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REVIEW

Biofortification of Durum Wheat with Zinc and Iron

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ABSTRACT

Micronutrient malnutrition affects over 2 billion people in the developing world. Iron (Fe) deficiency alone affects >47% of all preschool aged children globally, often leading to impaired physical growth, mental development, and learning capacity. Zinc (Zn) deficiency, like iron, is thought to affect billions of people, hampering growth and development, and destroying immune systems. In many micronutrient-deficient regions, wheat is the dominant staple food making up >50% of the diet. Biofortification, or harnessing the powers of plant breeding to improve the nutritional quality of foods, is a new approach being used to improve the nutrient content of a variety of staple crops. Durum wheat in particular has been quite responsive to breeding for nutritional quality by making full use of the genetic diversity of Fe and Zn concentrations in wild and synthetic parents. Micronutrient concentration and genetic diversity has been well explored under the HarvestPlus biofortification research program, and very positive associations have been confirmed between grain concentrations of protein, Zn, and Fe. Yet some work remains to adequately explain genetic control and molecular mechanisms affecting the accumulation of Zn and Fe in grain. Further, evidence suggests that nitrogen (N) nutritional status of plants can have a positive impact on root uptake and the deposition of micronutrients in seed. Extensive research has been completed on the role of Zn fertilizers in increasing the Zn density of grain, suggesting that where fertilizers are available, making full use of Zn fertilizers can provide an immediate and effective option to increase grain Zn concentration, and productivity in particular, under soil conditions with severe Zn deficiency.

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The increase of cereals and cash crops in modern cropping systems and full adoption of high-yielding cultivars have resulted in a dramatic reduction in food diversity and micronutrient intake. In developed market economies, this trend was caused by a combination of economic factors, which encouraged farmers to specialize on fewer crops and production technologies to maximize farm profits (Pfeiffer et al 2005). In developing countries, the introduction of short-statured, input-responsive wheat and rice cultivars catalyzed a "green revolution" with spectacular production increases. Farmers chose to grow more profitable, highly productive cereal crops, leading to a decline in the area under protein and micronutrient rich legumes. This tendency is evident in a proportional decrease in cereal prices and an increase in price for legumes, fruits, vegetables, and animal and fish protein. This has contributed significantly to a "hidden hunger" or micronutrient deficiency brought about by these less nutritious cereal crops becoming more affordable and available.

Production of various wild, traditional, or ancient food crops, which are genetically very diverse and rich in nutritional compounds such as micronutrients, has decreased and even disappeared. Out of 7,000 species ever cultivated by humans, currently only 30 plant species account for ≈95% of the world's food energy supply (FAO 1996; Cakmak et al 2002). Not only have the numbers of food crop species in use declined, but genetic variation within many traditional cultivated food crops has also decreased.

*The *e*-Xtra logo stands for "electronic extra" and indicates that Figs. 1 and 3 appear in color online.

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Among widely cultivated food crops, wheat plays a particularly important role in daily energy intake, especially in the developing world. For example, in many Central Asian and Middle Eastern countries, wheat provides ≈50% of the daily energy intake and the proportion can exceed 70% in rural areas (Cakmak 2008).

Widely cultivated modern wheat cultivars with a high-yield capacity are poor sources of micronutrients, especially Zn and Fe, for meeting daily requirements of humans. In addition, wheat is rich in antinutritional compounds such as phytic acid and phenolic compounds that reduce biological availability of Zn and Fe in the human digestive tract (Welch and Graham 2004). Generally, grain Zn and Fe concentrations in commercial wheat cultivars are 20–35 mg/kg (Rengel et al 1999; Cakmak et al 2004). These concentrations are not adequate for human nutrition in diets with wheat constituting the main source of essential minerals. Hence, such wheat-based diets consumed over a period of time can result in micronutrient malnutrition and related severe health complications such as anemia, high susceptibility to infectious diseases, disrupted brain function, and hampered physical development and stunting.

Importance of Durum Wheat

Durum wheat, the hardiest of all wheat species, has superior adaptation to semiarid climates and a comparative adaptive advantage over bread wheat and other small grains under hot dry conditions. Hot days and cool nights favor the development of very hard vitreous kernels most desirable for a wide variety of local and processed food products including couscous, bulgur, flat bread, and pasta, the most common durum end product in developed market economies (Elias and Manthey 2005).

Not surprisingly, traditional production and consumption developed in hot, dry regions of the Fertile Crescent, Mediterranean regions of North Africa, and Southern Europe. Driven by increasing popularity of pasta products and the development of an international export market, durum production expanded to additional

continents, countries and nontraditional agro-ecological zone. The world's durum wheat area and production are concentrated in the Middle East, North Africa, states of the Ex-Soviet Union, North America, Central India, and Mediterranean Europe. There are reliable web sites concerning the recent global statistics on area and production for the major global durum wheat producers such as www.fas.usda.gov/pecad/highlights/2005/07/durum2005/durum%20tables.htm and www.ers.usda.gov/data/wheat/yearbook/WheatYearbookTables-Recent.pdf. Other web sites are useful to collect information on recent growing cycles (www.fas.usda.gov/pecad/highlights/2005/07/durum2005/) and annually updated 10-year outlooks and forceasts of the world wheat industry (www.agecon-search. umn.edu/).

Based on data for 21 durum producing countries, the global durum production increased on average by 2.6% from 26.8 in 1994-1999 to 27.6 million metric tons during the 2000-2005 period. The increase in production was due to a 10.2% increase in yield, from 1.80 t ha^{-1} in 1994-1999 to 1.99 t/ha in 2000-2005 as the global durum area declined by 6.8% from 14.9 million hectares in 1994-1999 to 13.9 million hectares in 2000-2005.

However these statistics do no reflect the importance of durum, in particular in developing countries. Durum is a major staple in many countries that are not included in statistics compiled by the wheat industry. Frequently, statistics for these countries are not available or imprecise and commonly durum wheat, bread wheat, and other cultivated wheat species are not separated. Production and area figures are in general available for countries, but not for durum growing regions within countries and, hence, credible data to calculate statistics such as per capita intake does not exist. Developing reliable statistics is further complicated by farming practices. For example, in Ethiopia, several wheat species are grown in a mixture with durum as the dominant component. Ethiopia, a center of diversity for tetraploid wheat (Zohary 1970), may serve as example of a country where durum is the main staple in several regions and that, in general, is not included in durum statistics. Hence, proposing durum wheat for biofortification is difficult as justifications must rely on circumstantial evidence and information related to target areas where high durum consumption and micronutrient malnutrition coincidence is scant.

Durum wheat in developing countries is often grown under harsh, drought-prone, and even marginal conditions. These vulnerable environments are often subject to high production variability due to annual variation in rainfall. Poverty maps, which often mirror drought farming systems and drought occurrence, and maps of prevalence of micronutrient deficiency, can be used along with information on durum production area and per capita wheat intake to identify target zones where biofortification is likely to be a cost-effective intervention to alleviate micronutrient malnutrition. In these areas, wheat contributes >50% to the daily energy intake, compared with an average of ≈20% on a global scale. Pfeiffer and Payne (2005) give a classification and description of the global wheat agro-ecological zones, so-called mega-environments, with major breeding objectives and representative locations or regions used to target breeding.

Breeding Mineral Dense Durum Wheat

Biofortification is the process of increasing the micronutrient density of staple crops through conventional plant breeding and modern biotechnology to achieve a measurable and positive impact on human health. In contrast to conventional breeding, biofortification aims to positively affect human micronutrient status, an endeavor which entails merging breeding with nutrition and socioeconomics research to enhance traits whose value is measured as health outcomes. The mentioned disciplines have become an integral part of crop improvement strategies focused on developing micronutrient-dense crops (Nestel et al 2006; Pfeiffer and McClafferty 2007a,b). At the core of any biofortification breeding program is a product pathway driven by potential effects of research and nutrition (Pfeiffer and McClafferty 2007a). A critical step in developing biofortified crops, after identifying target regions and the target populations, entails setting nutrient target levels for breeding based largely on 1) bioconversion or bioavailability of ingested nutrients; 2) retention of micronutrients after storage, processing, and cooking; 3) micronutrient requirements of a population; and 4) potential levels of consumption by the target population. An overview of breeding micronutrient-dense crops including target level calculations and methods to analyze micronutrients is provided in Pfeiffer and McClafferty (2007a,b).

As in developing nonbiofortified durum cultivars, durum breeding for mineral density must develop attractive trait packages without compromising agronomic and end-use characteristics to trigger adoption by producers and consumers. High Zn and Fe are invisible and do not affect sensory traits, a characteristic that challenges the ability to distinguish biofortified cultivars from regular cultivars and raises issues such as product identity, branding, and procurement. As Zn and Fe concentration are not subject to genetic erosion, little maintenance breeding is required once genes have been incorporated in the gene pool (Pfeiffer and McClafferty 2007a). Hence, the cost of breeding for minerals decreases over time, and micronutrient density built into the gene pool will not affect future breeding for productivity traits.

In biofortification, breeders can achieve the mineral target increment by directly breeding for higher mineral concentration or breeding for increased bioavailability. For wheat, bioavailability of Zn can be assumed to be 25% and bioavailability of Fe can be assumed to be 5% as for other cereals with significant phytate concentrations (e.g., maize, sorghum, and pearl millet). Due to the substantially lower bioavailability of Fe when compared with Zn, significantly higher micronutrient increments have to be added to reach nutritional target levels and achieve a measurable impact on human health. Assuming a 300 g wheat intake/day and 90% micronutrient retention during processing, target increments for wheat, or concentrations to be added by breeding, for a measurable impact on micronutrient states are ≈11 mg/kg for Zn and 30 mg/kg for Fe. The bioavailability of Fe and Zn is associated with the presence of antinutrients such as phytate or the lack of promoter substances (Welch and Graham 2004; White and Broadley 2005). Because increasing bioavailability (or absorption) results in a proportional decrease in the required amounts of nutrient that need to be added, the strategies for breeding micronutrient-dense durum wheat could consider breeding for improved bioavailability and retention in addition to direct breeding for increased Zn and Fe.

Existing genetic variation, trait heritability, gene action, associations among traits, available screening techniques, and diagnostic tools are the criteria commonly used to identify selectable traits and estimate potential genetic gains. Crop improvement activities in breeding Zn/Fe dense wheat focus first on exploring the available genetic diversity for Fe and Zn. In parallel, agronomic and end-use features are characterized. The source of genetic variation is essential for the next breeding steps. In durum wheat, as discussed here, variation is present in the strategic gene pool (unadapted trait sources) and, in general, prebreeding is necessary before using the trait in final product development. If genetic variation is present in the tactical gene pool, materials can be used directly to develop competitive durum cultivars without first relying on the strategic gene pool and prebreeding activities.

In determining mineral concentration, precision methods such as Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES), Atomic Absorption Spectrometer (AAS), and X-ray Fluorescence Spectrometer (XRF) allow identifying a wide range of micronutrients. Inexpensive rapid screening methods, for use in prescreening, range from more qualitative colorimetric staining methods (Modified Perl's Prussian Blue, 2,2 Dipyridal, Dithizone, etc.) to semiquantitative methods, such as Near Infra-red Reflectance Spectrophotometry (NIRS). As Zn and Fe are present in

minute fractions, contamination from sources such as dust can result in false positives. To estimate the extent of the contamination, aluminum (Al) concentration of samples is being used as an indicator. Al concentrations >5 mg/kg are frequently associated with contaminant Fe (Pfeiffer and McClafferty 2007a).

Plant breeding for micronutrient density began to gain legitimacy when deficiencies in micronutrients such as iron, iodine, zinc, and vitamins were recognized as an issue of overwhelming global public health significance and one of the major development challenges of the 21st century. In July of 2003, the Consultative Group on International Agricultural Research (CGIAR) established HarvestPlus, the Biofortification Challenge Program to add food nutritional quality to its agricultural production research paradigm and capitalize on agricultural research as a tool for public health interventions. Growing evidence from HarvestPlus research supports findings that Fe and Zn concentration in wheat is controlled by several (2–5) relevant genes, and that heritability is of intermediate magnitude and additive gene action predominates. Transgressive segregation for Zn has been encountered in wheat. Although information on transgressive segregation is incomplete, results to-date suggest the presence of complementary genes, particularly in genetically distant sources such as wild relative wheat species. This is not unexpected, as in the past breeders did not select for micronutrients, and latent variation may have been lost in contemporary germplasm. Further, data from HarvestPlus research for numerous crops, including different wheat species, reveal a generic positive correlation between nitrogen (N) Fe and Zn concentrations. Positive association among minerals allows raising levels of a number of micronutrients simultaneously by direct selection for multiple micronutrients or by capitalizing on the indirect selection response. Significant associations between mineral concentrations (in particular Zn) and grain protein concentration in wheat, established by several researchers, are discussed below.

Considering the various elements that determine the probability of success in developing Zn and Fe dense durum wheat (existing genetic variation, trait heritability, gene action, associations among traits, and available screening techniques or diagnostic tools), it can be concluded that successful breeding mineral-dense durum wheat is feasible.

TABLE I
Range of Grain Concentration for Fe and Zn in Contemporary Wheat Cultivars and *Triticum dicoccoides* Accessions

No. of Genotypes	Fe (mg/kg dry wt)	Zn (mg/kg dry wt)	Reference
Modern wheat cultivars			
43	22–34	21–35	Tang et al (2008)
25	44–54	26–32	Pomeranz and Dikeman (1983)
27	35–56	26–40	Peterson et al (1986)
384	30–73	27–85	Welch (2001)
57	34–66	29–46	Ficco et al (2009)
14	30–38	26–34	Garvin et al (2006)
51	27–42	16–27	Qury et al (2006)
132	29–57	25–53	Graham et al (1999)
34[a]	29–38	8–12	Cakmak et al (2000)
Triticum dicoccoides			
19	24–49	20–159	Cakmak et al (2000)
20	28–78	43–107	Cakmak et al (2000)
22	52–80	69–139	Peleg et al (2008)
113	24–96	35–100	Cakmak et al (2004)
83	26–109	32–97	Cakmak et al (2004)
111[b]	21–91	14–190	Cakmak et al (2004)

[a] Wheat cultivars grown on severe Zn-deficient soil (DTPA Zn: 0.09 mg/kg) under field conditions in Central Anatolia in Turkey.
[b] Accessions grown in pots under greenhouse conditions.

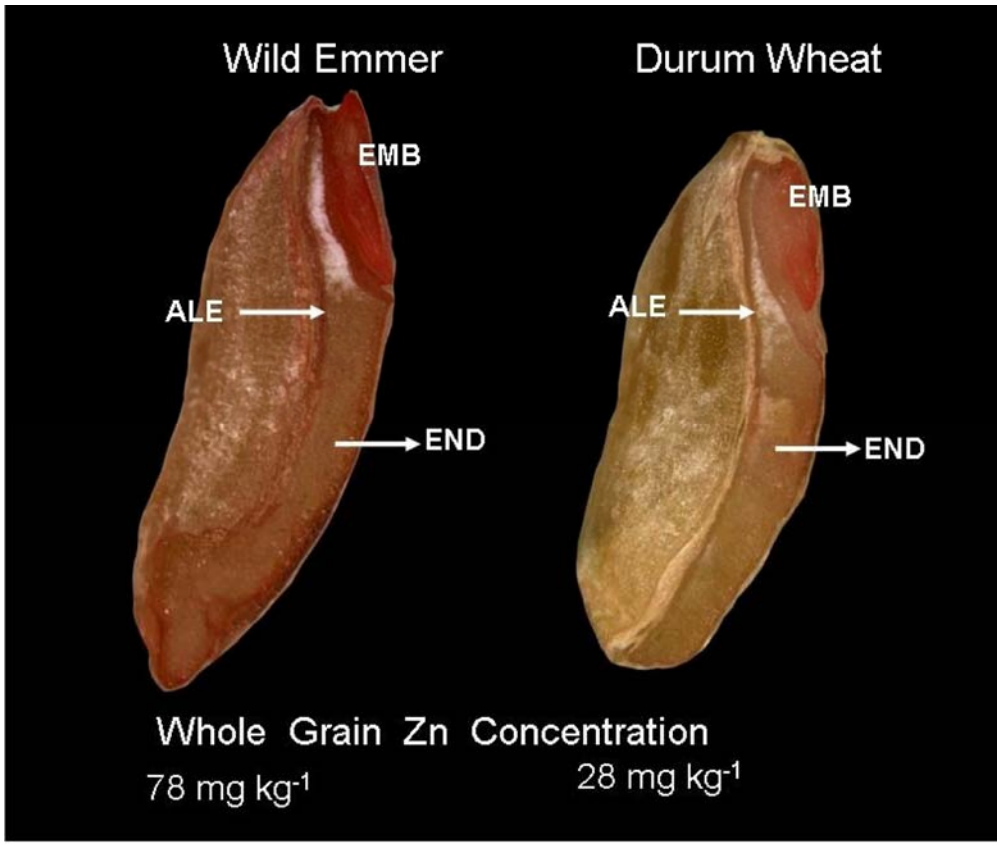

Fig. 1. Staining and localization of Zn in a wild emmer accession (left) and durum wheat cultivar (right). Seeds of wild emmer accession and Selcuklu durum wheat cultivar were stained by using the Zn dithizone (DTZ) method described by Ozturk et al (2006). DTZ forms a red colored complex with Zn; red color intensity is associated with high Zn concentration. END, endosperm; EMB, embryo; ALE, aleurone.

Grain Concentrations of Iron and Zinc

A large knowledge base regarding micronutrient concentration and genetic variation has been established for bread wheat. Assaying the genetic diversity spectrum for wheat has been a main objective in Zn and Fe gene and allele discovery research in Phase I (2004-2008) of the HarvestPlus project.

Compared with bread wheat, durum wheat tends to accumulate more Zn and Fe (Zubaidi et al 1999; Conti et al 2000) as well as Cd (Li et al 1997; Meyers et al 1982). It is still unknown why accumulation of Zn, Fe, and especially Cd occurs to a greater extent in durum than bread wheat. Durum wheat has also a higher protein concentration than bread wheat with grain protein representing a sink for Zn and other metals.

Average grain Zn concentration in modern wheat cultivars is low when compared with primitive and wild wheat (Ortiz-Monasterio and Graham 2000; Cakmak 2002) (Table I). Further recent research supports a hypothesis of a micronutrient dilution in wheat cultivars over time (Garvin et al 2006; Fan et al 2008). In developing countries, this trend is likely exacerbated by poor crop management and soil degradation. Besides low concentrations, genetic variation for micronutrients is very narrow in modern wheat cultivars. Screening of 57 modern durum wheat cultivars grown under field conditions in Italy showed little genetic variation for Zn (29–46 mg/kg with a mean of 34 mg/kg) and for Fe (34–67 mg/kg with a mean of 43 mg/kg) (Ficco et al 2009). When durum wheat is grown on soils with low plant availability of Zn due to adverse chemical conditions such as high pH and low organic matter, grain concentration of Zn is further reduced. For example, in Turkey, cultivated soils vary widely in the concentration of plant available Zn.

Durum wheat grain harvested at locations with very low plant availability of Zn in soils contains 8–12 mg of Zn/kg, whereas at locations with higher Zn availability grain Zn is 15–25 mg/kg (Cakmak et al 1999a; Erdal et al 2002). It is estimated that almost half of the cereal-cultivated soils in the world contain low amounts of plant available Zn, resulting in wheat grain Zn concentrations as low as 10 mg/kg (Graham et al 1992; Cakmak 2002, 2008). Such grain Zn concentrations are extremely low and inadequate to meet daily Zn requirements in diets where wheat is the predominant source of energy (Pfeiffer and McClafferty 2007b; Cakmak 2008).

In contrast to durum and bread wheat, wild emmer wheat (*Triticum dicoccoides*) contains a high amount of Zn and Fe and exhibits a useful and significant genetic variation for grain concentrations of micronutrients (Table I). As T. dicoccoides is the tetraploid wild progenitor of **durum** wheat and an ancestral constituent of bread wheat, genetic variation for micronutients and other nutritionally valuable traits has been lost in targeted breeding for productivity traits, abiotic and biotic stress tolerance, and the various end-use quality and market requirements. Screening of accessions of *T. monococcum*, *T. boeoticum*, *T. dicoccon*, *T. dicoccoides* and *Aegilops tauschii,* revealed and confirmed *T. dicoccoides* as most promising genetic resource for improving cultivated wheat in terms of magnitude of grain Zn and Fe (Cakmak et al 1999b, 2000; Ortiz-Monasterio and Graham 2000). Genotypes high in both micronutrient concentrations and micronutrient content (i.e., total amount of micronutrients per seed) have been identified among *T. dicoccoides* accessions. In screening 825 wild emmer accessions, the grain concentrations were 14–190 mg/kg under greenhouse conditions for Zn and 15–109 mg/kg for Fe (Cakmak et al 2004). Comparable large genetic variation could not be detected for other minerals in *T. dicoccoides* and for micronutrients in cultivated wheat. In addition, wild emmer accessions with high grain Zn and Fe concentrations exhibited large seed weight and size, resulting in a high total Zn and Fe per seed (maximum 7 μg of seed for Zn and 3.7 μg/seed for Fe). For cultivated wheat, grain content for Zn and Fe averages 1 μg/seed (Cakmak et al 2004). Hence, these screening results revealed that high grain concentration of Fe and Zn in wild emmer accessions was not due to a dilution effect caused by small grain size or low seed weight or test weight.

Higher Zn concentrations in wild emmer accessions compared with durum wheat cultivars have been demonstrated by using the dithizone (DTZ, diphenyl thiocarbazone) staining method described by Ozturk et al (2006). The colorimetric method is based on a DTZ reaction with Zn forming a red colored complex. As illustrated in Fig. 1, Zn is localized particularly in the embryo and aleurone of the kernel; the localization was pronounced in wild emmer accession containing 78 mg of Zn/kg.

Promising wild emmer accessions (MM5/4 and 24-39) have been identified for use as a micronutrient source and characterized by consistently high Zn (maximum 139 mg/kg), Fe (maxi-

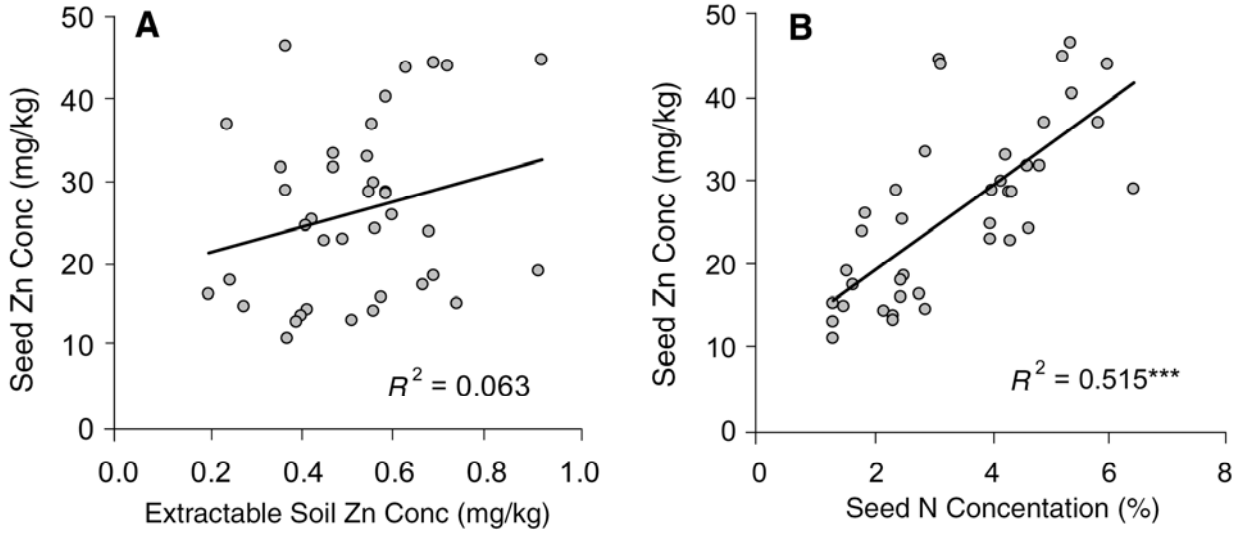

Fig. 2. Correlation between DTPA-extractable soil Zn and seed Zn concentrations (**A**), and seed Zn and seed N concentrations (**B**). Matured seeds tested for Zn and N concentrations collected from various cereal species (maize, sorghum, barley, and wheat) and dicotyledonous species (soybean, safflower, pea, common bean, canola, and common vetch) grown at the same location and year in farmers' fields in the Konya region of Central Anatolia, Turkey. Soil samples collected from the top 20 cm and subjected to inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis for Zn together with seed samples. Measurement of seed N by LECO CN analyzer (soil and seed samples collected by C. Balta, BD International Agricultural Research Institute, Konya).

mum 88 mg/kg) and protein (up to 26% w/w) under both water-limited and irrigated conditions (Peleg et al 2008). Both wild emmer accessions also showed high grain yield, and these results suggested that high accumulation of Zn and Fe in grain in MM5/4 and 24-39 was not a function of low grain yield but rather under genetic control. An inverse relationship between grain yield and grain micronutrient concentrations has been reported for different types of germplasm by McDonald et al (2008) and Murphy et al (2008). McDonald et al (2008) propose that for a reliable interpretation and successful exploitation of the genetic variation for Zn, any possible dilution and concentration effects resulting from differences in grain yield of genotypes should be taken into account. Screening genotypes for high grain Zn or Fe concentrations without considering the grain yield capacity of genotypes will not necessarily result in selecting the targeted genotypes with high genetic capacity for grain accumulation of Zn or Fe.

Wild emmer wheat has also a high concentration of amino acids and proteins, and is being increasingly used in breeding for improving grain protein concentration of modern cultivars (Nevo 2001; Shewry 2007). High levels of proteins and amino acids might be important for improved bioavailability of Zn and Fe in the human body. Several studies report positive effects of both high protein concentrations and certain amino acids such as metionine, cysteine, and histidine on Zn and Fe bioavailability in the diet (Lonnerdal 2000; Swain et al 2002). These findings suggest that wild emmer has high potential for use in durum wheat improvement to increase both grain micronutrient concentration and Zn and Fe bioavailability.

Grain Protein: A Sink for Zinc and Iron

A positive, frequently close, relationship between the grain concentrations of protein, Zn and Fe has been found plant species such as wheat (Peterson et al 1986; Morgounov et al 2007), triticale (Feil and Fossati 1995), maize (Banziger and Long 2000), soybean (Raboy et al 1994), and wild emmer wheat (Peleg et al 2008). This finding implies the existence of an association of a general nature and suggests that the genes controlling the concentration of protein, Zn, and Fe are possibly co-segregating.

A close relationship between grain Zn and N concentration was also found in a survey study in Central Anatolia on soils differing in diethylene triamine pentaacetic acid (DTPA)-extractable Zn concentrations. Seed samples (40) collected at harvest from sorghum (*Sorghum bicolor*), durum wheat (*T. turgidum*), bread wheat (*T. aestivum*), maize (*Zea mays*), barley (*Hordeum vulgare*), canola (*Brassica napus*), soybean (*Glycine max*), safflower (*Carthamus tinctorius*), pea (*Pisum sativum*), common bean (*Phaseolus vulgaris*), and common vetch (*Vicia sativa*) grown during the same year in fields in the Konya region of Central Anatolia were analyzed for Zn and N concentrations. Additionally, soil samples from the same fields were analyzed for DTPA-extractable (plant-available) Zn. The relationship between DTPA-extractable Zn concentration in soil and Zn concentration in seeds was nonsignificant, but seed Zn and N concentrations were significantly and positively correlated (Fig. 2).

The close association between grain Zn, Fe, and protein was also demonstrated by staining Zn, Fe, and protein in a durum wheat cultivar (cv. Selcuklu) (Fig. 3). Protein and Fe were stained by using Coomassie Brilliant Blue G-25 dye (Bradford 1976) and Perls Prussian blue (Perls 1867; Prom-u-thai et al 2003), respectively. Treatment of seeds with Coomassie Brilliant Blue G-25 dye resulted in blue color formation on the longitudinally excised seed surfaces. Similar to the localization of Zn (Figs. 1 and 3), blue color intensity was high in the aleurone and embryo, indicating co-localization of Zn and protein. Also, Fe concentrated in the same parts of grain and endosperm shows very slight staining for Fe (Fig. 3). The aleurone and in particular the embryo contain in addition high amounts of free amino acids (Fig. 3) as estimated by staining amino acids on cut seed surfaces by ninhydrin. Ninhydrin reacts with the amino groups of amino acids to form the purple dye (Moore and Stein 1949).

The co-localization of Zn, Fe, protein, and amino acids (Fig. 3) and very positive correlations between grain protein and Zn and Fe indicate that grain proteins represent a sink for Zn and Fe. There is further evidence supporting this idea. In the embryo, Zn is mainly concentrated in the protein bodies that contain ≤600 mg/kg of Zn (Mazzolini et al 1985). Given that Zn plays a particular role in protein synthesis (Cakmak et al 1989; Marschner 1995), enhancement in protein biosynthesis as a result of increased N applications may also increase the sink strength for Zn. Ozturk et al (2006) concluded that highest accumulation of Zn in wheat seed occurs in the early stage of seed formation, the same stage during which the highest protein synthesis takes place (Greene 1983; Martre et al 2003). In biological systems, proteins are highly dependent on Zn ions to maintain their activities. Zinc is needed for numerous proteins, having both a catalytic and a structural role (Anzellotti and Farrell 2008). Zinc is required by a larger number of proteins than other metals. Proteomic analysis revealed that ≈10% of the proteome in eukaryotic cells are Zn-binding proteins and 40% of these Zn-binding proteins are transcription factors (Andreini et al 2006). Generally, the biological samples with high protein concentrations (animal-based foods, legumes) also contain high Zn concentrations. Similar to protein, phytate represents an important sink in grain for both Zn and Fe. Aleurone and embryo fractions of grain with high Zn concentrations are high in phytate (Lott and Spitzer 1980; Lin et al 2005).

The presence of strong positive correlation between grain Zn, Fe, and protein within grain tissue and the co-localization of the relevant loci on the same chromosome indicate close links among molecular and physiological mechanisms contributing to the grain accumulation of Zn, Fe, and protein. When the genes encoding for high concentration of Zn, Fe, and protein are closely linked, selection for high protein concentration in breeding will result through a correlated selection response, in a simultaneous increase in Fe and Zn concentration.

Effect of Chromosome 6B on Grain Zinc and Iron Concentrations

The knowledge base concerning the genetic control and molecular and physiological mechanisms affecting the accumulation of Zn and Fe in grain of wild emmer and durum wheat is incomplete. Studies with Langdon (durum wheat)-dicoccoides and Chinese Spring (bread wheat)-dicoccoides chromosome substitution lines suggest that major genes affecting the accumulation of Zn and Fe are located on chromosomes 6B of T. dicoccoides (Cakmak et al 2004). In studies using Chinese Spring-*dicoccoides* chromosome substitution lines, 5B and 6A chromosomes also were associated with increased grain Zn and Fe concentrations.

Because 6B and 5B chromosome substitution lines were also highest in content (total amount) of Zn and Fe per seed, respectively, high Zn and Fe concentrations were not related to small seed size effects. In previous studies, chromosome 6B of *T. dicoccoides* carried additional genes affecting concentration of grain N or protein (Joppa et al 1997; Chee et al 2001). The 6B chromosome substitution lines, as a result, exhibited superior pasta quality properties and equivalent grain yield compared with the recipient parent Langdon (Cantrell and Joppa 1991; Steiger et al 1996). For further molecular studies Joppa et al (1997) developed a 6B-mapping population derived from the wild emmer 6B chromosome and showed that 6B substitution lines contain the high grain protein content (GPC) and a quantitative trait locus (QTL) named *QGpc.ndsu.6Bb*. In the previous studies, introgressing the locus into durum wheat genetic backgrounds resulted in increased grain protein concentrations under various environmental conditions (Joppa et al 1997; Chee et al 2001). In subsequent research, Olmos et al (2003) mapped the high grain protein QTL *QGpc.ndsu.6Bb* and designated the name GPC-B1.

Distelfeld et al (2007) tested the recombinant substitition lines (RSLs) carrying the GPC-B1 locus from *T. dicoccoides* for accumulation of micronutrients in the grain. RSLs carrying the *dicoccoides* GPC-B1 allele displayed on average increases in grain micronutrient concentrations of 12% for Zn, 18% for Fe, and a 38% increase in concentration of protein, when compared with the RSLs carrying the GPC-B1 allele from the durum wheat Langdon (Distelfeld et al 2007). The positive effect of the GPC-B1 locus on concentrations of grain micronutrients and protein was consistent when evaluated in five different environments, and the differences found for grain yield could not explain the higher protein and micronutrient concentrations (Distelfeld et al 2007).

It appeared that the positive effect of the GPC-B1 allele on micronutrient concentrations is related to early senescence of the flag leaves. The RSL lines carrying the *T. dicoccoides* GPC-B1 allele senesced three to four days earlier compared with lines carrying the durum allele, and there was a close positive relationship between the leaf chlorosis and grain protein concentration (Uauy et al 2006; Distelfeld et al 2007). Based on these observations, Uauy et al (2006) suggested that the GPC-B1 locus promotes degradation of leaf proteins and thus N remobilization in source organs. In good agreement with this suggestion, lines with the *T. dicoccoides* GPC-B1 allele had a greater amount of amino acids in flag leaves during the anthesis period and contained less N in straw and higher protein concentrations in grain at grain maturation compared with lines carrying the durum wheat allele. These findings point to a role of the GPC-B1 locus in stimulating N translocation from vegetative tissues into grain (Kade et al 2005).

Possibly, also Zn and Fe are effectively remobilized and transported into the grain by the gene action of the *T. dicoccoides* GPC-B1 allele in vegetative tissue during senescence (Fahima et al 2006; Distelfeld et al 2007).

The GPC-B1 locus encoded a NAC transcription factor (NAM-B1) that contributes to grain concentration of protein, Zn and Fe, assumingly by accelerating leaf senescence and thus remobilization of amino acids, Zn, and Fe from flag leaves into seeds (Uauy et al 2006). An inhibition in expression of NAM genes resulted in a corresponding decrease in grain concentration of micronutrients and associated delay in leaf senescence (Uauy et al 2006). Consequently, the NAM genes from *T. dicoccoides* were proposed as potentially useful candidate genes for improving commercial wheat for both micronutrient and protein concentrations. However, Uauy et al (2006) did not report grain yield data. And the extent to which changes in grain micronutrient concentration were due to differences in grain yield was not quantified. Additional research under different environmental conditions is warranted to better understand the role of NAM genes and determine the potential of NAM genes in applied breeding as accelerated senescence can negatively affect grain yield (Gregersen et al 2008). In a recently published annual report by Kidwell et al (2008), introgression of the *T. dicoccoides* GPC-B1 allele into two durum wheat cultivars did not result in a change in plant senescence and grain protein content under field conditions over two years. However, under greenhouse conditions, the *T. dicoccoides* GPC-B1 allele was effective in accelerating flag leaf senescence, suggestive of a significant interaction of the GPC-B1 gene with environmental conditions.

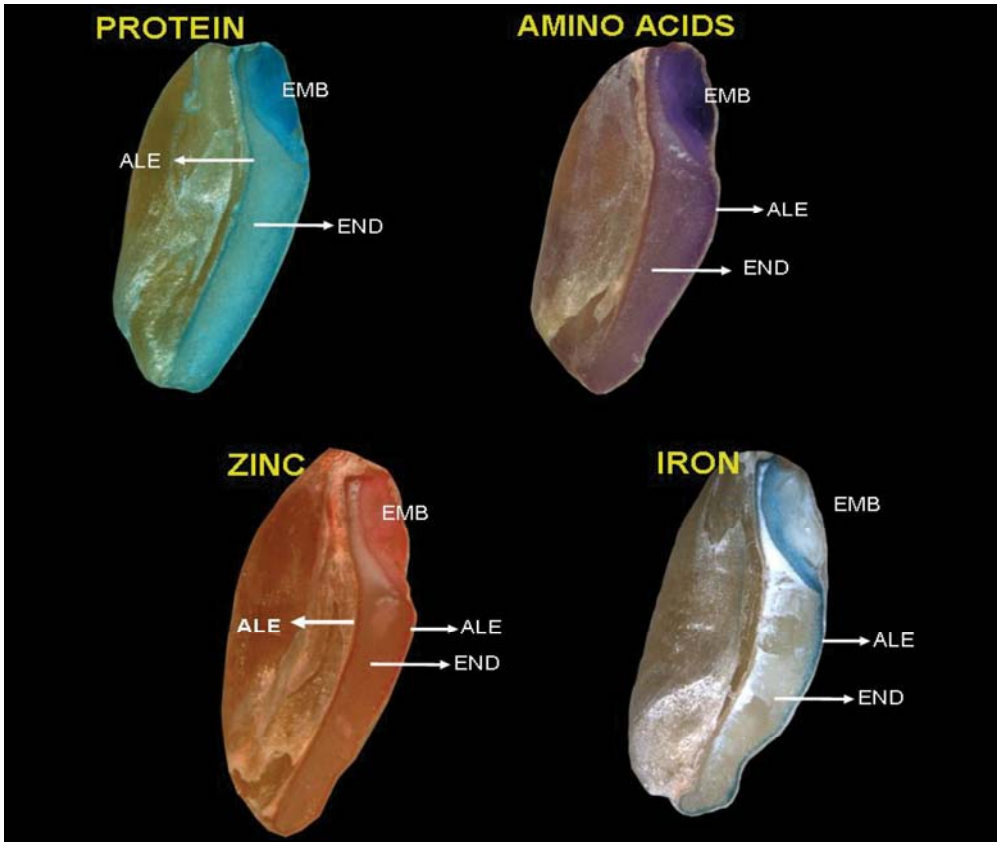

Fig. 3. Staining and localization of protein, amino acids, Zn and Fe in durum wheat cultivar Selcuklu containing 38 mg of Zn/kg, 54 mg of Fe/ kg and 18 g of protein/kg. Staining of longitudinally cut seed surface by Bradford reagent diluted by 2:1 (v/v) absolute ethanol (incubation at 70°C for 15 min) for protein, ninhydrin in 1% (w/v) ninhydrin dissolved in absolute ethanol (incubation at 70°C for 45 sec) for amino acids, dithizone in 500 mg/L 1,5-diphenyl thiocarbazone dissolved in absolute methanol (incubation at room temperature for 30 min) for Zn and Perls Prussian blue in 2% (w/v) potassium ferrocyanide dissolved in 2% (w/v) hydrocloric acid (incubation at room temperature for 30 min) for Fe. EMB, embryo; ALE, aleurone; END, endosperm.

Role of Nitrogen on Uptake and Transport of Zinc and Iron

Nitrogen nutritional status of plants may also exert positive effects on root uptake of Zn and Fe. Recently, we showed that positive correlations between grain Zn and protein concentrations occur under high soil applications of Zn and N (*unpublished*). The positive impact of improved N nutrition on Zn and Fe concentration in plants is relevant and demands further clarification. There are several steps during uptake and transport of Zn and Fe in plants which might be affected by N nutrition. By affecting root growth and stimulating root exudation of organic compounds (Marschner 1995; Paterson et al 2006), N may influence the mobility and root uptake of Zn and Fe from soils. In maize, increasing N application was effective in enhancing C partitioning into roots and promoting exudation of C-containing compounds from roots into rhizosphere (Liljeroth et al 1994). The expression level of Zn and Fe transporter proteins located on the root cell membranes such as ZIP family transporter proteins (Grotz and Guerinot 2006) might be affected by the plant N nutritional status. These transporter proteins greatly influence uptake and accumulation of Zn and Fe in plant cells. Expression of the genes encoding a Zn-transporter protein from *Arabidopsis thaliana* in roots of a barley genotype caused an increase in grain Zn concentration (Ramesh et al 2004). To our knowledge, there is no published data concerning the effects of increasing N nutrition on expression level or the amount of transporter proteins for Zn and Fe in root cells. Enhanced knowledge of the effects of N on the amount or expression levels of transporter proteins of Zn and Fe will contribute to better understanding the positive association between N, Zn, and Fe in seeds.

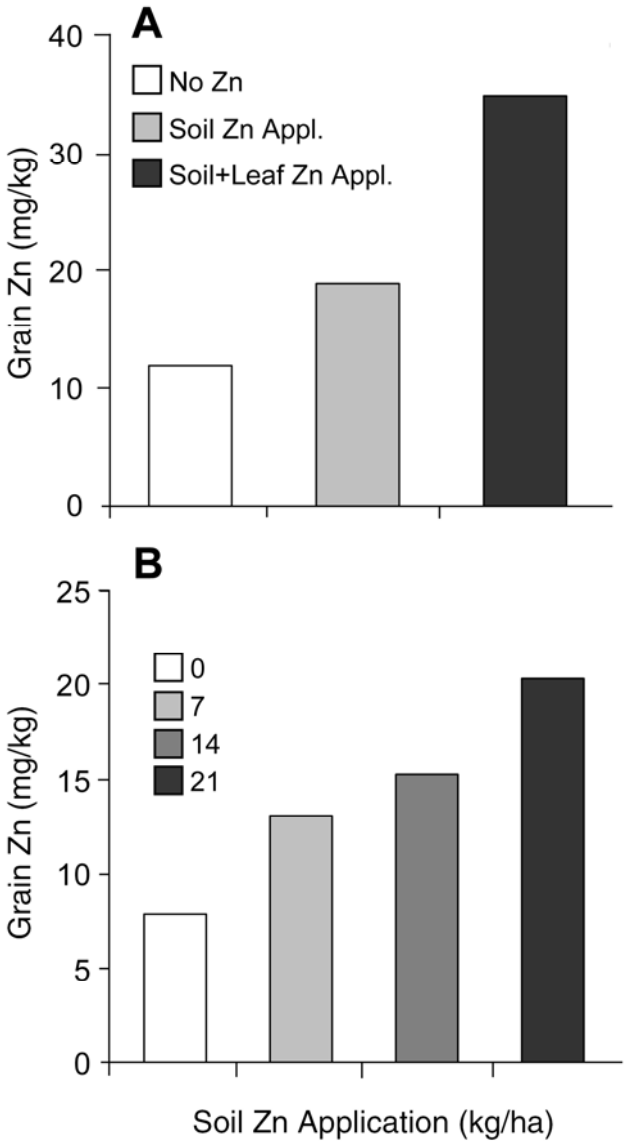

Fig. 4. Changes in grain Zn concentrations of durum wheat after soil and foliar application of $ZnSO_4$ (**A**) and application of increasing amount of Zn to soil (**B**). Eexperiments conducted on soil highly deficient in Zn under field conditions in Central Anatolia. Further details in Yilmaz et al (1997) and Ekiz et al (1998).

Following root absorption, Zn and Fe are transported into the shoot through xylem vessels either as free ions or chelated to low molecular-weight organic compounds. Similar to root uptake, root-to-shoot transport of Zn and Fe could be as well facilitated by N, either by affecting the levels of proteins contributing to xylem loading or chelation of Zn in the xylem by nitrogenous compounds for xylem transport by nicotianamine and mugineic acid family phytosiderophores (Mori et al 1991; Alam et al 2001; Curie et al 2009). Methionine, a sulfur-containing proteinogenic amino acid, is a critical precursor in the biosynthesis of nicotianamine and mugineic acid family phytosiderophores (Mori and Nishizawa 1987). To our knowledge, there is no published information on the subject of how reduced levels of methionine caused by low N supply affect biosynthesis and root release rate of phytosiderophores. Most likely low N supply may reduce the pool of methionine and in turn negatively affect the biosynthesis of phytosiderophores. In agreement with this hypothesis, Astolfi et al (2006) provided evidence of a sharp decrease in root release of phytosiderophores in barley plants treated with low sulfur. Phytosiderophores have a high ability to chelate Zn and Fe and contribute to their acquisitions by cereal crops (Marschner and Romheld 1994). Both biosynthesis and root release of phytosiderophores are stimulated by Zn and Fe deficiency in wheat plants (Mori et al 1991; Cakmak et al 1994; Suzuki et al 2006). Notably, the release of phytosiderophores under Zn deficiency is in general lower in durum wheat compared with bread wheat, a finding that has been discussed as major cause of higher susceptibility of durum wheat to Zn deficiency (Cakmak et al 1996a,b; Rengel and Romheld 2000). Recently, Suzuki et al (2008) established that deoxymugineic acid stimulated Zn transport from roots into shoot in rice plants. Similarly, Fe uptake and transport in plants is promoted by supply of Fe-deoxymugineic acid (Alam et al 2005; Tsukamato et al 2009).

It is important to emphasize that increased absorption of a micronutrient by roots does not always correlate with high grain accumulation of this micronutrient, as recognized for Fe in rice (Grusak et al 1999). As discussed previously, wild tetraploid wheat has a much higher grain Zn and Fe concentration than contemporary durum wheat cultivars. However, when grown under the same

TABLE II
Effects of Various Zn Application Methods[a]

Zn Application Method	Cultivar	Grain Zn (mg/kg)	Grain P (g/kg)	Phytic Acid (g/kg)	Phytate/Zn Molar Ratio
Control	Kunduru	11	4.2	11.8	112
	Dagdas	8	4.3	12.0	140
Soil	Kunduru	17	3.3	8.7	53
	Dagdas	16	3.3	9.5	62
Leaf	Kunduru	19	4.0	10.3	56
	Dagdas	28	3.5	9.8	35
Soil + Leaf	Kunduru	33	3.9	9.7	30
	Dagdas	36	3.8	10.0	29

[a] Data for grain concentrations of Zn, P, phytate, and phytate/Zn molar ratios in bread wheat (cv. Dagdas) and durum wheat (cv. Kunduru) grown on a highly Zn-deficient soil under field conditions in Central Anatolia (see also Yilmaz et al [1997] for details of Zn application methods; Erdal [1998]).

conditions, durum wheat cultivars and wild emmer wheat accessions have similar shoot Zn and Fe concentrations in the early growth stages (*unpublished results*).

These observations suggest that retranslocation of micronutrients deposited in shoot tissues plays a critical role in the grain accumulation of micronutrients. In wheat, ≤70% of the amount of Zn in vegetative plant parts is remobilized (Grewal and Graham 1999; Zubaidi et al 1999), whereas only 20% of Fe is remobilized in rice (Grusak et al 1999). Genotypic differences in grain yield may also affect grain concentrations of micronutrients despite a similar root uptake rate or shoot accumulation of micronutrients. These findings should be taken into consideration in evaluating plant genotypes for their capacity to accumulate Zn and Fe in the grain.

Remobilization and retranslocation of Zn and Fe from vegetative tissues into seeds through the phloem may be affected by the level of N nutrition. Zinc and Fe transporter proteins located on root cell membranes have also been identified on the plasma membranes of phloem cells, suggesting that these transporters are possibly involved in phloem transport of Zn and Fe into seeds (Haydon and Cobbettt 2007; Curie et al 2009). The transport forms of Fe and Zn in the phloem are still not well understood. A high pH of phloem sap is a major factor reducing solubility and translocation of Zn and Fe into sink organs. By affecting the pool of amino acids and peptides, N nutrition may contribute to chelation of Zn and Fe and promote their transport through phloem into seeds. Amino acids have been discussed as possible candidate ligands required for phloem transport of Fe and Zn (Grusak et al 1999). Nicotianamine (NA) is a well-documented chelating compound for Zn and Fe; it contributes to cellular distribution and phloem transport of Zn and Fe into seeds (Schmidke and Stephan 1995; von Wirén et al 1999; Haydon and Cobbett 2007). In phloem sap collected from *Ricinus* plants, the concentration of nicotianamine was estimated at ≈200 μ*M* by Stephan and Sholz (1993) and this concentration might be affected by N nutrition. Therefore, it would be revealing to further investigate the levels of nicotianamine in wild and modern tetraploid wheat contrasting in grain Zn and Fe concentration.

Enhancing Durum Wheat Grain Zinc and Iron Through Fertilizer Applications

Breeding biofortified wheat with high grain Zn and Fe concentration is a promising and cost-effective strategy to alleviate micronutrient malnutrition. The current HarvestPlus biofortification strategy exploits the genetic diversity for Zn and Fe reported for many crops, especially in wild wheat relative species (White and Broadley 2005) (Table I) using conventional breeding and more modern biotechnology. However, crop improvement to develop micronutrient dense wheat is a longer term breeding effort and requires extensive germplasm screening, crossing and developing early, intermediate, and final stage products, and intensive performance testing in target areas under different environmental conditions. In several target countries and target zones with high prevalence of Zn and Fe deficiency, soils cultivated to wheat are prone to adverse chemical and physical conditions that reduce the chemical solubility and plant availability of Zn and Fe. Low soil moisture, high soil pH, and high $CaCO_3$ content and low amount of organic matter decrease severely solubility and availability of Zn and Fe in the soil. As a consequence, absorption of Zn or Fe at amounts adequate for better crop production and higher grain mineral concentrations are significantly depressed (Cakmak 2008). For example, increasing soil from pH 6 to 7 reduces chemical solubility of Zn in soil by nearly 30-fold (Marschner 1993). Under such adverse chemical soil conditions, the genetic potential of biofortified wheat can only be partially expressed. Hence, it is critical to maintain an adequate amount of available Zn or Fe in the soil during plant growth because continued root uptake and transport into seeds during the seed-filling period is an important means of seed micronutrient accumulation (Waters and Grusak 2008). Further, maintaining adequate Zn transport from vegetative tissues into the seeds during reproductive growth stages (Hasslett et al 2001; Cakmak 2008) can be an important strategy in reducing variations in grain mineral concentration caused by environmental fluctuations and in producing and further enhancing grain Zn concentration. When feasible in a given target country, Zn fertilizer approach seems to be an effective way to improve grain Zn concentration in wheat and a useful complementary approach to on-going breeding programs.

Soil or foliar application of Zn-containing fertilizers greatly improved grain Zn concentrations in both durum and bread wheat (Cakmak 2008). In field trials in Central Anatolia, a well-known highly Zn-deficient region of Turkey (Cakmak et al 1996c), applying $ZnSO_4$ to soil enhanced both grain yield (Yilmaz et al 1997; Ekiz et al 1998) and grain Zn concentration of durum wheat (Fig. 4). An increase in grain Zn concentration by soil Zn application was almost twofold, whereas combined application of Zn through soil and foliarly was more effective and resulted in a more than threefold increase in Zn concentration in durum wheat grain (Fig. 4). Similar increases in grain concentrations of Zn in wheat following soil Zn application were also seen in Australia (Graham et al 1992) and India (Shiway et al 2008) under field conditions.

Furthermore, results revealed that the Zn nutritional status of wheat plants affected the grain concentrations of phosphorus (P) and phytic acid (PA). Table II displays the effect of various Zn application methods on grain P and phytic acid concentrations in bread and durum wheat. Under Zn-deficient soil conditions, Zn application dramatically increased grain Zn and simultaneously reduced grain P concentrations, especially for the soil Zn application treatment (Erdal 1998).

Decreases in grain P concentration by Zn applications are associated with a corresponding decrease in grain phytate concentration and the phytate to Zn molar ratios (Table II). Phytate is the major P storage compound in cereal grains and has a high potential for binding Zn and Fe, making them less soluble and less bioavailable for humans (Wise 1995; Lott et al 2000). Formation of insoluble phytate complexes of Zn and Fe are suggested to be a major reason for a high incidence of micronutrient deficiency in countries with diets high in phytate (Gibson 2006; Rimbach et al 2008). The phytate to Zn molar ratio is commonly used to estimate Zn bioavailability in food (Oberlas and Harland 2005; Hambidge et al 2008). In field trials in Central Anatolia, soil Zn application combined with foliar application significantly decreased phytate to Zn molar ratios in grain of both durum and bread wheat (Cakmak et al 1999a) (Table II) and these effects may result in significant effects on Zn nutritional status of populations relying on cereals as a micronutrient source.

Such important decreases in grain P and phytic acid after Zn fertilization might be a consequence of increasing grain yield (and thus a dilution effect). However, the decreasing effect of Zn fertilization on grain P and phytic acid was also found in rye that is highly tolerant to Zn deficiency and its yield was very slightly affected by Zn deficiency (Cakmak et al 1997; Erdal 1998). The effect of Zn on grain P under Zn-deficient conditions seems to be specific and most probably related to Zn-deficiency-induced root uptake of P (Loneragan et al 1982; Cakmak and Marschner 1986). In contrast to positive effects of Zn fertilization on grain Zn concentrations, recent results indicate that soil or foliar applications of various organic and inorganic forms of Fe fertilizers cannot influence grain Fe concentrations of durum wheat (B. Aciksoz et al, *unpublished results*). It should be borne in mind that, compared with Zn, Fe is less phloem mobile in cereals (Marschner 1995; Grusak et al 1999). The use of expensive Fe chelates, an otherwise suitable source of Fe to improve Fe concentration, is not cost-effective and does not appear as a feasible approach (Rengel et al 1999).

CONCLUSIONS

Growing evidence is now available indicating that wild and primitive wheats show large and useful genetic variation for grain concentrations of Zn and Fe. This genetic variation is now being intensively exploited under HarvestPlus program to improve modern wheat cultivars for both high concentrations and high bioavailability of Zn and Fe. Positive effects of N nutrition on uptake of Zn and Fe, a very close relationship between the concentrations of grain Zn, Fe, and protein, and co-localization of the genes on same chromosomes affecting their concentrations are highly important and relevant for further research. In designing breeding programs aimed at improving grain Zn and Fe concentrations, special attention should be paid to the association between Zn, Fe, and protein. Zinc fertilizer strategies can also provide an immediate and effective option to increase grain Zn concentration and productivity in wheat, particularly with severe soil Zn deficiency. However, fertilizer strategies must be practical and economically feasible. In low-income countries where resource-poor farmers do not have access to or cannot afford fertilizer, breeding for mineral density may remain the sole agricultural intervention to improve the nutritional content of staple crops.

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