cross section (white arrow). Four cross sections from four grains of each treatment were analyzed. Thus, the images shown are representative of four independent measurements of each treatment. See Materials and Methods for more details.

Zn concentration from the crease tissue to the external border of the endosperm. This gradient became steeper with the late application of Zn, indicating an important role of phloem and crease tissues in the delivery of Zn into the endosperm. A similar conclusion was drawn by Pearson et al. (*25*) in an experiment with wheat by using radiolabeled Zn (^{65}Zn) under controlled environmental conditions. Their data indicated that most of the ^{65}Zn was transported into the endosperm via the crease phloem.

In conclusion, application of Zn to soils and/or foliage represents a very useful and rapid approach in enrichment with Zn of wheat grains (particularly the endosperm and thus white wheat flour). In Zn-deficient location, grain Zn concentration increased from 11 mg kg^{-1} to 22 mg kg^{-1} with foliar Zn application and to 27 mg kg^{-1} with combined application of $ZnSO_4$ to soil and foliar. In locations without soil Zn deficiency, combination of high N application with two foliar Zn applications (e.g., at the booting and milk stages) increased grain Zn concentration, on average, from 28 mg kg^{-1} to 58 mg kg^{-1}. Timing of the foliar Zn application was found to be an important factor in increasing grain Zn concentration. An increase in grain Zn concentration by application of foliar Zn fertilizers was more pronounced when Zn was applied in the late compared with the early growth stages. This suggests that providing a large pool of Zn in the vegetative tissue during the reproductive growth stages (e.g., by foliar spraying of Zn fertilizers) is an important field practice for maximizing grain Zn accumulation. There is, now, an urgent need for research on bioavailability of grain Zn derived from late foliar Zn applications. High bioavailability of grain Zn is important for human nutrition, and this would make the fertilization strategy

an important solution to minimizing Zn-deficiency-related health problems in human populations. It is also important to mention that the ongoing breeding activities for developing high Zn genotypes should be integrated with Zn fertilization strategies in order to ensure sufficiently high Zn accumulation in grain, at least in countries where application of mineral fertilizers is feasible.

LITERATURE CITED

(1) Hotz, C.; Brown, K. H. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr. Bull.* **2004**, *25*, 94–204.

(2) Stein, A. J.; Netsel, P.; Meenakshi, J. V.; Qaim, M.; Sachdev, H. P. S.; Bhutta, Z. A. Plant breeding to control zinc deficiency in India: how cost-effective is biofortification? *Public Health Nutr.* **2007**, *10*, 492–501.

(3) Welch, R. M.; Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **2004**, *55*, 353–364.

(4) Black, R. E.; Lindsay, H. A.; Bhutta, Z. A.; Caulfield, L. E.; De Onnis, M.; Ezzati, M.; Mathers, C.; Rivera, J. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* **2008**, *371*, 243–260.

(5) Gibson, R. S. The role of diet- and host-related factors in nutrient bioavailability and thus in nutrient-based dietary requirement estimates. *Food Nutr. Bull.* **2007**, *28*, 77–100.

(6) White, P. J.; Broadley, M. R. Biofortification of crops with seven mineral elements often lacking in human diets - iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytol.* **2009**, *182*, 49–84.

(7) Cakmak, I. Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant Soil* **2008**, *302*, 1–17.

(8) Pfeiffer, W. H.; McClafferty, B. HarvestPlus: Breeding crops for better nutrition. *Crop Sci.* **2007**, *47*, 88–105.

(9) Brinch-Pedersen, H.; Borg, S.; Tauris, B.; Holm, P. B. Molecular genetic approaches to increasing mineral availability and vitamin content of cereals. *J. Cereal Sci.* **2007**, *46*, 308–326.

(10) Cakmak., I.; Pfeiffer, W. H.; McClafferty, B. Biofortification of durum wheat with zinc and iron. *Cereal Chem.* **2010**, *87*, 10–20.

(11) Yilmaz, A.; Ekiz, H.; Torun, B.; Gültekin, I.; Karanlik, S.; Bagci, S. A.; Cakmak, I. Effect of different zinc application methods on grain yield and zinc concentration in wheat grown on zinc-deficient calcareous soils in Central Anatolia. *J. Plant Nutr.* **1997**, *20*, 461–471.

(12) Graham, R. D.; Ascher, J. S.; Hynes, S. C. Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant Soil* **1992**, *146*, 241–250.

(13) Peck, A. W.; McDonald, G. K.; Graham, R. D. Zinc nutrition influences the protein composition of flour in bread wheat (*Triticum aestivum* L.). *J. Cereal Sci.* **2008**, *47*, 266–274.

(14) Rengel, Z.; Batten, G. D.; Crowley, D. E. Agronomic approaches for improving the micronutrient density in edible portions of field crops. *Field Crops Res.* **1999**, *60*, 27–40.

(15) Kutman, U. B.; Yildiz, B.; Ozturk, L.; Cakmak, I. Biofortification of durum wheat with zinc through soil and foliar applications of nitrogen. *Cereal Chem.* **2010**, *87*, 1–9.

(16) Grusak, M. A.; Pearson, J. N.; Marentes, E. The physiology of micronutrient homeostasis in field crops. *Field Crops Res.* **1999**, *60*, 41–56.

(17) Marschner, H. *Mineral Nutrition of Higher Plants*, 2nd ed.; Academic Press: London, U.K., 1995; pp 25−43.

(18) Uauy, C.; Distelfeld, A.; Fahima, T.; Blechl, A.; Dubcovsky, J. A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. *Science* **2006**, *314*, 1298–1301.

(19) Distelfeld, A.; Cakmak, I.; Peleg, Z.; Ozturk, L.; Yazici, A. M.; Budak, H.; Saranga, Y.; Fahima, T. Multiple QTL-effects of wheat Gpc-B1 locus on grain protein and Micronutrient concentrations. *Physiol. Plant.* **2007**, *129*, 635–643.

(20) Pearson, J. N.; Rengel, Z. Distribution and remobilization of Zn and Mn during grain development in wheat. *J. Exp. Bot.* **1994**, *45*, 1829–1835.

(21) Ozturk, L.; Yazici, M. A.; Yucel, C.; Torun, A.; Cekic, C.; Bagci, A.; Ozkan, H.; Braun, H. J.; Sayers, Z.; Cakmak, I. Concentration and localization of zinc during seed development and germination in wheat. *Physiol. Plant.* **2006**, *128*, 144–152.

(22) Persson, D. P.; Hansen, T. H.; Laursen, K. H.; Schjoerring, J. K.; Husted, S. Simultaneous iron, sulfur and phosphorus speciation analysis of barley grain tissues using SEC-ICP-MS and IPICP-MS. *Metallomics* **2009**, *1*, 418–426.

(23) Goto, F.; Yoshihara, T.; Shigemoto, N.; Toki, S.; Takaiwa, F. Iron fortification of rice seed by the soybean ferritin gene. *Nat. Biotechnol.* **1999**, *17*, 282–286.

(24) Wirth, J.; Poletti, S.; Aeschlimann, B.; Yakandawala, N.; Drosse, B.; Osorio, S.; Tohge, T.; Fernie, A. R.; Gunther, D.; Gruissem, W.; Sautter, C. Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotech. J.* **2009**, *7*, 631–644.

(25) Pearson, J. N.; Rengel, Z.; Jenner, C. F.; Graham, R. D. Dynamics of zinc and manganese movement in developing wheat grains. *Aust. J. Plant Physiol.* **1998**, *25*, 139–144.

(26) Becker, J. S.; Dietrich, R. C.; Matusch, A.; Pozebon, D.; Dressier, V. L. Quantitative images of metals in plant tissues measured by laser ablation inductively coupled plasma mass spectrometry. *Spectrochim. Acta, Part B* **2008**, *63*, 1248–1252.

(27) Tauris, B.; Borg, S.; Gregersen, P. L.; Holm, P. H. A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *J. Exp. Bot.* **2009**, *60*, 1333–1347.

(28) Shi, J.; Gras, M. A.; Silk, W. K. Laser ablation ICP-MS reveals patterns of copper differing from zinc in growth zones of cucumber roots. *Planta* **2009**, *229*, 945–954.

(29) Lindsay, W. L.; Norvell, W. A. Development of a DTPA soil test for zinc, iron, manganese and copper. *Soil Sci. Soc. Am. J.* **1978**, *42*, 421–428.

(30) Cakmak, I.; Yilmaz, A.; Ekiz, H.; Torun, B.; Erenoglu, B.; Braun, H. J. Zinc deficiency as a critical nutritional problem in wheat production in Central Anatolia. *Plant Soil* **1996**, *180*, 165–172.

(31) Fabig, W.; Ottow, J. C. G.; Mueller, F. Mineralization von 14Cmarkiertem Benzoat mit Nitrat als Wasserstoff-akseptor unter vollstaendig anaeroben Bedingungen sowie bei vermindertem Sauerstoffpartialdruck. *Landwirtsch. Forsch.* **1978**, *35*, 441–453.

(32) Hidalgo, A.; Brandolini, A. Protein, ash, lutein and tocols distribution in einkorn (*Triticum monococcum* L. subsp. *monococcum*) seed fractions. *Food Chem.* **2008**, *107*, 444–448.

(33) Welch, R. M. Importance of seed mineral nutrient reserves in crop growth and development. In *Mineral Nutrition of Crops: Fundamental Mechanisms and Implications*; Rengel, Z., Ed.; Food Products Press: New York, 1999; pp 205−226.

(34) Cakmak, I. Role of zinc in protecting plant cells from reactive oxygen species. *New Phytol.* **2000**, *146*, 185–205.

(35) Rengel, Z.; Graham, R. D. Importance of seed zinc content for wheat growth on zincdeficient soil. I. Vegetative Growth. *Plant Soil* **1995**, *173*, 259–266.

(36) Yilmaz, A.; Ekiz, H.; Gültekin, I.; Torun, B.; Barut, H.; Karanlik, S.; Cakmak, I. Effect of seed zinc content on grain yield and zinc concentration of wheat grown in zinc-deficient calcareous soils. *J. Plant Nutr.* **1998**, *21*, 2257–2264.

(37) Haris, D.; Rashid, D.; Miraj, G.; Arif, M.; Shah, H. 'On-farm' seed priming with zinc sulphate solution—A cost-effective way to increase the maize yields of resource-poor farmers. *Field Crops Res.* **2007**, *102*, 119–127.

(38) Finney, K. F.; Meyer, J. W.; Smith, F. W.; Fryer, H. C. Effect of foliar spraying of Pawnee wheat urea solutions on yield, protein content, and protein quality. *Agron. J.* **1957**, *49*, 341–347.

(39) Woolfolk, C. W.; Raun, W. R.; Johnson, G. V.; Thomason, W. E.; Mullen, R. W.; Wynn, K. J.; Freeman, K. W. Influence of late-season foliar nitrogen applications on yield and grain nitrogen in winter wheat. *Agron. J.* **2002**, *94*, 429–434.

(40) Grene, F. C. Expression of storage protein genes in developing wheat (*Triticum aestivum* L.) seeds. Correlation of RNA accumulation and protein synthesis. *Plant Physiol.* **1983**, *71*, 40–46.

(41) Martre, P.; Porter, J. R.; Jamieson, P. D.; Triböi, E. Modeling grain nitrogen accumulation and protein composition to understand the

sink/source regulation of nitrogen remobilization for wheat. *Plant Physiol*. **2003**, *133*, 1959–1967.
(42) Cakmak, I.; Marschner, H.; Bangerth, F. Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3- acetic acid and other phytohormones in bean (*Phaseolus vulgaris* L.). *J. Exp. Bot*. **1989**, *40*, 405–412.
(43) Andreini, C.; Banci, L.; Rosato, A. Zinc through the three domains of life. *J. Proteome Res*. **2006**, *5*, 3173–3178.
(44) Mazzolini, A. P.; Pallaghy, C. K.; Legge, G. J F. Quantitative microanalysis of Mn, Zn, and other elements in mature wheat seed. *New Phytol*. **1985**, *100*, 483–509.
(45) Morgounov, A.; Gomez-Becerra, H. F.; Abugalieva, A.; Dzhunusova, M.; Yessimbekova, M.; Muminjanov, H.; Zelenskiy, Y.; Ozturk, L.; Cakmak, I. Iron and zinc grain density in common wheat in Central Asia. *Euphytica* **2007**, *155*, 193–203.
(46) Peleg, Z.; Saranga, Y.; Yazici, A.; Fahima, T.; Ozturk, L.; Cakmak, I. Grain zinc, iron and protein concentrations and zinc-efficiency in wild emmer wheat under contrasting irrigation regimes. *Plant Soil* **2008**, *306*, 57–67.
(47) Zhao, F. J.; Su, Y. H.; Dunham, S. J.; Rakszegi, M.; Bedo, Z.; McGrath, S. P.; Sherwry, P. R. Variation in mineral micronutrient concentrations in grain of wheat lines of diverse origin. *J. Cereal Sci*. **2009**, *49*, 290–295.
(48) Haslet, B. S.; Reid, R. J.; Rengel, Z. Zinc mobility in wheat: Uptake and distribution of zinc applied to leaves or roots. *Ann. Bot*. **2001**, *87*, 379–386.
(49) Erenoglu, B.; Nikolic, M.; Romheld., V.; Cakmak, I. Uptake and transport of foliar applied zinc (^{65}Zn) in bread and durum wheat cultivars differing in zinc efficiency. *Plant Soil* **2002**, *241*, 251–257.
(50) Jiang, W.; Struik, P. C.; Lingna, J.; van Keulen, H.; Ming, Z.; Stomph, T. J. Uptake anddistribution of root-applied or foliar-applied 65Zn after flowering in aerobic rice. *Ann. Appl. Biol*. **2007**, *150*, 383–391.
(51) Waters, B. M.; Grusak, M. A. Whole-plant mineral partitioning throughout the life cycle in *Arabidopsis thaliana* ecotypes Columbia, Landsberg *erecta*, Cape Verde Islands, and the mutant line *ysl1ysl3*. *New Phytol*. **2008**, *177*, 389–405.
(52) Waters, B. M.; Uauy, C.; Dubcovsky, J.; Grusak, M. A. Wheat (*Triticum aestivum*) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain. *J. Exp. Bot*. **2009**, *60*, 4263–4274.
(53) Grewal, H. S.; Lu, Z. G.; Graham, R. D. Influence of subsoil zinc on dry matter production, seed yield and distribution of zinc in oil seed rape genotypes differing in zinc efficiency. *Plant Soil* **1997**, *192*, 181–189.
(54) Welch, R. M.; Webb, M. J.; Loneragan, J. F. Zinc in membrane function and its role in phosphorus toxicity. In *Proceedings of the Ninth Plant Nutrition Colloquium*; Scaife, A., Ed.; CAB International: Warwick, U.K., Wallingford, U.K., 1982; pp 710−715.
(55) Wang, H. L.; Offler, C. E.; Patrick, J. W. The cellular pathway of photosynthate transfer in the developing wheat grain. II. A structural analysis and histochemical studies of the pathway from the crease phloem to the endosperm cavity. *Plant Cell Environ*. **1995**, *18*, 373–388.

Received for review March 29, 2010. Revised manuscript received July 7, 2010. Accepted July 12, 2010. This study has been financially supported by the HarvestPlus Program (www.harvestplus.org) and the sponsors of the HarvestPlus Global Zinc Fertilizer Project (e.g., Mosaic Company, K+S Kali, International Zinc Association, Omex Agrifluids, International Fertilizer Industry Association and International Plant Nutrition Institute). The financial support by the German Research Foundation to W.J.H. (Grant HO 931/23-1) is also highly acknowledged.