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Biofortification of Durum Wheat with Zinc Through Soil and Foliar Applications of Nitrogen

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ABSTRACT

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Increasing zinc (Zn) concentration of cereal grains is a global challenge to alleviate Zn deficiency-related health problems in humans caused by low dietary Zn intake. This study investigated the effects of soil- and foliar-applied nitrogen (N) and Zn fertilizers on grain Zn accumulation of durum wheat (*Triticum durum*) grown on a Zn-deficient soil. In addition, localization of Zn and protein within durum wheat grain was studied by using Bradford reagent for protein and dithizone (diphenyl thiocarbazone) for Zn. Grain Zn concentration was greatly enhanced by soil or foliar applications of Zn. When Zn supply was adequately high, both soil and foliar N applications improved grain Zn concentration. Consequently, there was a significant positive correlation between grain concentrations of Zn and N, when Zn supply was not limiting. Protein and Zn staining studies showed co-localization of Zn and protein within grain, particularly in the embryo and aleurone. Results indicate that N and Zn fertilization have a synergistic effect on grain Zn concentration. Possibly, increasing N supply contributes to grain Zn concentration by affecting the levels of Zn-chelating nitrogenous compounds or the abundance of Zn transporters. Our results suggest that nitrogen management can be an effective agronomic tool to improve grain Zn concentration.

Zinc (Zn) deficiency is a common micronutrient deficiency occurring both in crop plants and human beings. Nearly half of the cereal-growing land in the world is affected by low availability of Zn to plant roots due to a variety of adverse chemical and physical conditions such as high pH level and low levels of organic matter and soil moisture (Alloway 2008; Cakmak 2008). Wheat grain is the most important source of calorie intake in a number of countries in the developing world, but wheat is inherently too low in Zn (generally 20–30 mg of Zn/kg of grain) to meet the recommended dietary allowance for Zn (Erdal et al 2002; Cakmak 2008). When grown on Zn-deficient soils, without supplemental Zn, grain Zn concentrations of wheat are further reduced to <10–15 mg of Zn/kg of grain, as shown on Zn-deficient field conditions in Iran, India, Turkey, and Australia (Graham et al 1992; Cakmak et al 1999; Erdal et al 2002; Alloway 2008).

Health complications associated with Zn deficiency include, among others, stunting in children, high susceptibility to infectious diseases, increased morbidity and mortality, impaired mental development, and poor birth outcomes in pregnant women (Hotz and Brown 2004; Black et al 2008). Recently, it has been reported that Zn and vitamin A deficiencies are globally the most serious nutrient deficiencies among children and represent major causes of death for children under 5 years of age (Black et al 2008). According to Black et al (2008), Zn deficiency is responsible for ≈450,000 deaths among children under 5 years of age, which is 4.4% of the worldwide child deaths in this age group. In countries where Zn deficiency is well documented as an important public health problem, cereal-based foods are the predominant source of daily calorie intake (Hotz and Brown 2004; Cakmak 2008; Gibson et al 2008).

Currently, there is a high and urgent need for increasing the Zn concentration in wheat grain and the edible parts of other staple food crops. Based on model studies, enrichment of cereal grains with Zn is a promising approach to reduce child deaths in India (Stein et al 2007). Breeding new genotypes with high grain Zn concentrations and applying Zn-containing fertilizers are two major agricultural strategies for improving grain Zn concentrations (Bouis 2003; Pfeiffer and McClafferty 2007; Cakmak 2008). Efforts for increasing the Zn concentration in wheat grain are, however, impeded by the lack of knowledge about the physiological factors affecting 1) root uptake, 2) root-to-shoot transport, 3) phloem loading, 4) remobilization of Zn from source tissues into developing seeds, and 5) seed deposition of Zn.

Increasing evidence in the literature suggests that these factors are affected by N fertilization or N metabolism of plants. Most of the Zn in the grain is thought to be localized in protein bodies in the form of globoid crystals, which are rich in phytate reserves (*myo*-inositol *bis*-hexaphoshate) (Lott and Buttrose 1978; Welch 1986). Protein-Zn-phytate complexes are probably the predominant Zn species in the wheat grain. In wheat or maize embryo, the Zn concentration in the protein bodies can reach up to 600 mg/kg (Mazzolini et al 1985; Marschner 1995). The protein-rich embryo and aleurone parts of wheat seeds are also rich in Zn, whereas at the same time the endosperm, which is low in protein and phytate, is low in Zn (Lott et al 1995; Welch and Graham 1999). By using a Zn-staining method, Ozturk et al (2006) demonstrated that Zn is particularly accumulated in the embryo and aleurone parts of seeds. One reason why pulses generally contain higher Zn than cereal grains might be related to the higher protein concentrations of pulses. In a study by Ehret (1985), whole wheat grains had 27 mg/kg of Zn and 14.2% protein, whereas the embryos of the same grains had 226 mg/kg of Zn and 42% protein. In biological systems, Zn and proteins are very closely associated. Among all metals, Zn is needed by the largest number of proteins for their catalytic functions and structural integrity. Proteomic analysis showed that ≤10% of the human proteome consists of Zn-binding

*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 4 appears in color online.

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proteins and ≈40% of these Zn-binding proteins are transcription factors, while the remaining 60% are enzymes and proteins involved in ion transport (Andreini et al 2006). Cysteine, histidine, aspartic acid, and glutamic acid residues seem to be common binding sites of Zn in proteins (Passerrini et al 2007; Shu et al 2008). Both the speciation and the localization data suggest that protein is a sink for Zn in the grain.

Grain proteins may contribute to the accumulation of Zn by increasing the storage capacity of the grain for Zn. This hypothesis is supported by the high positive correlations between seed protein and seed Zn (and also Fe) reported in several studies (Peterson et al 1986; Zebarth et al 1992; Feil and Fossati 1995; Morgounov et al 2007; Peleg et al 2008). Further support for the close relationship between Zn and N in grain comes from studies of *Gpc-B1* locus in tetraploid wheat, which is located on the short arm of chromosome 6B and affects the grain protein concentration. Recombinant chromosome substitution lines of durum wheat (*Triticum durum*) carrying the *Gpc-B1* allele from wild emmer wheat (*T. turgidum* ssp. *dicoccoides*) accumulated not only higher concentrations of protein but also higher concentrations of Zn (also Fe and Mn) in grain, as compared with lines carrying the allele from cultivated durum wheat (Distelfeld et al 2007). Because the *Gpc-B1* locus is associated with accelerated senescence, it was hypothesized that this locus contributes to the remobilization of micronutrients and N (amino acids) from senescing tissues into grain (Uauy et al 2005; Distelfeld et al 2007). However, delayed senescence can also increase the grain Zn accumulation. High N supply delays senescence and thereby extends the grain-filling period (Yang and Zhang 2006). Therefore, continued uptake of Zn during the extended grain-filling period may contribute to Zn accumulation in the grain.

The transport forms of Zn in phloem tissue are still unknown. In literature, several nitrogenous compounds such as amino acids and nicotianamine have been discussed as potential compounds contributing to chelation and transport of Zn in the phloem tissue (Grusak et al 1999; Von Wirén et al 1999). These nitrogenous compounds may also be involved in translocation of Zn from the root into the shoot. Increasing the N supply is known to increase the concentration of total free amino acids in leaves and stimulate the phloem export of amino acids (Caputo and Barneix 1997). It is also well documented that most of the grain N is derived from protein catabolism in senescing organs and particularly the degradation of chloroplast proteins during senescence (Feller et al 2008; Gregersen et al 2008). If the transport of Zn in the vascular system is really a limiting factor for the grain Zn accumulation, N might contribute to increase the grain Zn concentration by improving the Zn transport capacity of the vascular system.

To our knowledge, the effects of increasing soil and foliar N applications on the Zn accumulation of the wheat grain were not studied at deficient and sufficient Zn supply levels. As indicated above, the close linkage between grain Zn and protein concentrations as well as the existence of common genetic mechanisms affecting grain Zn and protein accumulation have been shown in durum wheat and emmer wheat (Cakmak et al 2004; Uauy et al 2006; Distelfeld et al 2007). Collecting information on how N fertilization affects accumulation of Zn in the shoot and the grain of durum wheat will further contribute to better understanding of the physiological and molecular mechanisms underlying the linkage between grain Zn and protein. Moreover, because durum wheat is extremely sensitive to Zn deficiency (Cakmak et al 1999), any contribution of N fertilization to Zn nutrition of durum wheat is of great importance in terms of both productivity and nutritional quality, especially under marginal conditions in semiarid regions, where durum wheat is widely cultivated (Elias and Manthey 2005).

From the present study, we report on 1) how increasing soil N fertilization affects shoot and grain concentrations of Zn in plants grown at low, adequate, and high Zn application rates, 2) how foliarly applied Zn and N fertilizers affect grain Zn accumulation under various soil applications of Zn and N, and 3) how grain concentrations of Zn and N are linked. The relationship between grain Zn and protein localization was also studied by using Zn and protein staining methods.

MATERIALS AND METHODS

Wheat plants (*Triticum durum*, cv. Balcali 2000) were grown under greenhouse conditions equipped with an evaporative cooling system under natural daylight (geographic coordinates: 40° 53' 24.5" N, 029° 22' 46.7" E). Seeds were sown in plastic pots containing 3.1 kg of Zn-deficient soil that was transported from a Zn-deficient location in Central Anatolia (Cakmak et al 1996). The soil characteristics were clay-loam texture, pH 7.6 in dH_2O, 1.5% organic matter, 18% $CaCO_3$. The diethylenetriamine pentaacetic acid (DTPA)-extractable Zn concentration was 0.1 mg/kg of soil.

Two separate pot experiments were conducted. Both were designed as factorial experiments. The first experiment studied the effects of varied soil N supply on the shoot Zn concentration and also Zn deficiency tolerance of plants at different levels of soil Zn supply. Before potting, the experimental soil was mixed homogeneously with nutrients (per kg of soil): 100 mg of P in the form of KH_2PO_4, 25 mg of S in the form of K_2SO_4, 2.5 mg of Fe in the form of Fe-EDTA, 50 mg of N (for low N plants), or 200 mg of N (for adequate N plants) in the form of $Ca(NO_3)_2$ $4H_2O$, and 0.05 mg of Zn (low Zn plants) or 2 mg of Zn (adequate Zn plants) or 10 mg of Zn (high Zn plants) in the form of $ZnSO_4$ $7H_2O$.

Ten seeds were sown in each pot. The seedlings were thinned to 5/pot shortly after emergence. The pots were watered daily using deionized water. After 35 days of growth under greenhouse conditions, shoots were harvested, washed with deionized water, and dried at 60°C for determination of shoot dry matter production. Dried shoot samples were ground and subjected to acid-digestion in a closed vessel microwave system (MarsExpress, CEM, Matthews, NC). Concentrations of the mineral nutrients including Zn were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian, Mulgrave, Australia). Measurements were checked by using the certified values of standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD).

The nutrient contents per plant (e.g., total amounts of nutrients) were calculated by multiplying the shoot dry weights by the shoot concentrations of the nutrients.

In the second experiment, which was conducted to investigate the effects of various soil and foliar applications of Zn and N on the grain Zn concentration and the yield, plants were grown until grain maturation at three different soil N supply levels. As basal N fertilization, N rates applied in the form of $Ca(NO_3)_2{\cdot}4H_2O$/kg of soil: 50 mg of N (for low N plants) and 200 mg of N (for adequate and high N plants). All other basal fertilizers, including Zn fertilizers, were applied at the same rates and in the same forms, as described above for the first experiment. Ten seeds were sown in each pot and the seedlings were thinned to 5/pot shortly after emergence. Pots were watered daily using deionized water. After six weeks of growth, 50 mg of P/kg of soil was added to all pots in the form of KH_2PO_4, and 200 mg of N/kg of soil was added to the pots of the high N plants in the form of $Ca(NO_3)_2{\cdot}4H_2O$. Finally, an additional 200 mg of N/kg of soil in the same form was added to the pots of the high N plants when plants were eight weeks old.

When the plants were in the flowering stage in the 9th week, the foliar applications of Zn and urea were started. The flowering was almost complete in low N plants, whereas high N plants were still at the beginning of the flowering stage. Three groups of plants (pots) were established. The first (control) group of plants was not sprayed with N or Zn, but only treated with deionized water. Group two was sprayed with a 0.5% (w/v) $ZnSO_4{\cdot}7H_2O$

solution. Group three was sprayed with a solution of 2% (w/v) urea solution. All foliar application solutions contained 200 mg/L of Tween20 as a surfactant. Plants were sprayed to the point of run-off using a hand-sprayer. Foliar applications were repeated after one week, when the low N plants were in the early milk development stage, and the flowering was completed in the high N plants.

When the plants senesced fully and the grains reached full maturity, the spikes and the straws were harvested separately and the shoot (straw) samples were dried at 60°C for determination of shoot (straw) dry matter production. Using a thresher, the grains were separated from the husks and weighed to determine the grain yield. Grain samples were acid-digested, and the concentrations of Zn and other nutrients including K, Fe, Mn, and Cu were determined by ICP-OES (Varian-Vista-Pro, Australia), as described above for the first experiment. Grain Zn yield was calculated by multiplying the grain yield by the grain Zn concentration. The grain N concentrations were determined by using a LECO TruSpec C/N Analyzer.

Protein staining used Bradford reagent containing Coomassie Brilliant Blue G-25 dye (Bradford 1976). Zn staining used dithizone reagent (Ozturk et al 2006). Seeds were initially incubated in water for 2 hr at room temperature and then excised longitudinally before treatment with the dye compounds. For protein staining, seeds were treated with diluted Bradford reagent (2:1 v/v dilution by absolute ethanol) and incubated at 70°C for 15 min. For Zn staining, seeds were treated with 500 mg/L of dithizone (1,5-diphenyl thiocarbazone) dissolved in absolute methanol and incubated at room temperature for 30 min (Ozturk et al 2006). Finally, the stained seeds were rinsed with water and analyzed qualitatively using a reflectance light microscope (Nikon SMZ1500) with a high-resolution digital camera (Diagnostic Instruments).

Each treatment consisted of four independent (pots) replicates in the first experiment and eight independent (pots) replicates in the second experiment. The significance of the effects of treatments and their interactions on the reported traits was evaluated by analysis of variance (ANOVA). Significant differences among means were determined by Fisher's least significant difference (LSD) test at the 5% level ($P \leq 0.05$).

RESULTS

In the first experiment, analysis of variance revealed significant effects of soil N and soil Zn applications as well as N × Zn interaction on the shoot dry weight, shoot Zn concentration, and shoot Zn content of five-week-old durum wheat plants grown under greenhouse conditions (Table I). Variation in N nutrition had a significant impact on the shoot dry matter yield of the plants at low Zn supply (Table II). Under Zn-deficient conditions, adequate N application greatly improved shoot growth as compared with low N application. Visual symptoms of Zn deficiency such as whitish-necrotic spots on the middle-older leaves developed only at low supply of Zn. However, these symptoms were more severe at low N than at adequate N supply. No N deficiency symptom was observed in the experimental plants under given conditions.

As shown in Table II, the shoot dry weight of plants at low Zn and adequate N treatments was 60% higher than the shoot dry weight of the plants grown at low Zn and low N treatments. With adequate (or high) Zn supply, the shoot dry weights of the plants with adequate N supply were only 11% higher than those of the plants grown with low N supply. When compared with the adequate Zn treatment, high Zn treatment did not make an extra contribution to biomass production, indicating that the Zn treatment of 2 mg/kg of soil was already sufficient to meet the Zn demand of wheat under given conditions.

As expected, the shoot Zn concentrations increased by increasing Zn treatments (Table III). At adequate or high Zn supply, the plants grown with an adequate N application had significantly greater concentrations and contents of Zn in the shoot than the plants grown with low N application. However, the positive effect of the N nutrition on the shoot Zn concentration could not be observed in plants grown with low Zn supply. The shoot Zn concentrations were not affected by the N treatments when Zn supply was limited. In contrast to the Zn concentration, the shoot Zn contents of plants at low Zn tended to increase by increasing N due to better growth, although the effect was not statistically significant.

In the second experiment, plants were grown to grain maturation with various soil and foliar applications of N and Zn. ANOVA calculated six traits including straw dry weight, grain yield, harvest index, grain Zn concentration, grain Zn yield, and grain N concentration (Table IV). Foliar applications of N or Zn had sig-

TABLE I
Analysis of Variance (ANOVA) of Effects of Soil N and Zn Applications on Shoot Dry Weight, Zn Concentration, and Zn Content[a,b]

Source of Variation (Applications)	df	Shoot Dry Weight		Shoot Zn Conc.		Shoot Zn Content	
		SS	*F* Pr	SS	*F* Pr	SS	*F* Pr
Soil N (A)	1	107,334	<0.001	813	<0.001	1452	<0.001
Soil Zn (B)	2	900,485	<0.001	36,231	<0.001	28820	<0.001
A × B	2	11,062	0.030	834	<0.001	1204	<0.001
Exp error	15	18,480		220		197	

[a] Data for five-week-old durum wheat (*Triticum durum* cv. Balcali 2000) plants (1st exp.) grown under greenhouse conditions.
[b] ANOVA test values: df, SS, and *F* Pr.

TABLE II
Effect of Low and Adequate N Treatment on Shoot Dry Weight of Plants Grown at Low, Adequate, and High Zn Levels

N Treatment[a]	Shoot Dry Weight (mg/plant)[b,c]		
	Low Zn	Adequate Zn	High Zn
Low	344 Aa	802 Ab	797 Ab
Adequate	537 Ba	896 Bb	912 Bb

[a] N treatments: low (50 mg of N/kg of soil) and adequate (200 mg of N/kg of soil).
[b] Data for five-week-old durum wheat plants (*Triticum durum* cv. Balcali 2000) grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions
[c] Values are means of four independent replicates. Mean values in a column followed by different uppercase letters and mean values in a row followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

TABLE III
Effect of Low and Adequate N Treatment on Shoot Zn Concentration and Shoot Zn Content

Zn Treatment[a]	Shoot Zn Concentration[b,d]		Shoot Zn Content[c,d]	
	Low N	Adequate N	Low N	Adequate N
Low	7.6 Aa	6.7 Aa	2.6 Aa	3.6 Aa
Adequate	36.4 Ba	44.8 Bb	29.1 Ba	40.0 Bb
High	87.3 Ca	114.8 Cb	69.8 Ca	104.5 Cb

[a] Data for five-week-old durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions
[b] N treatments: low (50 mg of N/kg of soil) and adequate (200 mg of N/kg of soil).
[c] Content measured as μg of Zn/plant.
[d] Values are means of four independent replicates. Mean values in a column followed by different uppercase letters and mean values in a row followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

nificant effects on all reported traits except grain yield. Soil applications of N and Zn as well as soil N × foliar applications interaction and soil N × soil Zn interaction significantly affected all reported traits. For soil Zn × foliar applications interaction, the effect turned out to be significant on all traits except for grain yield and harvest index. Finally, the triple interaction had significant effects on only grain Zn concentration, grain Zn yield, and grain N concentration.

The positive impact of high N supply on the shoot growth of plants under low Zn supply was also observed in this second experiment (Fig. 1). At low Zn supply, the plants with adequate and high N treatments grew better than the plants with low N treatment.

At all soil Zn treatments, soil N fertilization improved the vegetative growth of plants and resulted in significant increases in straw dry weights of plants (Table V). Soil Zn applications at adequate or high rates generally increased straw dry weight. Foliar urea application also improved shoot biomass production, especially at adequate and high soil N treatments, as compared with the plants without foliar treatment; whereas foliar Zn treatment had inconsistent effects on straw dry weight.

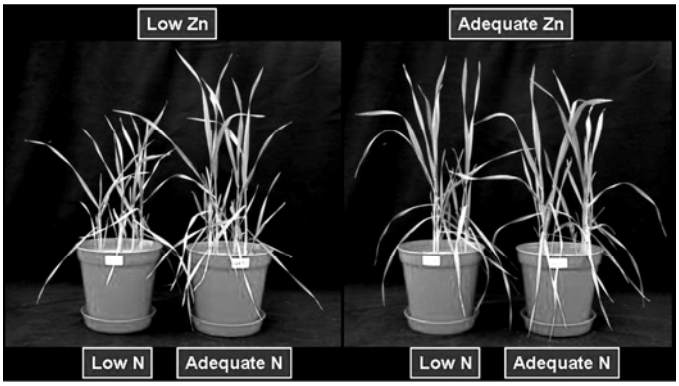

Fig. 1. Effect of low (50 mg of N/kg of soil) and adequate (200 mg of N/kg of soil) N treaments on the growth of six-week-old durum wheat (*Triticum durum* cv. Balcali 2000) plants at low (0.05 mg of Zn/kg of soil) and adequate (2 mg of Zn/kg of soil) Zn applications on a Zn deficient calcareous soil under greenhouse conditions.

The grain yield per plant varied at 0.8–3.2 g, depending on the Zn and N treatments (Table VI). At low soil Zn supply, the grain yield tended to decrease with increasing soil N supply, but at adequate or high Zn treatments, there were significant enhancements in grain yield by increasing soil N applications. Increases in the soil Zn applications enhanced the grain yield significantly, but foliar Zn application at low soil Zn supply could not rescue the yield, which might be explained by late foliar application of Zn. Similarly, increases in the soil N applications improved the grain yield significantly, but foliar urea application at low soil N supply could not prevent the yield losses caused by N deficiency, probably due to late foliar applications of urea.

At low soil Zn treatment, the harvest index was ≈48% under low soil N supply, but when the soil N supply was increased, the harvest index was drastically reduced to values of 20.0–27.4%

TABLE V
Effect of Low, Adequate, or High N Treatment on Straw Dry Weight

Foliar Application[a]	N Treatment[b]	Straw Dry Weight (g/plant)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	1.41 Aa	1.70 Ab	1.61 Ab
	Adequate	2.68 Ba	2.69 Ba	2.76 Ca
	High	2.87 Ca	3.21 Cb	3.28 Db
Urea	Low	1.45 Aa	1.83 Ab	1.79 Bb
	Adequate	3.08 Da	3.40 CDb	3.55 Ec
	High	3.06 Da	3.59 Eb	3.51 Eb
$ZnSO_4$	Low	1.39 Aa	1.68 Ac	1.58 Ab
	Adequate	3.04 Db	2.67 Ba	2.75 Ca
	High	3.13 Da	3.25 Cb	3.70 EFc

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N Treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil) or high (600 mg of N/kg of soil).
[c] Data for durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions. Foliar applications of urea and $ZnSO_4$ were realized by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[d] Values are means of eight independent replicates. Mean vlaues in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

TABLE IV
Analysis of Variance (ANOVA) of Effects of Foliar and Soil Applications of N and Zn on Straw Dry Weight and Grain[a,b]

Source of Variation (Applications)	df	Straw Dry Weight		Grain Yield		Harvest Index	
		SS	*F* Pr	SS	*F* Pr	SS	*F* Pr
Foliar (N/Zn) (A)	2	4.305	<0.001	0.269	0.459	312	0.002
Soil N (B)	2	114.753	<0.001	20.607	<0.001	4456	<0.001
Soil Zn (C)	2	2.866	<0.001	85.946	<0.001	10797	<0.001
A × B	4	2.434	<0.001	4.276	<0.001	1207	<0.001
A × C	4	1.015	0.018	0.701	0.397	155	0.169
B × C	4	1.408	0.003	27.405	<0.001	4909	<0.001
A × B × C	8	1.103	0.110	0.983	0.677	128	0.717
Experimental error	182	15.099		31.226		4330	
		Grain Zn Concentration		Grain Zn Yield		Grain N Concentration	
		SS	*F* Pr	SS	*F* Pr	SS	*F* Pr
Foliar (N/Zn) (A)	2	75971	<0.001	197284	<0.001	19.033	<0.001
Soil N (B)	2	16105	<0.001	209280	<0.001	41.308	<0.001
Soil Zn (C)	2	34800	<0.001	487309	<0.001	0.546	0.002
A × B	4	9471	<0.001	40346	<0.001	7.675	<0.001
A × C	4	20030	<0.001	18056	<0.001	0.991	<0.001
B × C	4	1895	<0.001	102196	<0.001	6.176	<0.001
A × B × C	8	2561	<0.001	12606	<0.001	1.403	<0.001
Experimental error	182	7561		55550		7.717	

[a] Data for straw dry weight, grain yield, harvest index, grain Zn concentration, grain Zn yield, and grain N concentration of mature durum wheat (*Triticum durum* cv. Balcali 2000) plants (2nd Exp.) grown under greenhouse conditions.
[b] ANOVA test values: df, SS, and *F* Pr.

(Table VII) due to a slight negative effect of increasing N supply on the grain yield (Table VI) and its strong positive effect on straw dry weight (Table V). Low soil Zn supply severely reduced the harvest index, when the soil N supply was adequate or high (Table VII). Generally, foliar applications of urea and Zn had inconsistent effects on the harvest index.

Both soil and foliar applications of Zn significantly enhanced grain Zn concentration of the plants, particularly the high soil N treatments (Table VIII). For example, at the low Zn supply, foliar application of Zn increased grain Zn concentration by 4.5-fold at the low N treatment, but 9-fold at the high N treatment. As expected, the lowest grain Zn concentrations were measured at the low soil Zn supply and in the absence of foliar Zn application. Under such Zn-limited conditions, the grain Zn concentration did not respond to increasing soil N supply or foliar application of urea. However, at the adequate or high soil applications of Zn or in foliar Zn application, increasing soil N supply had a significant positive impact on the grain Zn concentration. In foliar Zn fertilization, increasing soil N supply enhanced grain Zn concentrations by nearly twofold at all Zn treatments.

At adequate soil Zn supply and in the absence of foliar Zn application, the positive effect of increasing N supply on the grain Zn concentration could still be observed as a trend, but the effect was largely negated by yield increases (Tables VI and VIII). As expected, the total grain Zn yield per plant showed marked positive responses to increasing soil Zn application and foliar spray of Zn (Table IX). At almost all treatments except at low soil Zn supply, increasing N supply resulted in substantial increases in grain Zn yield. In general, the increases in grain Zn yield by N applications became more pronounced if the soil Zn supply increased from low to adequate level.

Depending on the treatment, the grain N concentrations varied at 1.61–3.49% (Table X). The lowest grain N concentrations were measured at the low soil N supply in the absence of foliar urea

TABLE VI
Effect of Low, Adequate, or High N Treatment on Grain Yield

Foliar Application[a]	N Treatment[b]	Grain Yield (g/plant)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	1.28 Ba	1.65 Ab	1.55 Ab
	Adequate	0.90 Aa	2.85 CDc	2.64 Cb
	High	1.01 ABa	3.06 Db	3.34 Ec
Urea	Low	1.52 Ca	1.93 Bb	1.82 Bb
	Adequate	0.76 Aa	2.67 Cc	2.44 Cb
	High	1.14 Ba	2.89 CDb	2.90 Db
$ZnSO_4$	Low	1.38 Ca	1.58 Aab	1.49 Aa
	Adequate	1.00 ABa	3.15 Dc	2.79 CDb
	High	1.05 ABa	2.59 Cb	2.54 Cb

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil), or high (600 mg of N/kg of soil).
[c] Data for durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.
[d] Values are means of eight independent replicates. Mean values in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

TABLE VII
Effect of Low, Adequate, or High N Treatment on Harvest Index

Foliar Application[a]	N Supply[b]	Harvest Index (%)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	47.2 Da	49.3 Bb	49.0 Cb
	Adequate	22.9 Ba	51.3 BCb	51.6 CDb
	High	27.4 Ca	47.7 Bb	49.1 Cb
Urea	Low	49.2 Da	51.2 BCab	50.5 Ca
	Adequate	20.0 Aa	42.2 Ac	40.5 Ab
	High	26.9 Ca	44.1 Ab	45.0 Bb
$ZnSO_4$	Low	48.3 Da	48.5 Ba	48.5 Ca
	Adequate	26.5 Ca	53.6 Cc	51.1 Cb
	High	25.7 Ca	44.2 Ac	40.6 Ab

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil), or high (600 mg of N/kg of soil).
[c] Data for durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.
[d] Values are means of eight independent replicates. Mean values in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

TABLE VIII
Effect of Low, Adequate, or High N Treatment on Grain Zn Concentration

Foliar Application[a]	N Treatment[b]	Grain Zn Concentration (mg/kg)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	10.1 Aa	23.2 Ab	36.6 Ac
	Adequate	11.3 Aa	25.6 Ab	59.6 Cc
	High	10.4 Aa	27.7 ABb	61.2 Cc
Urea	Low	9.2 Aa	24.6 Ab	47.0 Bc
	Adequate	10.3 Aa	28.8 ABb	61.6 Cc
	High	9.1 Aa	29.6 Bb	65.9 Dc
$ZnSO_4$	Low	45.0 Bb	41.0 Ca	50.3 Bc
	Adequate	94.4 Cc	60.3 Da	82.2 Eb
	High	92.4 Cc	78.6 Ea	89.9 Fb

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil), or high (600 mg of N/kg of soil).
[c] Data for durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.
[d] Values are means of eight independent replicates. Mean values in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

TABLE IX
Effect of Low, Adequate, or High N Treatment on Grain Zn Yield

Foliar Application[a]	N Treatment[b]	Grain Zn Yield (μg of Zn/plant)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	12.3 Aa	37.1 Ab	56.5 Ac
	Adequate	9.7 Aa	72.9 Cb	150.2 Dc
	High	10.4 Aa	83.7 Db	196.3 Ec
Urea	Low	15.2 Aa	47.5 Bb	85.5 Cc
	Adequate	7.1 Aa	70.9 Cb	148.3 Dc
	High	9.8 Aa	82.3 CDb	189.3 Ec
$ZnSO_4$	Low	60.9 Ba	64.4 Ca	75.0 Bb
	Adequate	92.5 Ca	184.5 Eb	228.7 Fc
	High	98.0 Ca	202.2 Fb	226.7 Fc

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil), or high (600 mg of N/kg of soil).
[c] Data for grain Zn yield (total amount of Zn in grains/plant) of durum wheat (*Triticum durum* cv. Balcali 2000) of fully mature plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.
[d] Values are means of eight independent replicates. Mean values in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

application. Increasing the soil N supply resulted in progressive increases in the grain N concentration. At the high N supply, the enhancements in soil Zn application generally improved grain N concentration of plants. Foliar application of urea was effective in increasing the grain N concentration.

The relation between the grain concentrations of Zn and N is shown in Fig. 2. In the absence of foliar Zn fertilization and at the low soil Zn supply, the grain Zn concentration did not show any relation to the grain N concentration (Fig. 2A), but at high soil Zn supply or with foliar Zn application, the grain Zn concentration exhibited a strong positive correlation with the grain N concentration (Fig. 2B–D). The positive impact of the N nutrition on the grain Zn concentration is dependent on high availability of Zn in soil or plant tissue. The slope of the line in Fig. 2D is higher than that of the line in Fig. 2C, which shows that the response of grain Zn to N nutrition at high soil Zn supply was strengthened when the Zn availability was further increased by foliar Zn application. As presented in Fig. 3, at high Zn availability due to either foliar or soil application of Zn, the increasing effect of N on grain Zn became more pronounced (Fig. 3A and B). The positive effect of increasing N supply was not restricted to the grain concentration of Zn. The grain Fe concentration also increased significantly with increasing soil N supply (Fig. 3C and D). For grain K concentration, however, there was no positive response to increasing N supply (Fig. 3E and F). At the adequate and high Zn supply, the grain K concentration even tended to slightly decrease with increasing N supply.

Dithizone method and Bradford reagent (Coomassie Brilliant Blue dye) were applied to study the localization and distribution of Zn and protein within the seeds, respectively. Figure 4 shows that both Zn and protein are concentrated in the embryo and aleurone regions of the grain. The endosperm part of seeds was also stained with the protein- and Zn-dye compounds, but less densely when compared with the embryo and aleurone regions.

TABLE X
Effect of Low, Adequate, or High N Treatment on Grain N Concentration

Foliar Application[a]	N Treatment[b]	Grain N Concentration (%)[c,d]		
		Low Zn	Adequate Zn	High Zn
None	Low	1.88 Bb	1.68 Aa	1.70 Aa
	Adequate	2.68 Db	2.47 Ba	2.72 Cb
	High	2.46 Ca	3.20 Db	3.37 EFc
Urea	Low	2.97 EFc	2.65 Ca	2.88 Db
	Adequate	3.16 Ga	3.28 Db	3.27 Eb
	High	2.80 Ea	3.49 Ec	3.39 Fb
$ZnSO_4$	Low	1.71 Ab	1.61 Aa	1.63 Aa
	Adequate	3.04 Fc	2.44 Ba	2.57 Bb
	High	2.89 Ea	3.24 Dc	3.15 Eb

[a] Foliar applications of urea and $ZnSO_4$ by spraying plants with 2% urea and 0.5% $ZnSO_4$, respectively.
[b] N treatments: low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil) or high (600 mg of N/kg of soil).
[c] Data for fully mature durum wheat (*Triticum durum* cv. Balcali 2000) plants grown at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.
[d] Values are means of eight independent replicates. Mean values in columns followed by different uppercase letters and mean values in rows followed by different lowercase letters are significantly different by Fisher's LSD test at the 5% level.

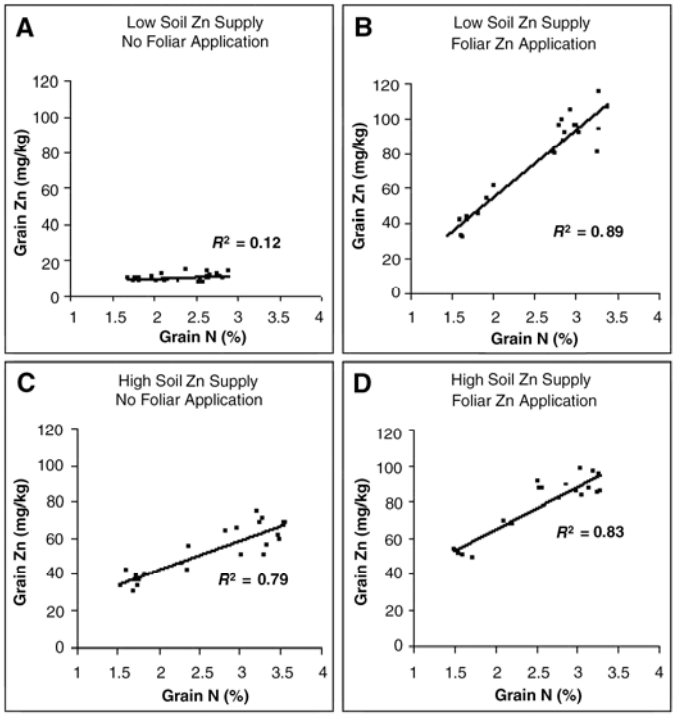

Fig. 2. Correlation between grain concentrations of Zn and N in durum wheat (*Triticum durum* cv. Balcalo 2000). Plants grown at low (**A** and **C**) or high (**B** and **D**) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions with (**C** and **D**) or without (**A** and **B**) foliar application of Zn.

DISCUSSION

Variation in N supply resulted in significant effects on Zn nutrition of wheat plants. During the vegetative growth, improving the N nutritional status of wheat had a significant ameliorative effect on shoot growth of plants under Zn-deficient conditions, but very

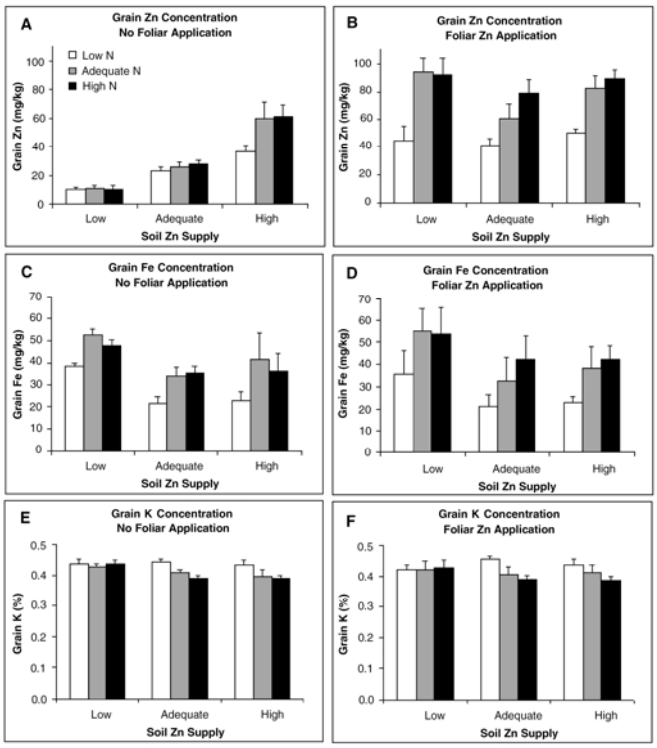

Fig. 3. Effect of increasing soil N treatments on grain concentrations of Zn, Fe, and K of durum wheat (*Triticum durum* cv. Balcali 2000) at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply. Plants were grown on a Zn-deficient calcareous soil with low (50 mg of N/kg of soil), adequate (200 mg of N/kg of soil), or high (600 mg of N/kg of soil) N treatments under green house conditions with (**B, D, F**) or without (**A, C, E**) foliar application of Zn. Vertical bars are ± SD of eight independent replicates.

little effect at adequate Zn supply (Table II and Fig. 1). This result suggested that the positive effect of high N on plant growth under Zn-deficient conditions cannot be explained with the correction of any hidden N deficiency (Fig. 1). Improving growth and alleviation of Zn-deficiency leaf symptoms in low-Zn plants by high N may involve increases in root uptake of Zn or the enhancement of internal Zn utilization. Increasing N treatments did not affect shoot Zn concentration, but tended to increase shoot content of Zn (Table III). Most probably, the increase in the shoot biomass of low-Zn plants by N resulted in the dilution of the Zn in plant tissue. It is a well-known phenomenon that lacking an increase in the concentration of a given nutrient despite its high total amount per plant reflects a dilution of the nutrient by enhanced growth (Marschner 1995). The same or similar shoot Zn concentrations despite significant differences in the expression of Zn-deficiency symptoms at varied N treatments (Fig. 1) may also indicate that high N results in higher mobility and physiological availability of Zn at the cellular level by affecting the level of Zn-chelating compounds (e.g., amino acids, peptides, or nicotianamine).

In contrast to the positive effects on shoot growth at early growth stages (Fig. 1), increasing the N supply did not result in a positive effect on grain yield of plants under low Zn supply and even reduced grain yield (Table VI). By stimulating the tillering and shoot biomass production (Ewert and Honermeier 1997; Salvagiotti and Miralles 2007), increasing N supply possibly resulted in severe Zn dilution and thus aggravated Zn-deficiency stress during the generative development under low-Zn supply (Table VI). Decreases in the grain yield due to increasing N treatments at low Zn may reflect Zn-deficiency-induced impairments in the development of reproductive organs (Sharma et al 1990; Cakmak and Engels 1999). Notably, although increasing the N supply reduced the grain yield at low Zn treatment (Table VI), it improved the straw yield (Table V) and thus reduced the harvest index significantly (Table VII). Unless the soil N supply was low, the harvest index was always reduced significantly by low soil Zn. Most probably, impaired development of reproductive organs due to Zn deficiency is associated with reduced demand for carbon allocation into seeds from shoot tissues, resulting in poor grain filling and low harvest index. The literature documents that the grain yield of wheat is much more sensitive to Zn deficiency than the straw yield under field conditions (Yilmaz et al 1997), probably due to reduced carbohydrate translocation from the source into the sink organs (Marschner and Cakmak 1989; Cakmak 2000).

Foliar applications of Zn and urea during grain-filling could not, or only could partially, prevent the yield losses associated with deficiencies of these elements. It appears that foliar applications of N and Zn during the grain-filling period are not useful in overcoming yield losses due to N and Zn deficiency, but they greatly contribute to the grain concentrations of N (Table X) (Woodard and Bly 1998; Varga and Svečnjak 2006) and Zn (Tables VI and VIII, respectively) (Yilmaz et al 1997; Cakmak 2008).

When sufficient Zn was supplied to the plants through soil or foliar applications, increasing the soil N application was highly effective in increasing shoot and grain Zn concentrations (Tables III and VIII). With low soil N supply, foliarly applied N (urea) was effective in improving the grain Zn concentration at high Zn treatment (Table VIII). Consequently, there was a strong positive relationship between the grain concentrations of Zn and N for high soil Zn supply or foliar Zn application (Fig. 2). These results lead us to suggest that N and Zn act synergistically in improving the grain Zn concentration when N and Zn exist at sufficient amounts either in the growth medium or in the source leaf tissues. There was also a close relationship between the grain concentrations of Zn and protein in their localization within the grain tissue. Protein and Zn staining studies demonstrated that Zn and protein are concentrated in the aleurone and embryo parts of the grain (Fig. 4). It seems very likely that N improves root uptake or retranslocation (remobilization) of Zn in wheat.

One plausible explanation for the positive impact of N on tissue Zn concentration could be related to the role of N in improving the plant growth, which may enhance the root uptake and shoot accumulation of Zn, an effect that is being exploited in phytoremediation of metal-contaminated soils (Schwartz et al 2003; Monsant et al 2008). Because both the grain Zn concentration and the grain yield were increased simultaneously by soil N applications, the total amount of grain Zn per plant (grain Zn yield) was enhanced by increasing soil N supply to a greater extent (Table IX) than the amount of Zn per unit tissue weight (Table VIII).

Nitrogen nutrition may affect the abundance of transporter proteins involved in the root uptake or root-to-shoot translocation or phloem loading of Zn, such as ZIP family proteins and YSL transporters (Waters et al 2006; Haydon and Cobbett 2007). The concentrations of compounds affecting chelation and translocation of Zn in plants such as nicotianamine, peptides, and amino acids might also be influenced by N nutrition of plants. High N supply may greatly increase the pool of nitrogenous compounds in leaf or phloem tissue as for amino acids (Caputo and Barneix 1997; Rubio-Covarrubias et al 2009). These compounds might be potential components of the phloem affecting the transport of Zn in the phloem tissue (Schmidke and Stephan 1995; Grusak et al 1999; Kruger et al 2002). The senescence-associated transport of N (amino acids and peptides) into seeds may stimulate the transport of Zn in chelated form. Nicotianamine (NA) is also an excellent chelator for Zn and contributes greatly to the cellular transport and phloem loading of Zn (von Wirén et al 1999; Haydon and Cobbett 2007). Interrupting biosynthesis of NA in tobacco plants impaired the Zn transport into reproductive organs and young leaves, whereas the overexpression of the NA synthase enzyme in tobacco plants increased Zn concentrations in young leaves and flowers (Takahashi et al 2003).

Increases in the grain accumulation of Zn after foliar application of Zn or urea suggest that Zn is easily translocated in phloem tissue, and the retranslocation of Zn from vegetative tissues into seeds is an important mechanism for Zn accumulation in the grain. High mobility of Zn in the phloem tissue has been also found in wheat by Haslett et al (2001) and Erenoglu et al (2002).

Very recently, under conditions of high availability of nutrients in growth medium, continued root uptake and translocation into

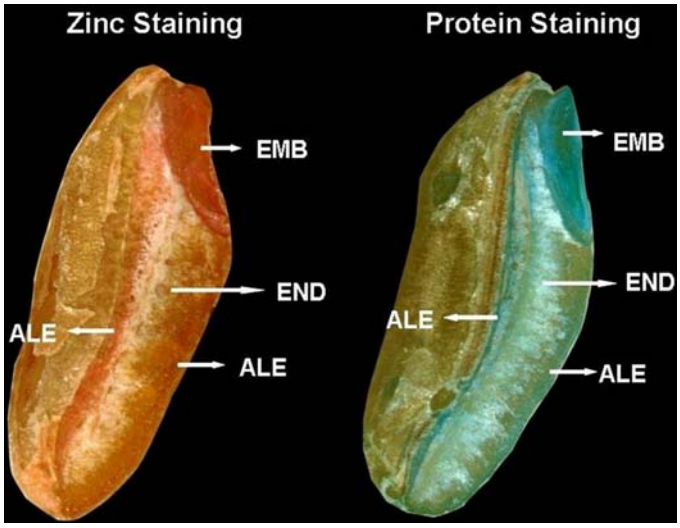

Fig. 4. Staining and localization of protein and Zn in a durum wheat cultivar (cv. Balcali 2000) containing 36 mg of Zn/kg and 11.6 g of protein/kg. Staining of longitudinally cut seed surface was done with Bradford reagent diluted 2:1 (v/v) in absolute ethanol (incubation at 70°C for 15 min) for protein and with dithizone reagent (500 mg/L of 1,5-diphenyl thiocarbazone dissolved in absolute methanol (incubation at room temperature for 30 min) for Zn.

seeds during the seed-filling period is an important way for seed micronutrient accumulation, which may be a more relevant process than remobilization of nutrients from source tissues (Waters and Grusak 2008). It is well documented that a high N nutritional status of plants extends the grain-filling period by delaying the senescence (Yang and Zhang 2006) and thereby prolongs the time that is available for the grain to accumulate Zn. In the current study, we also observed the same effect by high N rates in which the grain filling period was extended up to two weeks (data not shown).

Under such high N conditions, continued root uptake of Zn and its transport to seed during the extended grain-filling period could be a further major mechanism that contributes to the grain Zn accumulation. Accelerated senescence may also be important for grain Zn accumulation. Earlier leaf senescence increases both grain Zn and protein concentrations, possibly by increasing the levels of these nutrients available for remobilization from senescing tissues (Uauy et al 2006; Distelfeld et al 2007). When the root uptake of N and Zn is restricted due to drought or low availabilities of these nutrients in the soil during the grain-filling period, remobilization of the previously absorbed and stored Zn from source tissues (leaves, stems) may be a major contributing factor to the grain Zn accumulation.

Zinc is not the only essential nutrient whose grain concentration is positively affected by improved N nutrition. Grain concentrations of Fe (Fig. 3C) and also Mn and Cu (data not shown) also respond positively to increasing N supply, which suggests that these micronutrients may share similar N-dependent mechanisms with Zn for uptake or translocation to the grain or storage in the grain. In contrast to the grain concentrations of micronutrients, the grain K concentration does not respond to increasing N supply and even tends to decrease with increasing N supply (Fig. 3D), which clearly shows that the effect of N on grain concentrations of micronutrients seem to be rather a specific effect.

Micronutrient malnutrition is a growing global health problem caused mainly by low dietary intake of micronutrients, especially Zn and Fe (Bouis 2003; Pfeiffer and McClafferty 2007). Agricultural strategies including breeding and fertilization are widely accepted approaches to the problem, and the combination of the breeding with the fertilization approach seems to be the most cost-effective and sustainable approach (Cakmak 2008). The results of this study clearly demonstrate that the positive impact of N nutrition on the grain Zn accumulation should be considered in designing the fertilization and breeding programs. Selecting genotypes with higher grain protein concentrations and adapting appropriate N fertilization programs would be an effective strategy for maximizing the grain Zn accumulation in wheat.

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