ROLES OF NITROGEN AND ZINC NUTRITION IN
BIOFORTIFICATION OF WHEAT GRAIN

by
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ABSTRACT

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Keywords: Biofortification, iron, nitrogen, wheat, zinc

Deficiencies of zinc (Zn) and iron (Fe) are widespread nutritional problems, caused mainly by low dietary intake. Biofortification of cereal grains with Zn and Fe in order to alleviate the health problems associated with these deficiencies is a global challenge. Based on the hypothesis that nitrogen (N) nutrition may affect the transporter proteins and other nitrogenous molecules which are involved in root uptake, root-to-shoot transport, remobilization, phloem transport and grain accumulation as well as grain localization of Zn and Fe, the potential of N fertilization in biofortification of wheat grain was investigated in this project. For this purpose, wheat plants were grown with different N and Zn treatments under greenhouse or growth chamber conditions. Increasing N application improved the grain Zn and Fe concentrations by up to 100%. This impact of N on grain Zn concentration disappeared at low Zn supply, whereas the combination of high N and Zn treatments gave rise to synergistic results. Under high Zn availability, higher N supply increased the shoot Zn and Fe contents by up to 300%, indicating a tremendous enhancement in root uptake. Higher N application also led to a 240% increase in Zn and 70% increase in Fe remobilization to grains. Improving the N nutrition enhanced the Zn and Fe concentrations not only in the whole grain but also the endosperm, the most widely consumed part of wheat grain. As an agronomic biofortification tool, optimized N applications may rapidly and effectively contribute to the mitigation of Zn and Fe deficiency problems in developing countries.

ÖZET

BUĞDAY TOHUMUNUN BİYOFORTİFİKASYONUNDA
AZOT VE ÇİNKO BESLENMELERİNİN ROLLERİ

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Anahtar sözcükler: Azot, biyofortifikasyon, buğday, çinko, demir

Çinko (Zn) ve demir (Fe) eksiklikleri, genellikle yetersiz tüketimden kaynaklanan, yaygın beslenme sorunlarıdır. Bu eksikliklerle ilgili sağlık sorunlarının hafifletilmesi için, tahıl tohumlarının Zn ve Fe ile biyofortifikasyonu (zenginleştirilmesi), küresel bir meseledir. Azot (N) beslenmesinin Zn ve Fe'nin alımında, yeşil aksama taşınmasında, remobilizasyonunda, floem taşınmasında ve tohumda biriktirilmesi ile tohumdaki lokalizasyonunda rol oynayan taşıyıcı proteinleri ve başka azotlu molekülleri etkileyebileceği hipotezine dayanarak, bu projede, N beslenmesinin buğday tohumunun biyofortifikasyonuna yönelik potansiyeli araştırılmıştır. Bu amaçla, buğday bitkileri, farklı N ve Zn uygulamaları ile sera veya iklim odası koşullarında yetiştirilmiştir. Artan N uygulaması tohumun Zn ve Fe derişimlerini %100'e varan oranlarda arttırmıştır. Azotun tohum Zn derişimine olan bu etkisi yetersiz Zn uygulaması koşullarında kaybolmuştur; buna karşın, yüksek N ve Zn uygulamalarının kombinasyonu sinerjik sonuçlar doğurmuştur. Yüksek Zn varlığında, artan N uygulaması yeşil aksamın Zn ve Fe içeriklerini %300'e kadar çoğaltmıştır ki, bu da bu elementlerin kök alımında çok belirgin bir artışa işaret etmektedir. Ayrıca, artan N gübrelemesi, Zn'nin remobilizasyonunun %240, Fe'nin remobilizasyonunun ise %70 oranında arttırmıştır. Azot beslenmesinin iyileştirilmesi, Zn ve Fe derişimlerini sadece tohumun tamamında değil, buğday tohumunun en yaygın tüketilen bölümü olan endospermde de yükselmesini sağlamıştır. Bir tarımsal biyofortifikasyon aracı olarak, optimize edilmiş N uygulamaları, gelişmekte olan ülkelerdeki Zn ve Fe eksikliği sorunlarıyla mücadeleye hızlı ve etkin biçimde katkı sağlayabilir.

This work is dedicated

to my *Imzadi*, **Bahar Yıldız**,

without whose love and support I would not have accomplished it;

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA ....analysis of variance

B .... boron

ca ....circa (approximately)

$CaCO_3$ .... calcium carbonate

$Ca(NO_3)_2.4H_2O$ .... calcium nitrate tetrahydrate

$CaSO_4.2H_2O$ .... calcium sulfate dihydrate

Conc. ....concentration

Cont. ....continued

Cu .... copper

$CuSO_4.5H_2O$ .... copper sulfate pentahydrate

cv. .... cultivar

dH_2O ....distilled water

Discont. .... discontinued

DMA ....deoxymugineic acid

DTPA ....diethylenetriamine pentaacetic acid

eg. .... exempli gratia (for example)

FAO ....Food and Agricultural Organization

Fe ....iron

Fe^{2+} ....ferrous iron

Fe^{3+} .... ferric

Fe-EDTA .... iron ethylenediamine tetraacetic acid

FRD3.. ferric reductase defective 3

Gpc-B1 .... high grain protein content gene located on chromosome B1

H_3BO_3 .... boric acid

H_2O_2 ....hydrogen peroxide

HMA .... heavy metal ATPase

HNO_3 .... nitric acid

ICP-OES .... inductively coupled plasma optical emission spectrometry

ITP .... iron transport protein

K.. potassium

KCl...potassium chloride

KH_2PO_4 .... potassium dihydrogen phosphate

K_2SO_4 .... potassium sulfate

LSD .... least significant difference

MA .... mugineic acid

$MgSO_4.7H_2O$ .... magnesium sulfate heptahydrate

Mn .... manganase

$MnSO_4.H_2O$ .... manganese sulfate monohydrate

N .... nitrogen

NA .... nicotianamine

NAM-B1 .... the no apical meristem allele located on chromosome B1

$(NH_4)_6Mo_7O_{24}.4H_2O$ .... ammonium heptamolybdate (paramolybdate) tetrahydrate

$NiCl_2.6H_2O$ .... nickel chloride hexahydrate

NRAMP .... natural resistance-associated macrophage protein

P .... phosphorus

RDA .... recommended dietary allowance

S .... sulfur

SD .... standard deviation

Std. .... standard

v/v .... volume per volume

VIT .... vacuolar iron transporter

WHO .... World Health Organization

w/v .... weight per volume

YSL .... yellow stripe-like

ZIP .... zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like proteins

Zn .... zinc

$ZnSO_4.7H_2O$ .... zinc sulfate heptahydrate

(A) GENERAL INTRODUCTION

A.1. The Prevalence of Zinc and Iron Deficiencies and Associated Health Problems

Micronutrient malnutrition is a global health problem affecting over two billion people in developing countries and more than three billion people worldwide (Graham et al., 2001; Welch & Graham, 2004; Cakmak et al., 2010). Zinc (Zn) and iron (Fe) deficiencies are the most common micronutrient deficiencies (Welch & Graham, 2004; Cakmak et al., 2010). In the World Health Report published by the World Health Organization (WHO) in 2002, deficiencies of Zn and Fe rank fifth and sixth, respectively, in the list of leading causes of disease in developing high-mortality countries, and eleventh and ninth, respectively, among global health risk factors.

Health complications associated with Zn deficiency include, among others, stunting in children, high susceptibility to infectious diseases including pneumonia, diarrhea and malaria due to weakened immune system, impaired mental development, impaired wound healing, poor birth outcomes in pregnant women and increased morbidity and mortality (Hotz & Brown, 2004; Fraga, 2005; Black et al., 2008). According to a recent report, deficiencies of Zn and vitamin A are globally the most serious micronutrient deficiencies among children and represent major causes of child death (Black et al., 2008). Zinc deficiency alone is responsible for ca. 450,000 deaths among children under 5 years of age, which is 4.4% of the worldwide child deaths in this age group (Black et al., 2008).

Iron deficiency impairs physical growth, mental development and learning capacity in children, while it increases the frequency of childbirth complications and reduces stamina and productivity in adults (Bouis, 2003; Kennedy et al., 2003). Moreover, Fe deficiency is the most common cause of anemia (Kennedy et al., 2003; Fraga, 2005). Because of the high Fe demand during infancy and pregnancy, young

children and pregnant and postpartum women are the most commonly and severely affected population groups (WHO, 2002). In developing countries, one-fifth of perinatal and one-tenth of maternal mortality can be attributed to Fe deficiency (WHO, 2002).

A.2. The Reasons behind the Deficiencies of Zinc and Iron

Insufficient diversity in diet and low dietary intake of Zn and Fe have been discussed as major reasons for the high prevalence of micronutrient deficiencies in human populations (Bouis, 2003; White & Broadley, 2009; Cakmak et al., 2010). In countries, where Zn and Fe deficiencies are documented as a major public health concern, cereal-based foods are the predominant source of daily calorie intake (Hotz & Brown, 2004; Cakmak, 2008; Gibson et al., 2008). Among the staple food crops, wheat is the most important food crop in a number of developing countries with respect to its contribution to the daily calorie intake (FAO Database, 2003; Cakmak, 2008). Wheat accounts for 20% of the global daily calorie intake and over 50% of the calorie intake in many developing countries (FAO database, 2003). This percentage most probably exceeds 70% in rural regions (Cakmak, 2008).

Wheat is, however, inherently too poor in Zn and Fe to meet the demands of human beings for these micronutrients. Grains of commercial wheat cultivars generally contain 20-35 mg Zn or Fe per kg (Erdal et al., 2002; Cakmak et al., 2004; Cakmak et al., 2010). Nearly half of the cereal-growing land in the world is affected from low availability of Zn to plant roots due to a variety of adverse chemical and physical conditions, such as high level of pH, low levels of organic matter and soil moisture (Alloway, 2004; Cakmak, 2008). As a result, Zn deficiency is also commonly observed in crop plants including wheat. When grown on Zn-deficient soils without supplemental Zn, grain Zn concentration of wheat is reduced below 10-15 mg Zn per kg grain, as shown under field conditions in Iran, India, Turkey and Australia (Graham et al., 1992; Cakmak et al., 1999; Erdal et al., 2002; Alloway, 2004). Because wheat, like other cereals, releases phytosiderophores to acquire Fe (Marschner & Romheld, 1994), Fe deficiency is not observed in wheat under field conditions, but the present Fe concentration of wheat grain is usually inadequate for human nutrition.

In addition to inadequate intake, low bioavailability of Zn and Fe in wheat grain is a further major factor behind Zn and Fe deficiency in human beings. Wheat grain is poor in bioavailability promoters (e.g. organic acids) and rich in antinutrients including phytate (myo-inositol bis-hexaphoshate), polyphenolics, fibers and lectins, which result in poor absorption in the gut and thus effectively reduce the bioavailability of Zn and Fe (Welch & Graham, 2004; White & Broadley, 2005; White & Broadley, 2009).

A.3. How to Tackle the Global Zinc and Iron Deficiency Problem

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 has been shown to be a promising way to reduce child deaths in India (Stein et al., 2007). Food fortification and supplementation are effective interventions for tackling micronutrient malnutrition and widely applied in some countries, but these are expensive strategies and cannot easily access people living in rural regions of developing countries, i.e. people who need them the most (Bouis, 2003; Stein et al., 2007; Pfeiffer & McClafferty, 2007). The most promising strategy for alleviating the global micronutrient deficiency problem is biofortification, which is the biological enrichment of staple food crops with micronutrients. Agronomic biofortification coupled to breeding for high Zn and Fe content and bioavailability in edible parts of staple foods appears to be the most sustainable and cost-effective approach (White & Broadley, 2005; Pfeiffer & McClafferty, 2007; Cakmak, 2008; White & Broadley, 2009). Nitrogen nutrition of plants appears to be a critical component for an effective biofortification of food crops with Zn and Fe due to several physiological and molecular mechanisms which are under the influence of N nutritional status (Cakmak et al., 2010).

A.4. The Link between Nitrogen and Zinc

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 up to 10% of the human proteome consists of Zn-binding proteins and nearly 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 speciation and localization data, as discussed below, suggest that protein is a sink for Zn.

Grain proteins may contribute to the accumulation of Zn by increasing the sink strength of the grain for Zn. This hypothesis is supported by the high positive correlations between seed protein and seed Zn (and also seed Fe) found in several wheat germplasms (Peterson et al., 1986; Zebarth et al., 1992; Feil & Fossati, 1995; Morgounov et al., 2007; Peleg et al., 2008). One reason why pulses generally contain higher Zn than cereal grains might be related to the higher protein concentrations of pulses. Most of the Zn in cereal 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 & Buttrose, 1978; Welch, 1986). Protein-Zn-phytate complexes are probably the predominant Zn species in wheat grain (Lott et al., 1995). In barley grain, Zn is mainly bound to peptides (Persson et al., 2009). In wheat or maize embryo, the Zn concentration in the protein bodies can reach up to 600 mg kg^{-1} (Mazzolini et al., 1985; Marschner, 1995). The protein-rich embryo and aleurone of wheat seeds are also rich in Zn, whereas the endosperm, which is low in protein and phytate, is at the same time low in Zn (Lott et al., 1995; Welch & 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. In a study by Ehret (1985), whole wheat grains had 27 mg kg^{-1} Zn and 14.2% protein, whereas the embryos of the same grains had 226 mg kg^{-1} Zn and 42% protein.

A 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 (*Triticum turgidum ssp. dicoccoides*) accumulated not only higher concentrations of protein but also higher concentrations of Zn and Fe in the grain, as compared to lines carrying the allele from cultivated durum wheat (Distelfeld et al., 2007). The *Gpc-B1* locus has been shown to be responsible for the remobilization of Zn, Fe and N (amino acids) from senescing leaf tissues into grain through the action of *NAM-B1* gene (Uauy et al., 2006a, b; Waters et al., 2009). These results indicate that at least some genes affecting the grain accumulations of Zn, Fe and protein are closely linked as shown in *Triticum dicoccoides* (Cakmak et al., 2004; Distelfeld et al., 2007; Uauy et al., 2006a, b).

A.5. The Questions Addressed in this Project

The first step was the investigation of the effects of varied N nutrition on the shoot and grain concentrations of Zn and Fe in wheat. In Chapter I, the great potential of soil and foliar applications of N combined with soil and foliar applications of Zn in biofortifying wheat with Zn and Fe is documented based on the results of soil culture experiments conducted under greenhouse conditions.

As the second step, a nutrient balance experiment was carried out in order to study the impact of N nutritional status on the dynamics of Zn and Fe throughout the ontogenesis of wheat. The critical data collected in this soil culture experiment about how N affects the total shoot content, partitioning and remobilization of Zn and Fe at various stages of wheat development are reported in Chapter II. These results have important implications for the mechanisms underlying biofortification through N fertilization.

Under field conditions, the uptake of nutrients from the soil is often restricted during the grain-filling stage due to various stress factors such as drought. Chapter III reports and discusses the results of a model nutrient solution experiment, carried out in growth chamber in order to study the effects of N nutrition on the Zn distribution and grain allocation under conditions of restricted uptake of Zn after anthesis.

Finally, Chapter IV focuses on the endosperm of wheat grain, which is much more relevant than the whole grain from the point of view of human nutrition, because this part is the most widely consumed portion of the grain in many developing countries including Turkey. The effects of N nutrition on the Zn and Fe concentrations of different grain fractions including the endosperm were examined in the final soil culture experiment, the results of which are reported and discussed in the final chapter.

(B) GENERAL MATERIALS AND METHODS

In all experiments documented here, Balcali2000, a Turkish durum wheat cultivar (*Triticum durum* cv. Balcali2000) was cultivated as described below.

B.1. Plant Growth Facilities

B.1.1. Greenhouse

All soil culture experiments were conducted in a greenhouse under natural daylight. The geographic coordinates of the greenhouse are 40° 53' 24.5" N and 029° 22' 46.7" E. The greenhouse is equipped with a heating system and an evaporative cooling system, which keep the temperature inside the greenhouse in the range of 15-25˚C depending on the season and day time.

B.1.2. Growth Chamber

The solution culture experiment was carried out in a growth chamber under controlled climatic conditions (light/dark periods: 16/8 h; temperature (light/dark): 22˚C/18˚C; relative humidity (light/dark): 60%/70%; photosynthetic flux density: 400 $\mu mol\ m^{-2}\ s^{-1}$).

B.2. Soil Culture

Seeds were sown in plastic pots containing 3.1 kg Zn-deficient soil that was transported from a Zn-deficient location in Eskisehir, Central Anatolia (Cakmak et al., 1996a). The soil used is a calcareous (18% $CaCO_3$) and alkaline (pH 8.0 in dH_2O) soil with clay-loam texture and low organic matter content (1.5%). The diethylenetriamine pentaacetic acid (DTPA)-extractable Zn concentration was 0.1 mg kg^{-1} soil as determined by using the method described by Lindsay and Norvell (1978).

Before sowing the seeds, the following nutrients were homogeneously incorporated in the experimental soil (per kg dry soil): 100 mg phosphorus (P) in the form of KH_2PO_4, 25 mg sulfur (S) in the form of K_2SO_4 and 2.5 mg Fe in the form of Fe-EDTA, N in the form of $Ca(NO_3)_2.4H_2O$ and Zn in the form of $ZnSO_4.7H_2O$. Different amounts of N and Zn were used, depending on the experimental design. When the plants reached the Zadoks stage 49 (first awns visible) in ca. 45 days old, 50 mg P per kg soil was added to all pots in the form of KH_2PO_4. Ten seeds were sown in each pot. The seedlings were thinned to 4 or 5 per pot, depending on the experiment, shortly after emergence. The pots were watered daily with deionized water. Each pot had an independent saucer for avoiding the uncontrolled loss of nutrients dissolved in water flowing out.

B.3. Solution Culture

Seeds were imbibed in saturated $CaSO_4.2H_2O$ solution for half an hour and germinated in perlite moisturized with saturated $CaSO_4.2H_2O$ solution for 4-5 days at room temperature before being transferred to solution culture. Seedlings were grown in plastic pots containing 3 L of nutrient solution consisting of 0.9 mM K_2SO_4, 0.2 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 0.1 mM KCl, 100 μM Fe-EDTA, 1 μM H_3BO_3, 0.5 μM $MnSO_4.H_2O$, 0.2 μM $CuSO_4.5H_2O$, 0.2 μM $NiCl_2.6H_2O$ and 0.14 μM $(NH_4)_6Mo_7O_{24}.4H_2O$. Zinc was supplied in the form of $ZnSO_4.7H_2O$, and different concentrations of N were established by adding $Ca(NO_3)_2.4H_2O$. Lower N pots were

supplemented with $CaSO_4.2H_2O$ for complementing missing Ca. Nutrient solutions were continuously aerated and refreshed every 3-4 days.

B.4. Harvest

All harvested plant samples were washed in deionized water and dried at 60℃. Grains were manually separated from husk. Dried samples were weighed at room temperature for biomass and yield determination.

B.5. Element Analysis

Dry samples were ground to fine powders in an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany). For the analysis of mineral nutrients other than N, ground samples were subjected to acid-digestion (ca. 0.2 g sample in 2 ml 30% H_2O_2 and 5 ml 65% HNO_3) in a closed vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). After digestion, the total sample volume was finalized to 20 ml by adding double-deionized water. Concentrations of mineral nutrients including boron (B), potassium (K), P, Cu, Fe, Mn and Zn were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia). The N concentrations in the samples were determined by using LECO TruSpec C/N Analyzer (Leco Corp., St Joseph, MI, USA). Measurements were checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

B.6. Calculations

The mineral concentrations other than N in the samples were calculated by multiplying the values measured by ICP-OES with the dilution factor, which is calculated for each sample separately by dividing the total sample volume (ml) by the dry weight (g) of the digested sample.

For calculating the mineral contents for a given plant part, the calculated mineral concentrations were multiplied by the measured total dry weights of the concerned plant part. Similarly, the grain mineral yields, i.e. the total amounts of minerals of interest deposited in the grains, were determined by multiplying the grain yield by the grain mineral concentrations.

The harvest index (%) was calculated by dividing the grain yield by the sum of the grain yield and dry straw biomass.

B.7. Statistical Analysis

All experiments had factorial designs and 3-16 replicates in each treatment group. The GenStat software (Release 6.2) was used for statistical analysis. The significance of the effects of the treatments and their interactions on the reported traits was evaluated by analysis of variance (ANOVA). Then, significant differences between means were determined by Fisher's protected least significant difference (LSD) test at the 5% level ($p \leq 0.05$).

CHAPTER 1

BIOFORTIFICATION OF WHEAT WITH ZINC
THROUGH SOIL AND FOLIAR APPLICATIONS OF NITROGEN

1.1. Introduction

Zinc deficiency is a widespread public health concern, most often caused by low dietary intake of bioavailable Zn (Welch & Graham, 2004; White & Broadley, 2009; Cakmak et al., 2010). The recommended dietary allowance (RDA) for Zn is 11 mg/day for adult males (19+ years of age) and 8 mg/day for adult females (19+ years of age), with the value increasing to 11 mg/day and 12 mg/day during pregnancy and lactation, respectively (Institute of Medicine, 2001). Wheat, the most important staple crop in many countries (FAO Database, 2003), is inherently poor in Zn (Cakmak, 2008). When wheat is grown on Zn-deficient soils, the concentration of Zn in the grain may be reduced below 10 mg per kg (Cakmak et al., 1999; Erdal et al., 2002). Moreover, not only the concentration, but also the bioavailability of Zn is low in wheat grain due to the very low amounts of promoters (such as organic acids) and high concentrations of antinutrients like phytate (Welch & Graham, 2004; White & Broadley, 2005; White & Broadley, 2009). Therefore, people whose diets are heavily based on wheat products are at high risk of Zn deficiency (Hotz & Brown, 2004; Gibson et al., 2008).

Biofortification, as compared to food fortification, supplementation and diet diversification, appears to be the most applicable and the most feasible strategy for the alleviation of Zn deficiency problem, especially in rural areas of the developing world (Pfeiffer & McClafferty, 2007; Cakmak, 2008). Breeding of wheat cultivars with high grain Zn by conventional methods is an important component of the biofortification approach (Welch & Graham, 2004). However, the genetic biofortification approach has

two main limitations: There is low genetic variation for grain Zn in cultivated wheat, which can be utilized in breeding programs. In contrast to cultivated wheat, however, wild relatives of wheat including wild emmer wheat (*Triticum turgidum ssp. dicoccoides*) and *Aegilops tauschii* are promising genetic resources for genetic biofortification (Calderini & Ortiz-Monasterio, 2003; Cakmak et al., 2004). Another limitation is the fact that the availability of Zn to the plants is very low due to adverse soil and environmental conditions such as high pH and low soil moisture in many wheat-growing areas (Alloway, 2004; Cakmak, 2008). At this point, agronomic biofortification appears to be a critical component of the biofortification approach. Among the agronomic strategies, application of Zn fertilizers is an effective and well-documented tool for the biofortification of wheat with Zn. Several studies demonstrated that applying Zn fertilizers to cereal crops improves not only the productivity, but also the grain Zn concentration (Cakmak, 2008). Under field conditions, the grain Zn concentration can be increased by Zn fertilization by up to four-fold, depending on the soil conditions and application method (Yilmaz et al., 1997).

Efforts for increasing the Zn concentration in wheat grain are impeded by the lack of knowledge on the physiological factors affecting the major steps on the route of Zn from the soil to the grain: root uptake, root-to-shoot translocation (via xylem), phloem transport, remobilization (retranslocation) of Zn from source tissues into developing seeds and seed deposition of Zn. Increasing evidence in the literature suggests that there is a close link between N and Zn in biological systems (*see Section A.4*) and that the steps mentioned above may be affected by the N nutritional status of plants, and therefore, by N fertilization (Cakmak et al. 2010).

Collecting information on how N fertilization affects accumulation of Zn in the shoot and the grain of durum wheat grown at different Zn availabilities will contribute to our understanding of the physiological mechanisms underlying the linkage between grain Zn and N. Moreover, since durum wheat is extremely sensitive to Zn deficiency (Cakmak et al., 1999), any contribution of N fertilization to Zn nutrition of durum wheat will be of great importance in terms of both yield and nutritional quality, especially under marginal conditions in semi-arid regions, where durum wheat is widely cultivated (Elias & Manthey, 2005).

In the study presented in this chapter, the following questions were addressed:

i) How does increasing soil N fertilization affect the shoot and grain concentrations of Zn in wheat when grown at low, adequate or high Zn availability?

ii) How do foliar applications of Zn and N affect the grain Zn accumulation under various soil Zn and N conditions?

iii) How are the concentrations of Zn and N in the grain linked in durum wheat?

In this chapter the relationship between grain Zn and protein localization was also studied by using Zn and protein staining methods.

1.2. Materials and Methods

Two separate pot experiments were conducted under greenhouse conditions as described below:

1.2.1. First Experiment

The first experiment was carried out to study 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. The experiment had a factorial design with four independent (pot) replicates.

The soil was prepared as described in "General Materials and Methods". The soil in low N pots was fertilized with 50 mg.kg^{-1} N, whereas that in adequate N pots was fertilized with 200 mg.kg^{-1} N. Three different Zn levels were established by adding 0.05 mg.kg^{-1} Zn (low Zn supply), 2 mg.kg^{-1} Zn (adequate Zn supply), or 10 mg.kg^{-1} Zn (high Zn supply) to the soil.

After 35 days of growth, shoots were harvested, dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods".

1.2.2. Second Experiment

The second experiment was conducted to investigate the effects of various soil and foliar applications of N and Zn on the grain Zn concentration and yield. The experiment had a factorial design with eight independent (pot) replicates.

Plants were grown until grain maturation at three different soil N supply levels. At the beginning, the following N rates were applied per kg soil: 50 mg N for low N plants and 200 mg 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. After 45 days of growth, 200 mg N per kg soil was added to high N pots in the form of $Ca(NO_3)_2.4H_2O$, together with secondary P fertilization. Two weeks later, additional 200 mg N per kg soil was added in the same form to high N pots, which received, in total, 600 mg N per kg soil.

When the plants were at 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 (Zadoks stage 69), whereas high N plants were still at the beginning of the flowering stage (Zadoks stage 60). 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, while the second group was sprayed with a 0.5% (w/v) $ZnSO_4.7H_2O$ solution, and the third group was sprayed with a solution 2% (w/v) urea solution. All foliar application solutions contained 200 mg L^{-1} of Tween20 as surfactant. Plants were sprayed to the point of run-off by using a hand-sprayer. The foliar applications were repeated after one week, when the low N plants were in the early milk development stage (Zadoks stage 73), and flowering was completed in the high N plants (Zadoks stage 69).

When the plants senesced fully and the grains reached full maturity, the spikes and the straws were harvested separately. They were dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods".

1.2.3. Staining

Protein staining was carried out by using the Bradford reagent containing Coomassie Brilliant Blue G-25 dye (Bradford, 1976), and Zn staining was carried out with the dithizone reagent (Ozturk et al., 2006). Seeds were initially incubated in water for 2 h at room temperature and then excised longitudinally prior to treatment with the dye compounds. For protein staining, seeds were treated with diluted Bradford reagent (i.e. 2:1 [v/v] dilution by absolute ethanol) and incubated at $70^{o}C$ for 15 min. For Zn staining, seeds were treated with 500 mg L^{-1} 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 Inc.).

1.3. Results

In the first experiment, analysis of variance revealed significant effects of soil N and soil Zn applications as well as N x 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 1.1).

Table 1.1: Analysis of variance (ANOVA) of the effects of soil N and Zn applications on the shoot dry weight, Zn concentration and Zn content of five-week-old durum wheat (*Triticum durum* cv. Balcali2000) plants (1st Exp.) grown under greenhouse conditions

Source of Variation	DF	Shoot Dry Weight		Shoot Zn Conc.		Shoot Zn Content	
		SS	F Pr.	SS	F Pr.	SS	F Pr.
Soil N	1	107334	<0.001	813	<0.001	1452	<0.001
Soil Zn	2	900485	<0.001	36231	<0.001	28820	<0.001
Soil N x Soil Zn	2	11062	0.030	834	<0.001	1204	<0.001
Exp. Error	15	18480		220		197	
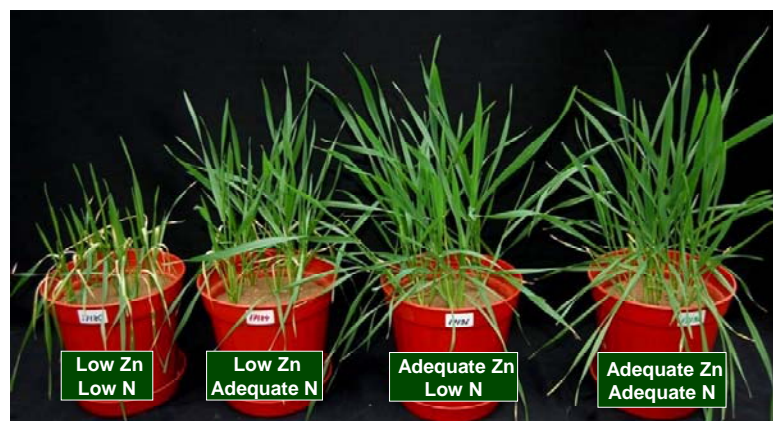
Fig. 1.1: Effect of low (50 mg N kg^{-1} soil) and adequate (200 mg N kg^{-1} soil) N treatments on growth of four-week-old durum wheat (*Triticum durum* cv. Balcali 2000) plants at low (0.05 mg Zn kg^{-1} soil) and adequate (2 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

Variation in N nutrition had a significant impact on the shoot dry matter yield of the plants at low Zn supply (Fig. 1.1; Table 1.2). Under Zn-deficient conditions, adequate N application greatly improved shoot growth as compared to 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. These symptoms were, however, more severe at low N than at adequate N supply (Fig. 1.1). No N deficiency symptom was observed in the experimental plants under given conditions.

Table 1.2: Effect of low (50 mg N kg^{-1} soil) and adequate (200 mg N kg^{-1} soil) N treatments on the shoot dry weight of five-week-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions

N Treatments	Shoot Dry Weight, mg plant^{-1}		
	Low Zn	Adequate Zn	High Zn
Low	344 Aa*	802 Ab	797 Ab
Adequate	537 Ba	896 Bb	912 Bb

* Values are means of four independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

As shown in Table 1.2, 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. In the case of 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 to 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^{-1} 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 1.3). At adequate or high Zn supply, the plants grown with 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 (Table 1.3). The shoot Zn concentrations were not affected from 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 (Table 1.3).

Table 1.3: Effect of low (50 mg N kg^{-1} soil) and adequate (200 mg N kg^{-1} soil) N treatments on the shoot Zn concentration and shoot Zn content of five-week-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn treatments on a Zn-deficient calcareous soil under greenhouse conditions.

Zn Treatments	Shoot Zn Concentrations mg Zn kg^{-1} dry wt		Shoot Zn Content µg Zn plant^{-1}	
	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

* Values are means of four independent replicates. Means in columns followed by different uppercase letters and means in rows (independent for Zn concentration and Zn content) followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

In the second experiment, plants were grown until grain maturation with various soil and foliar applications of N and Zn. Analysis of variance was carried out for six traits including straw dry weight, grain yield, harvest index, grain Zn concentration, grain Zn yield and grain N concentration (Table 1.4). Foliar applications of N or Zn had significant effects on all reported traits except grain yield (Table 1.4). Soil applications of N and Zn as well as soil N x foliar applications interaction and soil N x soil Zn interaction affected all reported traits significantly (Table 1.4). In the case of soil Zn x foliar applications interaction, the effect turned out to be significant on all traits except for grain yield and harvest index (Table 1.4). Finally, the triple interaction had significant effects on only grain Zn concentration, grain Zn yield and grain N concentration (Table 1.4).

Table 1.4: Analysis of variance (ANOVA) of the effects of foliar and soil applications of N and Zn on the straw dry weight, grain yield, harvest index, grain Zn concentration, grain Zn yield and grain N concentration of mature durum wheat (*Triticum durum* cv. Balcali2000) plants (2nd Exp.) grown under greenhouse conditions

Source of Variation	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 x B	4	2.434	<0.001	4.276	<0.001	1207	<0.001
A x C	4	1.015	0.018	0.701	0.397	155	0.169
B x C	4	1.408	0.003	27.405	<0.001	4909	<0.001
A x B x C	8	1.103	0.110	0.983	0.677	128	0.717
Exp. Error	182	15.099		31.226		4330	

Source of Variation	DF	Grain Zn Conc.		Grain Zn Yield		Grain N Conc.	
		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 x B	4	9471	<0.001	40346	<0.001	7.675	<0.001
A x C	4	20030	<0.001	18056	<0.001	0.991	<0.001
B x C	4	1895	<0.001	102196	<0.001	6.176	<0.001
A x B x C	8	2561	<0.001	12606	<0.001	1.403	<0.001
Exp. Error	182	7561		55550		7.717	

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. 1.2). At low Zn supply, the plants with adequate and high N treatments grew better than the plants with low N treatment.
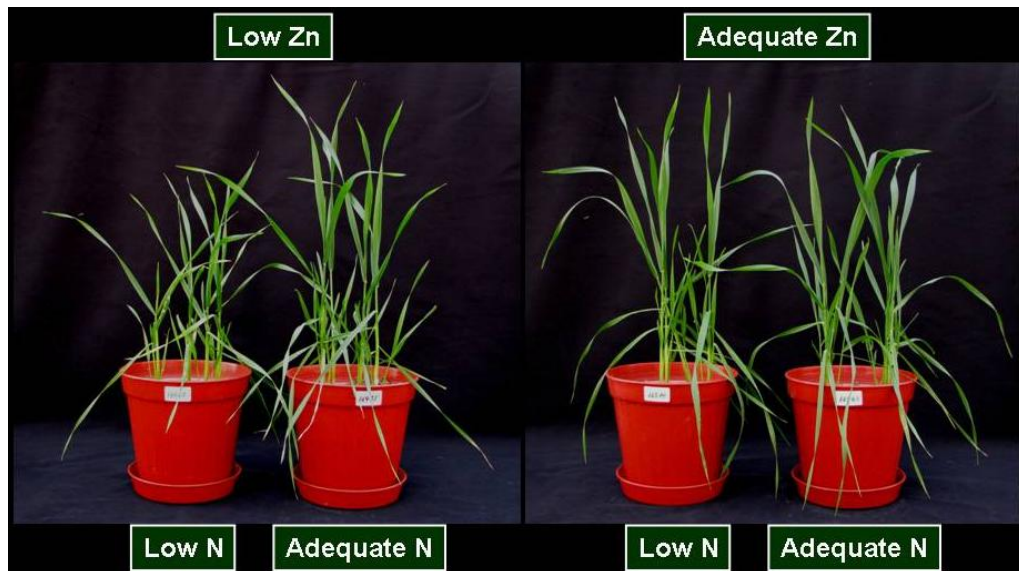
Fig.1.2: Effect of low (50 mg N kg^{-1} soil) and adequate (200 mg N kg^{-1} soil) N treatments on growth of four-weeks-old durum wheat (*Triticum durum* cv. Balcali2000) plants at low (0.05 mg Zn kg^{-1} soil) and adequate (2 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

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 1.5). Soil Zn applications at adequate or high rates generally increased straw dry weight (Table 1.5). Foliar urea application also improved shoot biomass production, especially at adequate and high soil N treatments, as compared to the plants without foliar treatment; whereas foliar Zn treatment had inconsistent effects on straw dry weight (Table 1.5).

Table 1.5: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on the straw dry weight of durum wheat (*Triticum durum cv.* Balcali2000) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions. Foliar applications of urea and $ZnSO_4$ were realized by spraying the plants with 2% urea and 0.5% $ZnSO_4$, respectively.

Foliar Application	N Treatments	Straw Dry Weight, g $plant^{-1}$		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p<0.05$).

The grain yield per plant varied between 0.76 g and 3.34 g, depending on the Zn and N treatments (Table 1.6). 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 (Table 1.6). Increases in the soil Zn applications enhanced the grain yield significantly, but foliar Zn application at low soil Zn supply could not prevent yield losses, 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.

Table 1.6: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on the grain yield of durum wheat (*Triticum durum cv. Balcali2000*) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

Foliar Application	N Treatments	Grain Yield, g $plant^{-1}$		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$).

At low soil Zn treatment, the harvest index was around 48% under low soil N supply, but when the soil N supply was increased, the harvest index was drastically reduced to values between 20.0 and 27.4% (Table 1.7) due to the slight negative effect of increasing N supply on the grain yield (Table 1.6) and its strong positive effect on straw dry weight (Table 1.5). Low soil Zn supply severely reduced the harvest index, when the soil N supply was adequate or high (Table 1.7). Generally, foliar applications of urea and Zn had inconsistent effects on the harvest index (Table 1.7).

Table 1.7: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on the harvest index of durum wheat (*Triticum durum cv. Balcali2000*) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

Foliar Application	N Supply	Harvest Index (%)		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

Both soil and foliar applications of Zn significantly enhanced grain Zn concentration of the plants, particularly in the case of high soil N treatments (Table 1.8). 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 the case of foliar Zn application, increasing soil N supply had a significant positive impact on the grain Zn concentration. In the case of foliar Zn fertilization, increasing soil N supply enhanced grain Zn concentrations by nearly 2-fold at all Zn treatments (Table 1.8).

Table 1.8: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on grain Zn concentration of durum wheat (*Triticum durum cv.* Balcali2000) plants grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions. Foliar applications of urea and ZnSO$_4$ were realized by spraying the plants with 2% urea and 0.5% ZnSO$_4$, respectively.

Foliar Application	N Treatments	Grain Zn Concentration, mg kg^{-1}		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

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 there, the effect was largely negated by yield increases (Tables 1.6 & 1.8). As expected, the total grain Zn yield per plant showed marked positive responses to increasing soil Zn application and foliar spray of Zn (Table 1.9). 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 (Table 1.9).

Table 1.9: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on the grain Zn yield (total amount of Zn in grains per plant) of durum wheat (*Triticum durum* cv. Balcali2000) plants at full maturity. Plants were grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

Foliar Application	N Treatments	Grain Zn Yield, μg Zn plant^{-1}		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$).

Depending on the treatment, the grain N concentrations varied between 1.61% and 3.49% (Table 1.10). The lowest grain N concentrations were measured at the low soil N supply in the absence of foliar urea application. Increasing the soil N supply resulted in progressive increases in the grain N concentration. At the high N supply, enhancements in the soil Zn application generally improved grain N concentration of plants. Foliar application of urea was effective in increasing the grain N concentration.

Table 1.10: Effect of low (50 mg N kg^{-1} soil), adequate (200 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N treatments on the grain N concentration of durum wheat (*Triticum durum* cv. Balcali2000) plants at full maturity. Plants were grown at low (0.05 mg Zn kg^{-1} soil), adequate (2 mg Zn kg^{-1} soil) or high (10 mg Zn kg^{-1} soil) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions.

Foliar Application	N Treatments	Grain N, (%)		
		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

* Values are means of eight independent replicates. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$).

The relation between the grain concentrations of Zn and N is shown in Fig. 1.3. In the absence of foliar Zn fertilization and at the low soil Zn supply, there was no corrleation between the grain Zn and N concentrations (Fig. 1.3A), but at high soil Zn supply and/or in the case of foliar Zn application, the grain Zn concentration exhibited a strong positive correlation with the grain N concentration (Fig. 1.3B-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. 1.3D is higher than that of the line in Fig. 1.3C, 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.
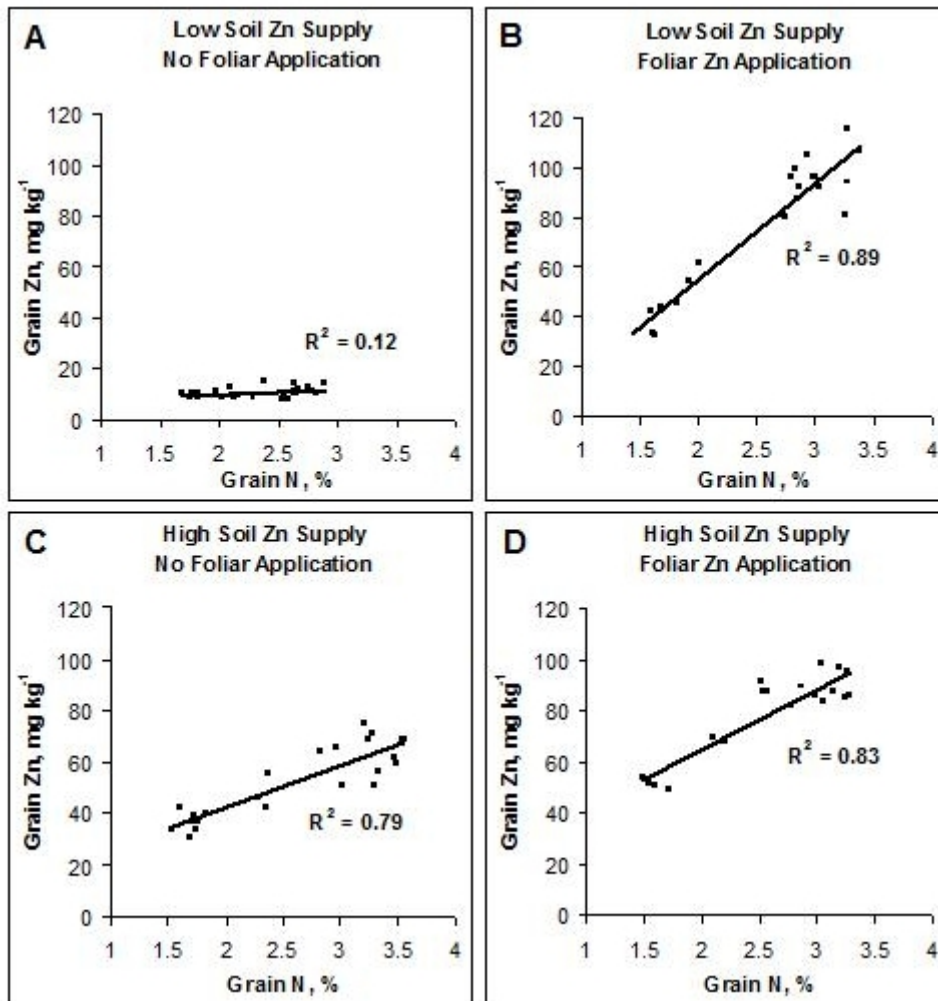
Fig. 1.3: Correlation between grain concentrations of Zn and N in durum wheat (*Triticum durum* cv. Balcali2000). Plants were grown at low (A and C) or high (B and D) Zn supply on a Zn-deficient calcareous soil under greenhouse conditions with (C and D) or without (A and B) foliar application of Zn.

As presented in Fig. 1.4, at high Zn availability due to either foliar or soil application of Zn, the increasing effect of N on grain Zn became more pronounced (Figs. 1.4A & 1.3B). 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. 1.4C-D). In the case of grain K concentration, however, there was no positive response to increasing N supply (Fig. 1.4E-F). At the adequate and high Zn supply, the grain K concentration even tended to slightly decrease with increasing N supply.
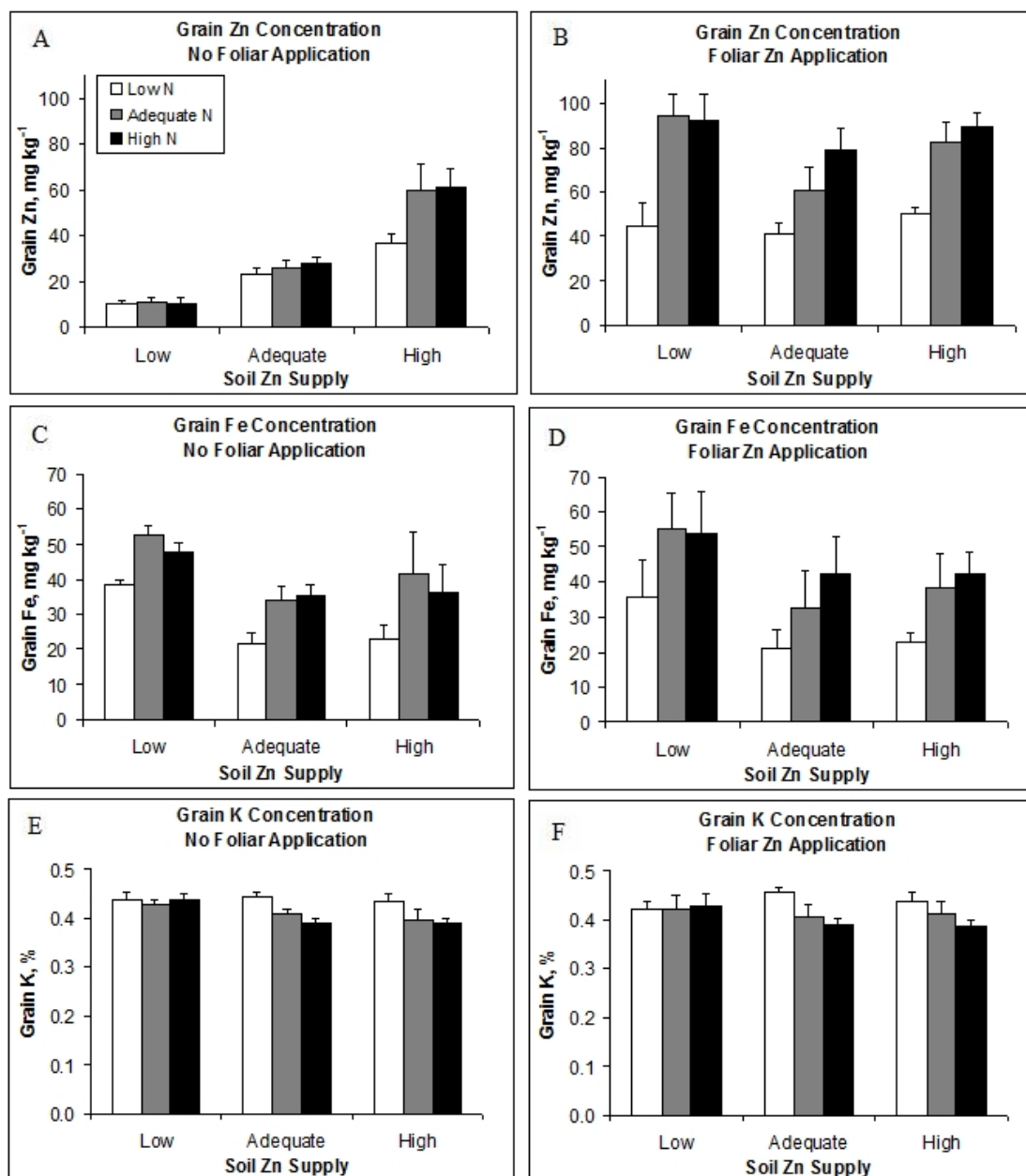
Fig. 1.4: Effect of increasing soil N treatments on grain concentrations of Zn, Fe, and K of durum wheat (*Triticum durum* cv. Balcali2000) at low (0.05 mg of Zn/kg of soil), adequate (2 mg of Zn/kg of soil), or high (10 mg of Zn/kg of soil) Zn supply. Plants were grown on a Zn-deficient calcareous soil 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.

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. Fig. 1.5 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 to the embryo and aleurone regions.
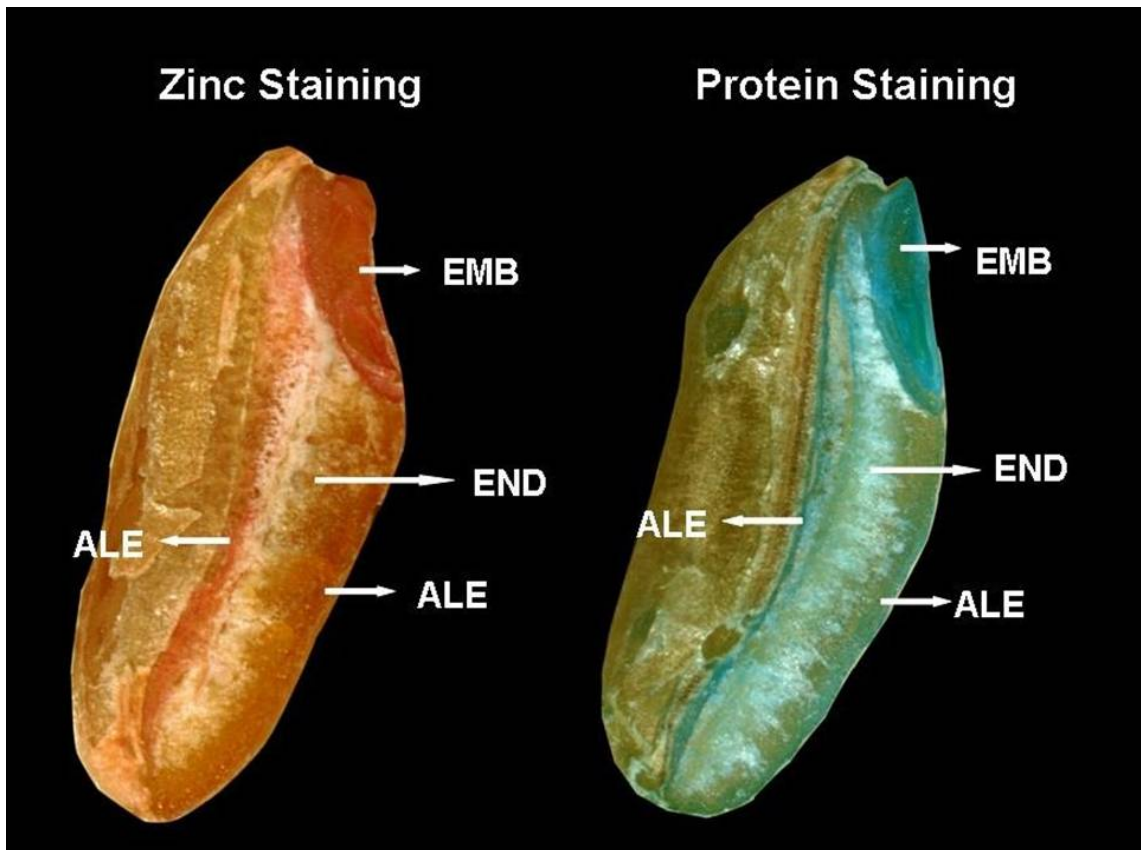
Fig. 1.5: Staining and localization of protein and Zn in durum wheat (*Triticum durum* cv. Balcali2000) grain containing 36 mg.kg^{-1} of Zn and 11.6 g.kg^{-1} of protein. Staining of longitudinally cut seed surface was done with Bradford reagent diluted 2:1 (v/v) in absolute ethanol (incubation at 70°C 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.

1.4. Discussion

Variation in N supply resulted in significant impacts on Zn nutrition of wheat plants. During early 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 little effect at adequate Zn supply (Table 1.2; Figs. 1.1 & 1.2). 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. Improving growth and alleviation of Zn deficiency leaf symptoms in low-Zn plants by high N may involve increases in root uptake of Zn and/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 1.3). 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 lack of an increase in the concentration of a given nutrient despite its high total amount per plant is a reflection of the dilution of the nutrient by enhanced growth (Marschner, 1995). Having the same or similar shoot Zn concentrations despite significant differences in the expression of Zn deficiency symptoms at varied N treatments (Figs. 1.1 & 1.2) 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) (see below).

In contrast to the positive effects on shoot growth at early growth stages (Table 1.2), 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 1.6). By stimulating the tillering and shoot biomass production (Ewert & Honermeier, 1999; Salvagiotti & 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 1.6). 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 & Engels, 1999). Notably, although increasing the N supply reduced the grain yield at low Zn treatment (Table 1.6), it improved the straw yield (Table 1.5) and thus reduced the harvest index significantly (Table 1.7). Unless the soil N supply was low, the harvest index was always reduced significantly by low

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soil Zn (Table 1.7). 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. Supporting this suggestion, it has been documented 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 & Cakmak, 1989; Cakmak, 2000).

Foliar applications of Zn and urea, which were carried out during the grain-filling period, could not or only partially prevent the yield losses associated with deficiencies of these elements (Table 1.6). It appears that foliar applications of N and Zn during the grain-filling period are not useful to overcome yield losses due to N and Zn deficiency, but they greatly contribute to the grain concentrations of N (Table 1.10; Woodard & Bly, 1998; Varga & Svečnjak, 2006) and Zn (Table 1.8; Yilmaz et al., 1997; Cakmak, 2008), respectively.

When sufficient Zn was supplied to the plants via soil and/or foliar applications, increasing the soil N application was highly effective in increasing the shoot and grain Zn concentrations (Tables 1.3 & 1.8). In the case of the low soil N supply, foliar-applied N (urea) was effective in improving the grain Zn concentration at high Zn treatment (Table 1.8). Consequently, a strong positive relationship between the grain concentrations of Zn and N was found in the case of high soil Zn supply and/or foliar Zn application (Fig. 1.3). 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. The close relationship between the grain concentrations of Zn and protein was also found in terms of 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. 1.5).

It seems very likely that N improves the root uptake and/or retranslocation (e.g., 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). Since both the grain Zn concentration and the grain yield

were increased simultaneously by soil N applications, the total amount of grain Zn per plant (e.g., grain Zn yield) was enhanced by increasing soil N supply to a greater extent (Table 1.9) than the grain Zn concentration (Table 1.8).

Nitrogen nutrition may affect the abundance of transporter proteins involved in the root uptake and/or root-to-shoot translocation and/or phloem loading of Zn such as Transporter proteins including zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like proteins (ZIP) family proteins and yellow stripe-like (YSL) transporters (Waters et al., 2006; Haydon & 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 found for amino acids (Caputo & Barneix, 1997; Rubio-Covarrubias et al., 2008). These compounds might be potential components of the phloem affecting the transport of Zn in the phloem tissue (Schmidke & Stephan, 1995; Grusak et al., 1999; Kruger et al., 2002). 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). The senescence-associated transport of N (e.g., 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 & Cobbett, 2007). Interrupting the 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 following 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, it has been shown that under conditions of high availability of nutrients in growth medium, continued root uptake and translocation into 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 & Grusak, 2008). It is well

documented that a high N nutritional status of plants extends the grain-filling period by delaying the senescence (Yang & Zhang, 2006) and thereby prolongs the time which 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 is known to increase both grain Zn and protein concentrations, possibly by increasing the levels of these nutrients available for remobilization from senescing tissues (Uauy et al., 2006b; Distelfeld et al., 2007). When the root uptake of N and Zn is restricted due to drought and/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. 1.4C) 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 their uptake and/or their translocation to the grain and/or their 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. 1.4D), which clearly shows that the effect of N on grain concentrations of micronutrients seem to be rather specific.

1.5. Conclusions

Micronutrient malnutrition is a growing global health problem, caused mainly by low dietary intake of micronutrients, especially Zn and Fe (Bouis, 2003; Pfeiffer & 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 N and Zn fertilization have a synergistic positive effect on the grain Zn concentration. Possibly, increasing N supply contributes to the grain Zn concentration by elevating the levels of Zn-chelating nitrogenous compounds and/or the abundance of Zn transporters. 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.

CHAPTER 2

EFFECT OF NITROGEN ON
UPTAKE, REMOBILIZATION AND PARTITIONING OF ZINC AND IRON
THROUGHOUT THE DEVELOPMENT OF DURUM WHEAT

2.1. Introduction

In Chapter I, a great potential of soil and foliar applications of N fertilizers for the biofortification of wheat grain with Zn and also Fe has been clearly demonstrated. Furthermore, it has been shown that the positive effects of N applications on the grain Zn are dependent on the Zn availability to the plant, and N and Zn act synergistically in improving the grain Zn concentration.

The trafficking of Zn and Fe cations from rhizosphere into grains involves several transport processes including uptake, root-to-shoot translocation and remobilization from source tissues. These transport processes are dependent on various proteins and nitrogenous chelators including amino acids:

1. Cereals are strategy II plants with respect to root iron acquisition, i.e., they release mugineic acid (MA) family phytosiderophores to chelate and take up ferric (Fe^{3+}) iron (Marschner & Romheld, 1994). The amino acid methionine is the precursor for the synthesis of nicotianamine, which is, in turn, used in the biosynthesis of the mugineic acid family of phytosiderophores as a key substrate (Mori & Nishizawa, 1987). Phytosiderophores contribute not only to Fe but also Zn uptake (Marschner & Romheld, 1994), and their biosynthesis and release are generally stimulated by the deficiencies of both Fe and Zn in wheat (Cakmak et al., 1994), although Zn deficiency does not significantly stimulate phytosiderophore release in durum wheat (Cakmak et al., 1996b; Rengel & Romheld, 2000).

2. Transporter proteins including zinc-regulated transporter (ZRT)/iron-regulated transporter (IRT)-like proteins (ZIP), YSL transporters, heavy metal ATPase (HMA) family proteins, ferric reductase defective 3 (FRD3) and vacuolar iron transporter (VIT) proteins are implicated in the root uptake, xylem loading and unloading, xylem-to-phloem exchange, phloem loading and unloading, and grain deposition of Zn and Fe (Waters et al., 2006; Haydon & Cobbett, 2007; Borg et al., 2009; Curie et al., 2009; Palmer & Guerinot 2009).

3. Nicotianamine (NA) is required for the phloem loading, translocation and unloading of Fe and Zn (Waters et al., 2006; Borg et al., 2009; Curie et al., 2009). MA family phytosiderophores as well as proteins like the iron transport protein (ITP) in castor bean (*Ricinus communis*) can also facilitate metal translocation (Kruger et al., 2002; Suzuki et al., 2008; Tsukamato et al., 2009).

Since all of these molecules are nitrogenous in nature, N is a critical raw material for their biosynthesis. Any improvement in the N nutritional status may, therefore, enhance their biosynthesis and thus improve the root uptake, transport and grain allocation of Zn and Fe (Cakmak et al., 2010).

When compared to nitrogen (e.g., amino acids), Zn and Fe have, generally, a lower phloem mobility in plants (Marschner, 1995). However, in some plant species, Zn may exhibit relatively high phloem mobility. For example, in a study by Miller et al. (1994), over 78% of the total shoot Zn in wheat was allocated to grains. Moreover, foliar-applied Zn has been documented to be retranslocated to the sink organs of plants such as young parts of roots and shoots (Hasslet et al., 2001; Erenoglu et al., 2002). It seems that Fe might also be quite phloem-mobile in wheat. The published reports show that up to 77% of shoot Fe can be transported into grain (Garnett & Graham, 2005). Nitrogen remobilization ratio into grain ranges from 70% to 89% in wheat, depending on the genotype (Kichey et al., 2007). In *Arabidopsis thaliana*, the reductions in the N, Fe and Zn levels of vegetative tissues during leaf senescence were 85%, 57% and 56%, respectively (Himelblau & Amasino, 2001). The results of Uauy et al. (2006b) indicate the existence of a close linkage between remobilization of N in the form of amino acids and remobilization of Fe and Zn from senescing tissues into grains in wheat.

In the literature, to our knowledge, there is no information about how and to what extent N nutrition affects the partitioning of Zn and Fe among the vegetative and generative shoot tissues during the ontogenesis of a wheat plant, and how soil Zn deficiency interacts with the effects of N nutrition on nutrient partitioning. The study reported in this chapter has been conducted to collect information about how the uptake, remobilization and grain allocation of Zn, Fe and N as well as their partitioning among vegetative and generative shoot tissues are affected by Zn and N supply during the development of durum wheat grown on a Zn-deficient soil. This report also gives important clues about the mechanisms by which improved N application can increase grain Zn and Fe concentrations and may aid in the utilization of the "nitrogen approach" in biofortification efforts.

2.2. Materials and Methods

In the experiment, which was designed as a factorial experiment with 8 harvest groups and 4 pot replicates in each harvest group, durum wheat plants were cultivated under either low or high N supply and either low or high Zn supply conditions.

The soil was prepared as described in "General Materials and Methods". The soil in low N pots was fertilized with 50 mg.kg^{-1} N, whereas that in high N pots was fertilized with 250 mg.kg^{-1} N. The low Zn condition was established by adding 0.2 mg Zn kg^{-1} to the soil, and the high Zn condition by adding 5 mg Zn kg^{-1}. The N and Zn supply levels, which are referred to as high levels in this study, represent normal levels which are applied commonly in our greenhouse experiments, and are not excessively high or potentially toxic levels.

Plants in different harvest groups were harvested at different developmental stages, as presented in Table 2.1. As plants growing under different Zn and N supply conditions did not reach a given developmental stage at the same time, the harvests were always carried out, when the main stems of high Zn-high N plants reached the developmental stage of interest. The final group was harvested, when plants under all Zn x N conditions totally senesced and all grains ripened. At the first two harvest stages, the shoots were harvested as whole, but starting from the 3rd stage, the leaves, stems,

main spike and tiller spikes (if available) were harvested separately (Table 2.1). From the 5th stage on, spikes contained grains, which were separated manually from the husk.

Table 2.1: Age, developmental stage and harvested shoot parts of durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions at different harvest stages (I-VIII)

Harvest Stage	Age (Days)	Zadoks Stage[a]		Harvested Shoot Parts				
				Whole Shoot	Main Spike	Tiller Spikes	Leaves	Stems
I.	38	3.0	Stem Elongation	✓	N.A.[b]	N.A.	x	x
II.	48	3.5	Stem Elongation	✓	N.A.	N.A.	x	x
III.	55	4.9	End of Booting	X	✓	N.A.	✓	✓
IV.	62	6.5	Mid-Anthesis	X	✓	✓	✓	✓
V.	69	7.1	Watery Ripe	X	✓	✓	✓	✓
VI.	79	7.7	Late Milk	X	✓	✓	✓	✓
VII.	90	8.5	Soft Dough	X	✓	✓	✓	✓
VIII.	108	9.9	Ripe Seed	X	✓	✓	✓	✓

[a] Developmental stage of the main stem of high Zn-high N plants according to the
[b] Not available

Throughout this chapter:

i. The term "husk" refers to the vegetative parts of the spike, which include the rachis, rachilla, grain stalk, glume, lemma and palea (Pearson et al. 1995).

ii. The term "shoot" refers to all above-ground parts of wheat plants including the grains.

iii. The term "straw" refers to all vegetative parts of the shoot, including the leaves, stems and husk.

The harvested materials were dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods". The mineral content data were used to make further calculations:

i. The harvest index for a mineral was determined by dividing the grain yield for that mineral by the total shoot content at full maturity.

ii. For a mineral nutrient, the net amount remobilized from the straw was calculated by subtracting the straw content at maturity from the highest straw content reached during the development. Then, the net amount remobilized from the

straw was divided by the highest straw content reached during the development in order to calculate the "straw remobilization ratio" for that mineral nutrient.

iii. The remobilized amount of a mineral from pre-anthesis stores was calculated by subtracting the straw content at full maturity from the straw content at the IV. harvest stage (mid-anthesis).

iv. The amount of grain mineral provided by post-anthesis shoot uptake was determined by subtracting the amount of a mineral remobilized to grains from pre-anthesis stores from the grain yield of that mineral at full maturity.

2.3. Results

Analysis of variance was carried out in order to determine the significance of the effects of Zn supply, N supply, harvest stage as well as their double and triple interactions on 18 selected traits of durum wheat plants grown under greenhouse conditions (Table 2.2). The multivariate analysis revealed that Zn supply and harvest stage affected all traits analyzed significantly. Nitrogen supply had significant effects on all traits analyzed except husk dry weight, main stem grain Fe concentration and tiller grain Fe concentration.

Table 2.2: Multivariate analysis of variance (ANOVA) of the effects of Zn supply, N supply, harvest stage and their interactions on selected traits of durum wheat (*Triticum durum* cv. Balcali2000) grown under greenhouse conditions

Source of Variation	Leaf DW[a]	Stem DW	Husk DW	MS[b] Grain Yield	TL[c] Grain Yield	Tot. Grain Yield
Zinc Supply (A)	<0.001*	<0.001	<0.001	<0.001	<0.001	<0.001
Nitrogen Supply (B)	<0.001	<0.001	0.331	0.015	<0.001	<0.001
Harvest Stage (C)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A x B	<0.001	<0.001	<0.001	0.039	<0.001	<0.001
A x C	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
B x C	<0.001	<0.001	0.009	<0.001	<0.001	<0.001
A x B x C	<0.001	<0.001	0.024	0.012	<0.001	<0.001

Source of Variation	Shoot Zn Content	Shoot Fe Content	Shoot N Content	MS Grain Zn Conc.	TL Grain Zn Conc.	Tot. Grain Zn Yield
Zinc Supply (A)	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
Nitrogen Supply (B)	<0.001	<0.001	<0.001	<0.001	0.004	<0.001
Harvest Stage (C)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A x B	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A x C	<0.001	0.004	0.011	0.777	<0.001	<0.001
B x C	<0.001	<0.001	<0.001	0.003	<0.001	<0.001
A x B x C	<0.001	<0.001	<0.001	<0.001	0.151	<0.001

Source of Variation	MS Grain Fe Conc.	TL Grain Fe Conc.	Tot. Grain Fe Yield	MS Grain N Conc.	TL Grain N Conc.	Tot. Grain N Yield
Zinc Supply (A)	<0.001	<0.001	<0.001	<0.001	0.048	<0.001
Nitrogen Supply (B)	0.243	0.339	<0.001	<0.001	<0.001	<0.001
Harvest Stage (C)	<0.001	<0.001	<0.001	0.001	<0.001	<0.001
A x B	<0.001	0.758	<0.001	<0.001	0.310	<0.001
A x C	0.002	<0.001	<0.001	0.121	0.156	<0.001
B x C	0.383	<0.001	<0.001	<0.001	0.031	<0.001
A x B x C	0.002	0.242	<0.001	0.345	0.184	<0.001

* Values are F probabilities.
[a] Dry weight; [b] Main Stem; [c] Tiller

2.3.1. Dry Weight and Grain Yield

High N application encouraged tillering and vegetative growth under both low Zn and high Zn condition (Fig. 2.1). As senescence was delayed by high N treatment, especially at high Zn supply, the high Zn-low N plants reached full maturity almost two weeks earlier than the high Zn-high N plants.
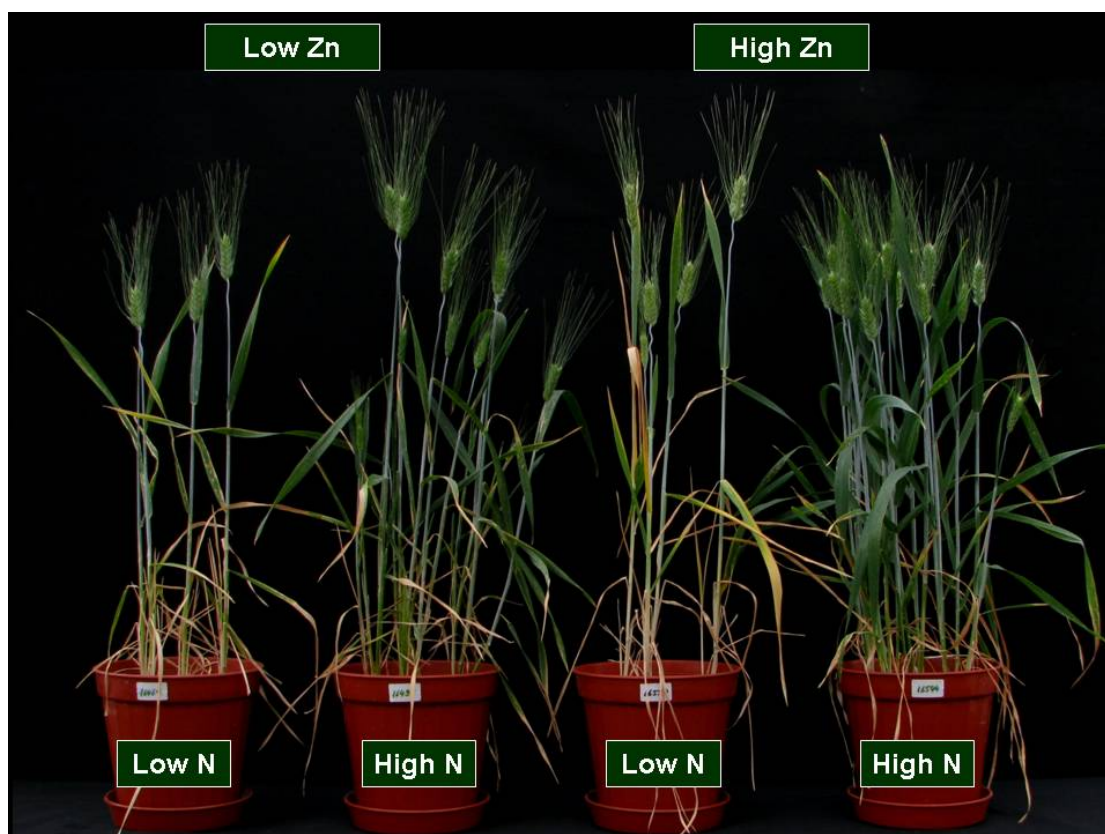
Fig. 2.1: 79-day-old (at the VI. harvest stage) durum wheat (*Triticum durum* cv. Balcali2000) plants grown with low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions

The straw dry weight was significantly improved by high Zn and N supplies at all harvest stages except the first (Table 2.3). At high N conditions, the straw biomass increased steadily, peaked at the VII. harvest stage and then decreased significantly at the latest stage of grain-filling. The stem and leaf dry weight responded positively to high N and high Zn in a similar way as the straw dry weight. The trends of stem dry weight with respect to harvest stages were similar to those of straw biomass. In the case of husk dry weight, neither N nor Zn had consistent effects.

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Table 2.3: The straw, leaf, stem and husk dry weight of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (I-VIII)

Harvest Stage	Straw Dry Weight, g plant^{-1}				Leaf Dry Weight, g plant^{-1}			
	Low Zn		High Zn		Low Zn		High Zn	
	Low N	High N	Low N	High N	Low N	High N	Low N	High N
I	0.30 Aa*	0.38 Aa	0.42 Aab	0.39 Aa	*N.A.*	*N.A.*	*N.A.*	*N.A.*
II	0.78 Ba	1.09 Bb	1.20 Bc	1.41 Bd	*N.A.*	*N.A.*	*N.A.*	*N.A.*
III	1.25 Ca	1.97 Cb	1.99 CDb	2.46 Cc	0.52 Ca	0.86 Ac	0.68 Db	1.30 Bd
IV	1.71 Da	2.66 Db	2.60 Fb	3.75 Dc	0.52 Ca	1.02 Cc	0.66 Db	1.57 Dd
V	1.84 DEa	2.85 Ec	2.49 Fb	3.78 Dd	0.47 Ba	0.89 Ac	0.55 Cb	1.36 Cd
VI	1.90 Ea	3.04 Fc	2.16 Eb	5.04 Ed	0.52 Ca	0.93 Bc	0.56 Cb	1.54 Dd
VII	1.68 Da	3.59 Hc	1.89 Cb	5.16 Ed	0.52 Ca	1.01 Cb	0.50 Ba	1.56 Dc
VIII	1.66 Da	3.32 Gb	1.75 Ca	3.81 Dc	0.37 Aa	0.90 ABc	0.46 Ab	0.89 Ac

Harvest Stage	Stem Dry Weight, g plant^{-1}				Husk Dry Weight, g plant^{-1}			
	Low Zn		High Zn		Low Zn		High Zn	
	Low N	High N	Low N	High N	Low N	High N	Low N	High N
I	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
II	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
III	0.51 Aa	0.81 Ab	0.88 Ab	0.91 Abc	0.22 Aa	0.30 Ac	0.43 Ad	0.25 Ab
IV	0.81 BCa	1.24 Bb	1.26 Cb	1.57 Bc	0.37 Ba	0.40 Ba	0.68 Ec	0.60 BCb
V	0.94 CDa	1.50 Cc	1.35 Cb	1.88 Cd	0.43 Ca	0.46 Cb	0.59 Dd	0.54 Bc
VI	0.91 Ca	1.62 Dc	1.01 Bb	2.91 Ed	0.48 Da	0.49 Ca	0.59 Db	0.59 BCb
VII	0.69 Ba	2.06 Fc	0.85 Ab	3.03 Fd	0.47 Da	0.53 Db	0.54 Cb	0.57 Bbc
VIII	0.74 Ba	1.85 Eb	0.80 Aa	2.24 Dc	0.49 Da	0.58 Eb	0.49 Ba	0.57 Bb

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

At all stages of grain-filling, the total grain yield per plant was significantly increased by high Zn supply with the effects being more dramatic at high N level (Table 2.4). The positive effects of Zn supply on grain yield were further enhanced by high N supply. Similarly, the total grain yield responded to high N fertilization positively, and the responses were more marked at high Zn supply. As found with the total grain yield, the main stem grain yield was also significantly increased by high Zn and high N supply, but the positive responses of the main stem grain yield to high Zn and N

supplies were far less pronounced than those of the total grain yield. Under high N conditions, the tiller grain yield constituted a significant portion of the total grain yield and was dramatically enhanced by high Zn supply (Table 2.4).

Table 2.4: The total, main stem and tiller grain yield of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (V-VIII)

| Harvest Stage | Total Grain Yield, g plant^{-1} | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	0.13 Aa*	0.24 Aa	0.35 Aab	0.32 Aab
VI	0.46 Ba	0.71 Bb	1.05 Bc	1.47 Bd
VII	1.10 Ca	1.77 Cc	1.59 Cb	3.08 Cd
VIII	1.18 Ca	2.04 Dc	1.59 Cb	5.49 Dd

| Harvest Stage | Main Stem Grain Yield, g plant^{-1} | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	0.13 Aa	0.17 Aa	0.34 Ab	0.15 Aa
VI	0.46 Ba	0.54 Ba	1.05 Bc	0.74 Bb
VII	1.07 Ca	1.37 Cb	1.54 Cd	1.51 Cc
VIII	1.14 Ca	1.57 Db	1.51 Cb	2.12 Dc

| Harvest Stage | Tiller Grain Yield, g plant^{-1} | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	0.00 Aa	0.07 Aa	0.01 Aa	0.16 Aab
VI	0.00 Aa	0.17 Ab	0.00 Aa	0.73 Bc
VII	0.03 Aa	0.40 Bc	0.06 Ab	1.58 Cd
VIII	0.05 Aa	0.47 Bb	0.08 Aa	3.37 Dc

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

2.3.2. Shoot Contents and Grain Deposition of Zn, Fe and N

The shoot Zn content, i.e. the sum of the straw Zn content and grain Zn yield, was highly responsive to Zn fertilization at each development stage (Table 2.5). High N also increased the shoot Zn content and doubled the positive effect of high Zn application on the shoot Zn content at later stages of the grain-filling. The shoot Zn content continued to increase steadily until the final harvest at high N supply, but the increase ceased at earlier stages at low N supply. The trends exhibited by the shoot Fe content during the development of durum wheat were similar to those of the shoot Zn content. Moreover, high Zn lowered the shoot Fe content during the grain-filling period when the N supply was low, but improved it significantly when the N supply was high. Nitrogen fertilization itself was highly effective in increasing the shoot Fe content, irrespective of the Zn supply. As expected, N fertilization also enhanced the shoot N content significantly, and its effect was more pronounced at high Zn supply. At low N treatment, the increase in the shoot N content ceased almost totally as early as at the II. harvest stage, but at high N treatment, it continued to rise until the plants reached maturity.

Table 2.5: The shoot Zn, Fe, and N content per plant of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different stages (I-VIII)

Harvest Stage	Shoot Zn Content, µg plant^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
I	3 Aa*	4 Aa	26 Ab	30 Ab
II	5 Aa	8 Aa	46 Bb	88 Bc
III	8 Aa	13 ABb	56 Cc	124 Cd
IV	15 Ba	21 Cb	66 Dc	187 Dd
V	12 Aba	25 Cb	74 Ec	189 Dd
VI	13 ABa	33 Db	78 Ec	241 Ed
VII	18 Ba	30 CDb	79 Ec	273 Fd
VIII	21 BCa	40 Eb	78 Ec	318 Gd

Harvest Stage	Shoot Fe Content, µg plant^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
I	24 Aa	26 Aa	28 Aa	27 Aa
II	61 Ba	79 Ab	57 Ba	89 Bc
III	73 Cb	116 Bc	64 Ba	128 Cd
IV	85 Da	159 Cb	80 Ca	176 Dc
V	105 Eb	176 Dc	82 Ca	185 Dd
VI	120 Fb	210 Ec	81 Ca	271 Ed
VII	124 Fb	259 Fc	85 Ca	332 Fd
VIII	124 Fb	251 Fc	95 CDa	356 Gd

Harvest Stage	Shoot N Content, mg plant^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
I	15 Aa	21 Ab	22 Ab	21 Ab
II	27 Ba	52 Bc	31 Bb	73 Bd
III	28 Ba	65 Cb	29 Ba	88 Cc
IV	27 Ba	73 Db	29 Ba	109 Dc
V	30 Ba	79 Eb	31 Ba	111 Dc
VI	31 Ba	92 Fb	32 Ba	135 Ec
VII	35 BCa	97 Gb	33 Ba	152 Fc
VIII	33 BCa	113 Hb	32 Ba	162 Gc

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 2.6: The main stem grain Zn concentration, tiller grain Zn concentration and total grain Zn yield of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (V-VIII)

Harvest Stage	Main Stem Grain Zn Conc., mg kg^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	16 Ba*	16 Ca	42 Cb	41 BCb
VI	11 Ab	8 Ba	33 Bc	37 Ad
VII	9 Ab	6 Aa	29 Ac	39 Bd
VIII	10 Ab	8 Ba	28 Ac	40 Bd

Harvest Stage	Tiller Grain Zn Conc., mg kg^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	*N.A.*	13 Ca	37 Bb	37 Ab
VI	*N.A.*	10 Ba	*N.A.*	38 Ab
VII	12 Bb	6 Aa	29 Ac	50 Bd
VIII	7 Aa	9 Ba	29 Ab	51 Bc

Harvest Stage	Total Grain Zn Yield, µg plant^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	2 Aa	3 Aa	14 Ab	12 Ab
VI	5 Aa	6 Aa	35 Bb	55 Bc
VII	9 ABa	11 Ba	46 Cb	137 Cc
VIII	12 Ba	17 Cb	44 Cc	254 Dd

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$).

High Zn supply enhanced the main stem grain Zn concentration dramatically during the grain-filling at both N levels (Table 2.6). High N application lowered the main stem grain Zn concentration in Zn-deficient plants, but improved it significantly in high-Zn plants. Consequently, the highest main stem grain Zn concentration was obtained under high Zn-high N condition. Moreover, the high Zn-high N condition was the only condition under which the Zn concentration of main stem grains did not decrease during the grain-filling but remained more or less at the same level. Under all

other conditions, the Zn concentration of main stem grains was at its maximum at the beginning of the grain-filling period and tended to decrease until the final harvest. The tiller grain Zn concentration responded to both high N and high Zn treatments in similar ways as the main stem grain Zn concentration. The total grain Zn yield per plant exhibited impressive positive responses to high Zn application, and the effect of high Zn was strongly augmented by high N.

Table 2.7: The main stem grain Fe concentration, tiller grain Fe concentration and total grain Fe yield of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different stages (V-VIII)

| Harvest Stage | **Main Stem Grain Fe Conc., mg kg^{-1}** | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	40 Ac*	36 Ab	26 Ba	28 Aa
VI	46 Bd	39 Bc	23 Aa	29 Ab
VII	49 Bd	35 Ac	21 Aa	28 Ab
VIII	54 Cd	42 Cc	21 Aa	34 Bb

| Harvest Stage | **Tiller Grain Fe Conc., mg kg^{-1}** | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	*N.A.*	36 Ab	36 Bb	25 Aa
VI	*N.A.*	48 Bb	*N.A.*	36 Ca
VII	57 Bb	58 Cb	28 Aa	30 Ba
VIII	46 Ac	62 Dd	31 Aa	43 Db

| Harvest Stage | **Total Grain Fe Yield, µg plant^{-1}** | | | |
| | Low Zn | | High Zn | |
	Low N	High N	Low N	High N
V	5 Aa	8 Aa	9 Aa	8 Aa
VI	21 Ba	29 Bab	24 Ba	48 Bc
VII	46 Cb	70 Cc	34 Ca	90 Cd
VIII	64 Db	95 Dc	34 Ca	215 Dd

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

The Fe concentrations of both the main stem and tiller grains were always higher at low Zn supply than that at high Zn supply (Table 2.7). High N reduced the main stem grain Fe concentration in Zn-deficient plants, but increased it in high-Zn plants. The Fe concentration of mature tiller grains was markedly increased by high N, irrespective of the Zn supply. From the VI. stage on, the total grain Fe yield per plant responded positively to both high Zn and high N fertilization. The most dramatic effect of high N treatment on the total grain Fe yield was observed in mature plants supplied with high Zn, where the total grain Fe yield at high N was more than 6 times as high as that at low N.

The grain N concentration was significantly increased by high N application in both the main stem and the tillers, as expected (Table 2.8). In contrast, high Zn application resulted in lower main stem and tiller grain N concentration throughout the grain-filling when the N supply was low. When the N supply was high, high Zn did not affect the N concentrations of developing grains and decreased only the N concentrations of mature grains. The total grain N yield per plant was always higher at high N supply than at low N supply. High Zn fertilization also improved the total grain N yield at almost all stages of the grain-filling.

Table 2.8: The main stem grain N concentration, tiller grain N concentration and total grain N yield of durum wheat (*Triticum durum* cv. Balcali2000), grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (V-VIII)

Harvest Stage	Main Stem Grain N Conc., %			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	2.4 Bb*	2.3 Ab	2.1 Ba	2.3 ABb
VI	2.3 Ab	2.2 Ab	1.7 Aa	2.1 Ab
VII	2.2 Ab	2.2 Ab	1.6 Aa	2.2 Ab
VIII	2.1 Ab	2.6 Bd	1.7 Aa	2.4 Bc

Harvest Stage	Tiller Grain N Conc., %			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	*N.A.*	2.5 Aa	*N.A.*	2.5 Aa
VI	*N.A.*	2.6 Aa	*N.A.*	2.3 Aa
VII	2.3 Bb	2.5 Ab	1.4 Aa	2.5 Ab
VIII	1.6 Aa	3.6 Bc	1.5 Aa	2.5 Ab

Harvest Stage	Total Grain N Yield, mg plant^{-1}			
	Low Zn		High Zn	
	Low N	High N	Low N	High N
V	3 Aa	7 Ab	7 Ab	8 Ab
VI	10 Ba	16 Bb	17 Bb	33 Bc
VII	20 Ca	40 Cc	25 Cb	73 Cd
VIII	25 Da	57 Db	26 Ca	137 Dc

* Values are means of 4 independent pot replicates, each containing 4 plants. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05).

2.3.3. Distribution of Zn, Fe and N among Shoot Parts

The Zn contents of different vegetative and generative shoot parts are shown in Fig. 2.2 at various developmental stages under different Zn and N supply conditions. Both the leaves and the stems responded positively to high N and/or high Zn fertilization in terms of their Zn contents. When the Zn supply was low, the leaves

contained more Zn than the stems, which contained more Zn than the husk, before the onset of grain-filling. Under the low Zn and low N condition, the Zn contents of the leaves, stems and husk tended to decrease, while the grain Zn yield was increasing progressively during the grain-filling. However, under the low Zn and high N condition, only the husk exhibited a decreasing trend in the Zn content during the generative development, while other vegetative parts did not show consistent trends. In contrast to low Zn conditions, not the leaves but the stems contained more Zn at the end of the vegetative period under high Zn conditions. Moreover, the stems remained the Zn-richest vegetative shoot parts until the VII. stage. The Zn contents of all vegetative shoot parts decreased during the grain-filling, but the sharpest decreases were observed in the stems and leaves, especially when both Zn and N supplies were high.
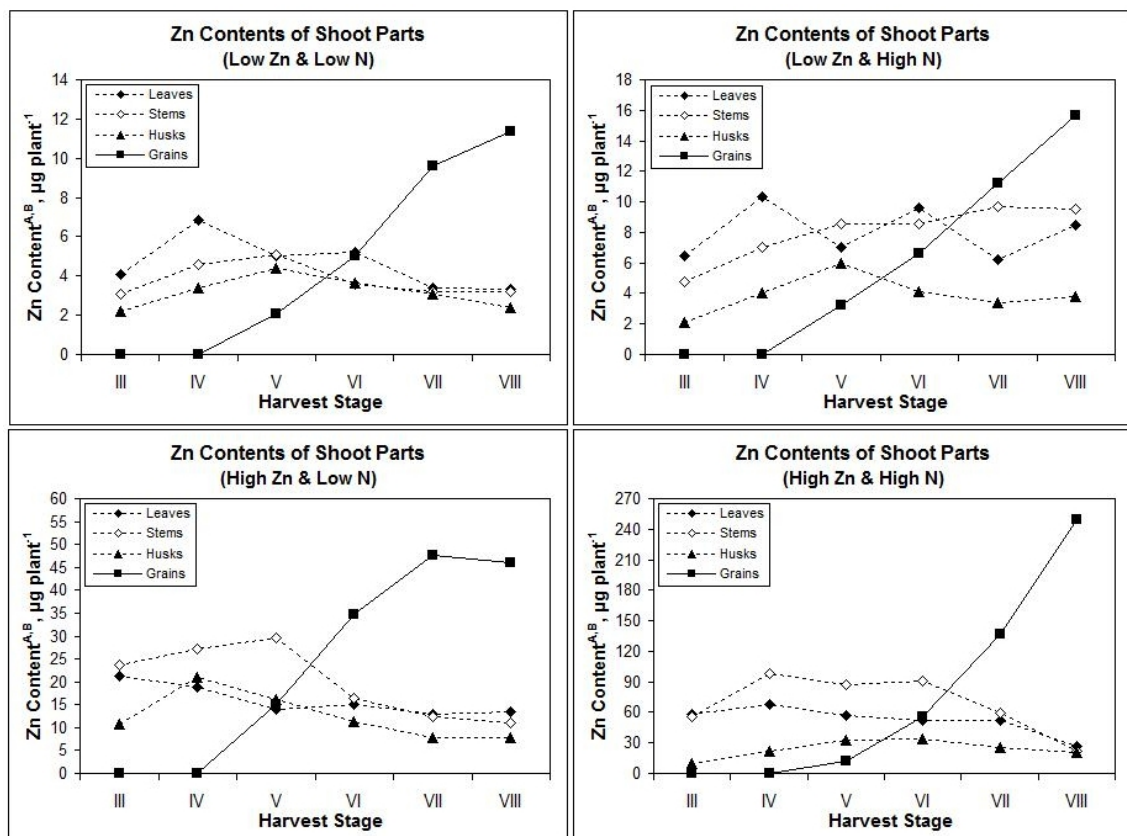
[A] For main stem and tiller grains: Zn Content = Grain Zn Yield

[B] Scales are different in all graphs.

Fig. 2.2: Zn contents of vegetative (leaves, stems and husk) and generative (main stem grains and tiller grains) shoot parts of durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (III-VIII)

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During the grain-filling period, the shares of the vegetative shoot parts in the shoot Zn content tended to decrease, whereas the shares of the grains increased under all conditions (Fig. 2.3). Only under the high Zn-high N condition, the grain Zn yield of the tillers had a greater share than that of the main stem in the total grain Zn yield of mature plants. The Zn harvest index, i.e. the ratio of the total grain Zn yield to the shoot Zn content, was reduced in mature plants by high N fertilization at low Zn treatment, while high N application increased the Zn harvest index at high Zn treatment.
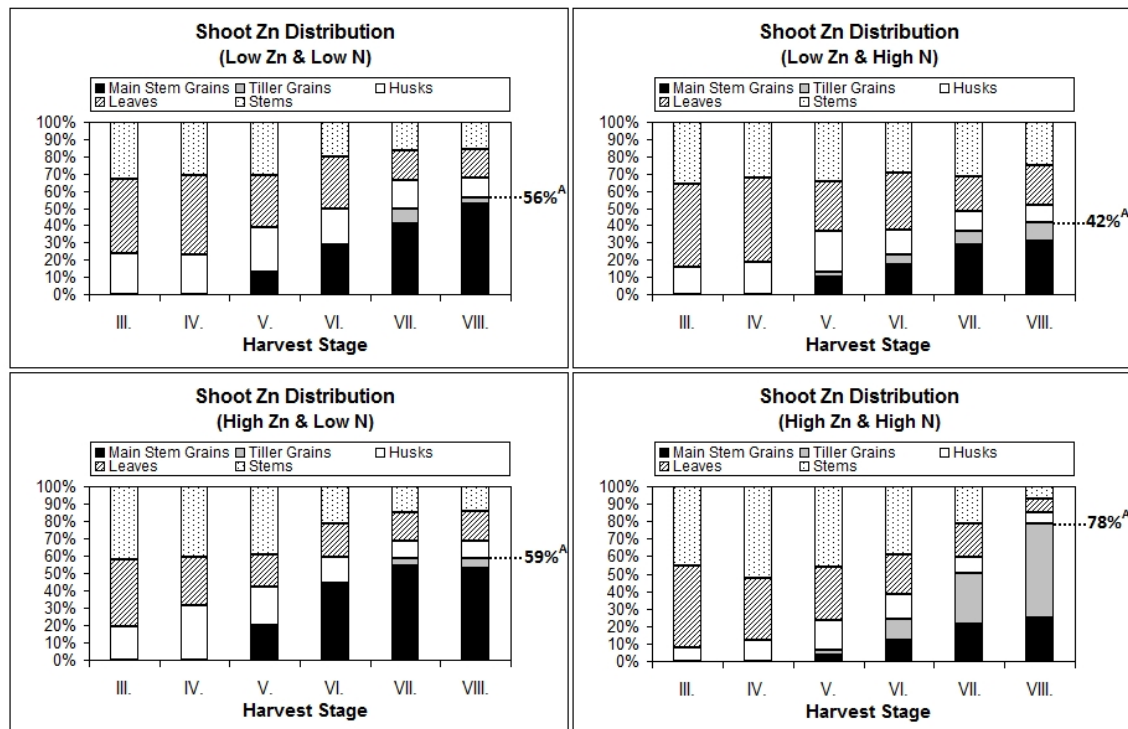
[A] Zinc harvest index at the final stage

Fig. 2.3: Relative distribution of Zn among generative (main stem grains and tiller grains) and vegetative (husk, stems, leaves) shoot parts of durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (III-VIII)

In contrast to Zn, for which the leaf and stem contents were comparable, Fe was mainly allocated to the leaves throughout the development under high Zn conditions (Fig. 2.4). The Fe content of the leaves was not only the highest among the Fe contents of all vegetative shoot parts, but also higher than the grain Fe yield at all stages except the final stage under the high Zn-high N condition. At all stages, the stems and husk

contained relatively low and comparable amounts of Fe. Under the high Zn-low N condition, the leaf Fe content did not change significantly during the grain-filling, but the stem and husk Fe contents tended to decrease. In contrast, when both the Zn and N supplies were high, the leaf Fe content continued to increase at the early stages of the grain-filling, but then exhibited a very sharp decrease at the very end of the development. High N fertilization enhanced the Fe harvest index tremendously.
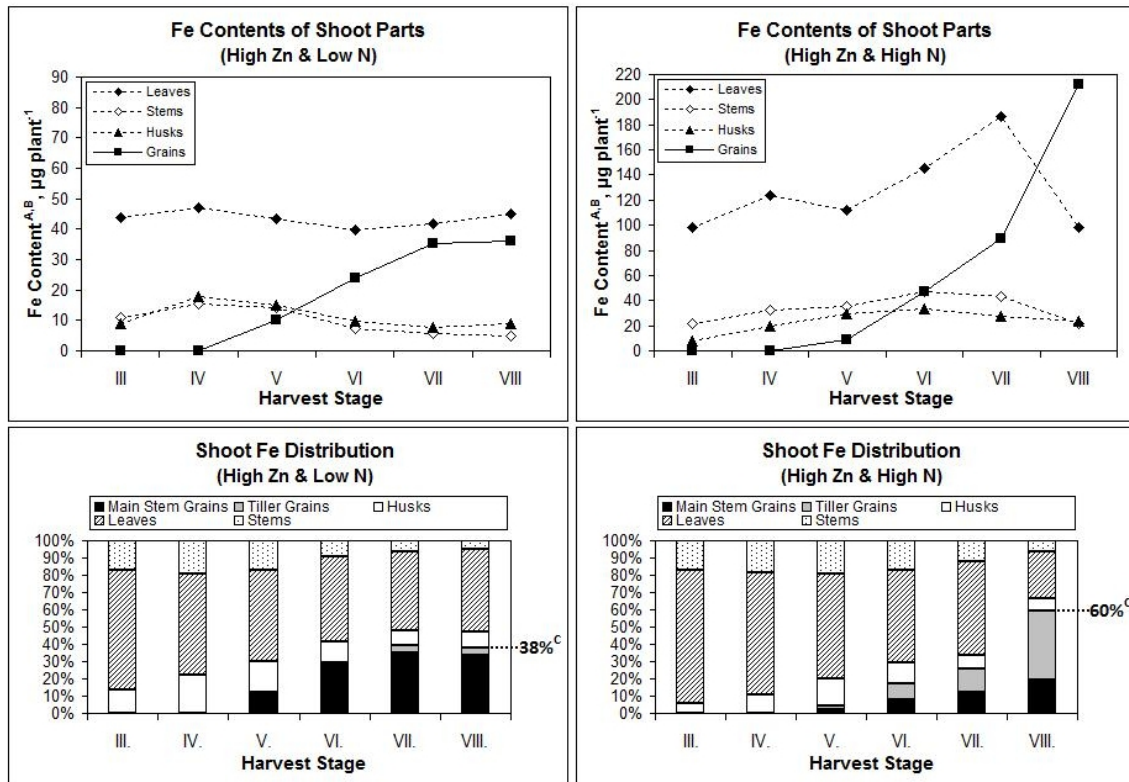
[A] For main stem and tiller grains: Fe Content = Grain Fe Yield

[B] Scales of the graphs are different.

[C] Iron harvest index at the final stage

Fig. 2.4: Iron contents of vegetative (leaves, stems and husk) and generative (main stem grains and tiller grains) shoot parts and relative distribution of Fe among the same parts of durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (III-VIII)

N Contents of Shoot Parts
(Low Zn & Low N)

N Contents of Shoot Parts
(Low Zn & High N)

N Contents of Shoot Parts
(High Zn & Low N)

N Contents of Shoot Parts
(High Zn & High N)
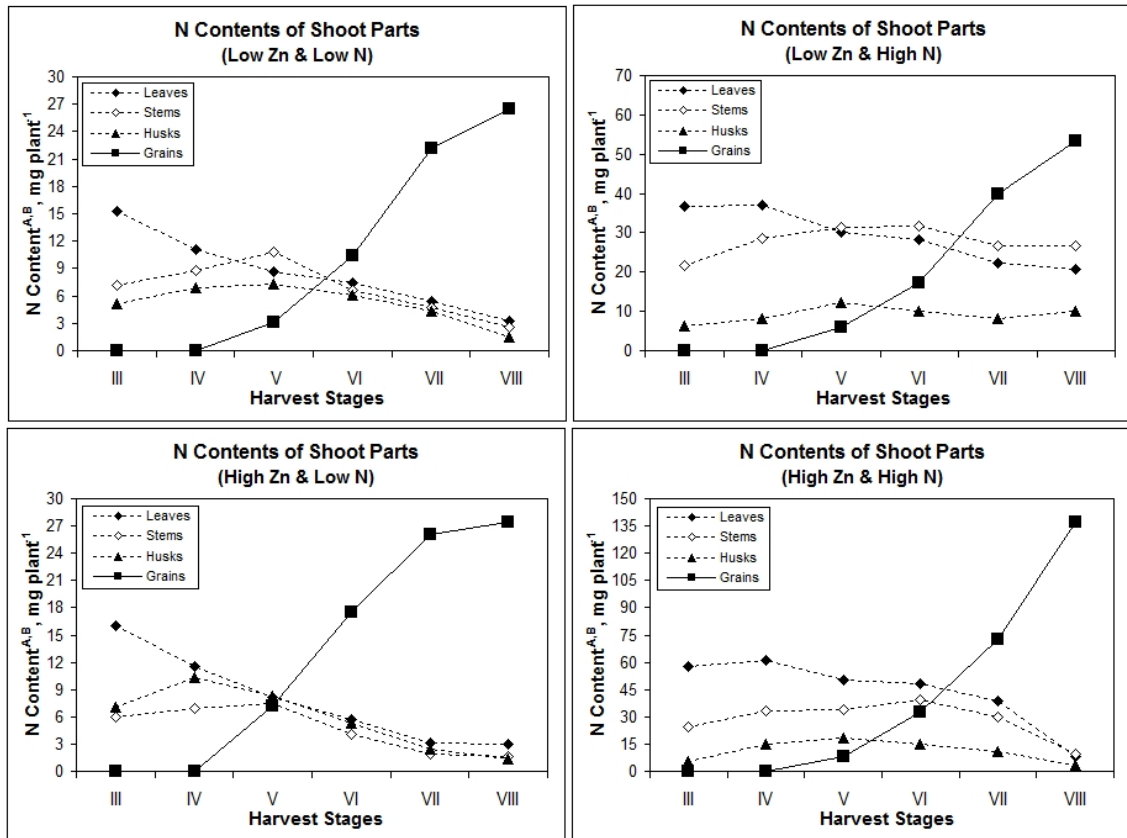
[A] For main stem and tiller grains: N Content = Grain N Yield

[B] Scales are different in all graphs.

Fig. 2.5: Nitrogen contents of vegetative (leaves, stems and husk) and generative (main stem grains and tiller grains) shoot parts of durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (III-VIII)

High N application increased the N contents of all vegetative and generative shoot parts significantly at all developmental stages (Fig. 2.5). Under low N conditions, the N contents of vegetative shoot parts were not significantly affected by the Zn supply level. When the N supply was low, the N content of the leaves started to decrease as early as at the III. stage, and the N contents of all vegetative shoot parts decreased during the grain-filling. Under high N condition, when the Zn application was low, only minor decreases were observed in the N contents of the vegetative shoot parts during the grain-filling, but when the Zn application was high, they were severely depleted of Zn after the VI. stage. At low Zn supply, the N harvest index dropped sharply when the

N application was increased (Fig. 2.6). By contrast, the N harvest index rose when the N application was increased at high Zn supply. The tiller grains had the greatest share in the shoot N content of the high Zn-high N plants at the final stage.
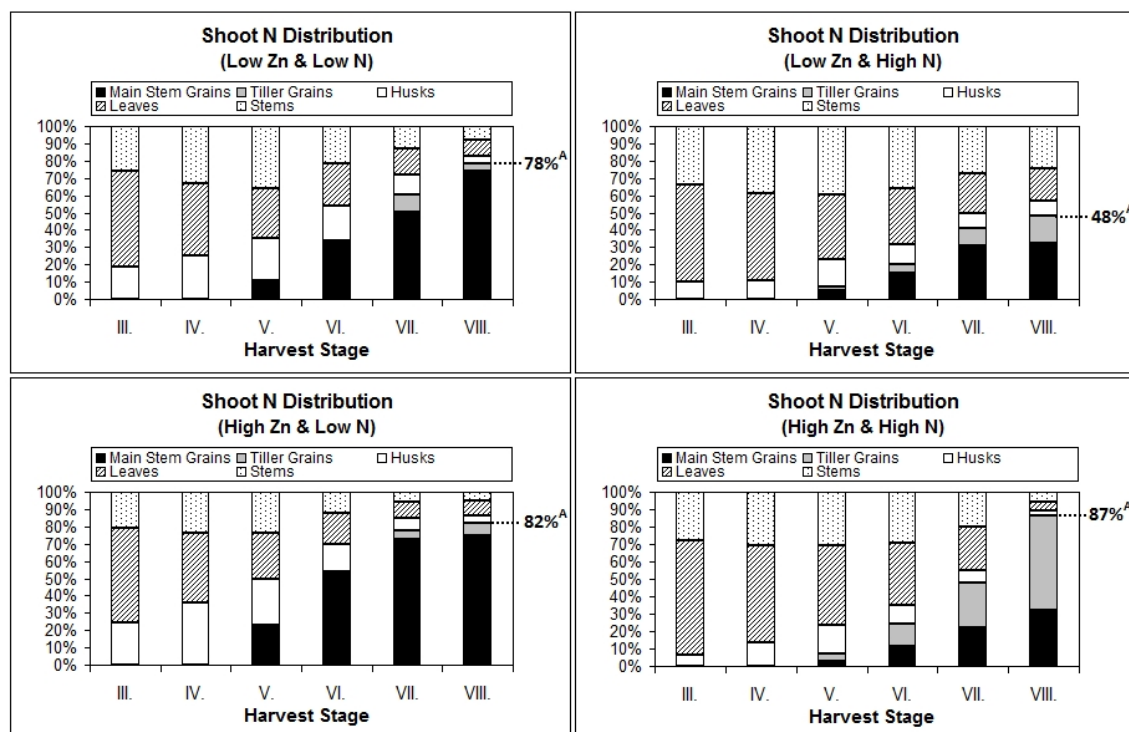
A Nitrogen harvest index at the final stage

Fig. 2.6: Relative distribution of N among shoot organs (main spike, tiller spikes, stems and leaves) of durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions and harvested at different developmental stages (III-VIII)

2.3.4. Remobilization and Shoot Uptake of Zn, Fe and N during the Grain-Filling

Table 2.9 shows the straw remobilization ratios of Zn, Fe and N under different Zn and N supply conditions as well as the relative contributions of straw remobilization and shoot uptake during the grain-filling to their grain deposition. When the Zn supply was low, the straw Zn remobilization ratio was reduced by high N treatment. In contrast, high N treatment increased the straw Zn remobilization ratio in high-Zn plants. Under both low N and high N conditions, the straw Zn remobilization ratio was lower in Zn-deficient plants.

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Table 2.9: Straw remobilization ratios and contributions of remobilization of pre-anthesis stores and post-anthesis shoot uptake to grain deposition of Zn, Fe, and N in durum wheat (*Triticum durum* cv. Balcali2000) grown at low (0.2 mg Zn kg^{-1} soil) or high (5.0 mg Zn kg^{-1} soil) Zn and low (50 mg N kg^{-1} soil) or high (250 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil under greenhouse conditions

Remobilization and Shoot Uptake during Grain Filling

Zinc	Low Zn		High Zn	
	Low N	High N	Low N	High N
Straw Zn Remobilization Ratio[a]	34%	15%	51%	62%
Amount of Grain Zn (µg plant^{-1}) Provided by:				
Remobilization of Pre-Anthesis Zn[b]	5	0	34	116
Post-Anthesis Shoot Zn Uptake	7	17	10	137
Share of Grain Zn Provided by:				
Remobilization of Pre-Anthesis Zn	44%	0%	78%	46%
Post-Anthesis Shoot Zn Uptake	56%	100%	22%	54%

Iron	Low Zn		High Zn	
	Low N	High N	Low N	High N
Straw Fe Remobilization Ratio[a]	40%	17%	27%	42%
Amount of Grain Fe (µg plant^{-1}) Provided by:				
Remobilization of Pre-Anthesis Fe[b]	25	3	21	36
Post-Anthesis Shoot Fe Uptake	39	92	12	179
Share of Grain Fe Provided by:				
Remobilization of Pre-Anthesis Fe	39%	3%	63%	17%
Post-Anthesis Shoot Fe Uptake	61%	97%	37%	83%

Nitrogen	Low Zn		High Zn	
	Low N	High N	Low N	High N
Straw N Remobilization Ratio[a]	70%	26%	79%	77%
Amount of Grain N (µg plant^{-1}) Provided by:				
Remobilization of Pre-Anthesis N[b]	19	17	23	84
Post-Anthesis Shoot N Uptake	6	40	3	53
Share of Grain N Provided by:				
Remobilization of Pre-Anthesis N	74%	30%	88%	62%
Post-Anthesis Shoot N Uptake	26%	70%	12%	38%

[a] Ratio of net Zn (Fe, N) remobilized from the straw to the highest straw Zn content

[b] Assume: ~100% of Zn (Fe, N) remobilized from the straw is translocated to grains.

The straw Fe remobilization ratio also responded to high N positively in high-Zn plants but negatively in Zn-deficient plants (Table 2.9). In the case of N, the straw remobilization ratio was again reduced by high N treatment at low Zn supply, but was not affected by high N treatment at high Zn supply. When Zn was not limiting, the straw remobilization ratio of N was significantly higher than that of Zn, which was significantly higher than that of Fe, irrespective of the N supply.

Any mineral nutrient deposited in the grains during the grain-filling period is either remobilized from the straw or taken up by the shoot during the same period. The relative contributions of these two sources depend on the conditions, as shown in Table 2.9. In the case of grain Zn, the amount provided by remobilization of pre-anthesis Zn from the straw was higher at high Zn supply than at low Zn supply. High N fertilization also increased Zn remobilization markedly, when the Zn application was high. But nevertheless, high N application decreased the share (percentage) of grain Zn provided by remobilization of pre-anthesis stores and increased the share provided by shoot uptake during the grain-filling at both Zn supply levels. Moreover, this effect of high N was not only observed in grain Zn deposition, but also in grain Fe and N depositions. Consequently, the relative contribution of shoot uptake during the grain-filling increased for all elements, when the N supply was increased. Under the high Zn-high N condition, 54% of the grain Zn was provided by post-anthesis shoot uptake. This percentage was markedly higher for Fe (83%) and significantly lower for N (38%) under the same condition.

2.4. Discussion

The leaf, stem and straw dry weights were significantly enhanced by high N supply (Fig. 2.1, Table 2.3), which is known to stimulate the straw biomass production and tillering in wheat (Ewert & Honermeier, 1999; Salvagiotti & Miralles, 2007). These vegetative parts were also responsive to high Zn supply, but to a lesser extent. Under all conditions, significant decreases in the dry weights of these vegetative parts were observed during the later stages of the grain-filling as a result of senescence and retranslocation to developing grains. This decrease in vegetative biomass started

relatively later under high N condition, possibly due to delayed senescence in wheat under high N supply (Scalet et al., 1991; Yang & Zhang 2006).

Both high N and high Zn treatments also increased the total grain yield significantly (Table 2.4). Their positive effects on the total grain yield were not additive but synergistic. When the low N – low Zn condition is considered as reference, high N application increased the yield by 73%, while high Zn application enhanced the yield by 35%. But the combined effect of high N and high Zn treatments on the yield was over 350%. This result indicates reduced N use efficiency under Zn-deficient conditions.

The shoot Zn content was significantly enhanced by both high Zn and high N treatments (Table 2.5). Under high Zn condition, the shoot Zn content continuously increased until maturity at high N, whereas it ceased to rise at the beginning of the grain-filling at low N. The shoot Fe content was also highly responsive to high N treatment, and as in the case of Zn, shoot Fe uptake continued during the grain-filling only under high N condition. The increases in shoot Zn and Fe contents by N were much greater than the increases in shoot dry weight by N. These results suggest that Zn and Fe uptake is maintained and enhanced by high N supply. In the absence of foliar nutrient uptake, the increase in the shoot content of any mineral is due to either root uptake and root-to-shoot translocation or retranslocation from root stores (Garnett & Graham, 2005; Waters & Grusak, 2008).

The answer to the question, whether the positive effect of high N on Zn uptake is related to the internal N-nutritional status of the plant or external N availability lies in Table 2.5. In the case of high Zn-low N plants, the shoot N content reached its maximum as early as at the 2nd harvest stage and remained constant afterwards. At this stage, the plants had already absorbed over 90% of the N fertilizer added to the otherwise N-poor potting soil (data not shown). While the N concentrations of vegetative tissues were decreasing due to dilution and retranslocation after the 2nd harvest stage, the plants continued to take up Zn until at least the 5th harvest stage. This result suggests that the external availability of N does at least not directly affect root Zn uptake. Rather, the internal N status of the plant may be critical in maintaining and enhancing root Zn uptake and root-to-shoot translocation. Zinc uptake and translocation depends on the activity of Zn transporters such as ZIP, YSL transporters and HMA family proteins (Guerinot, 2000; Waters et al., 2006; Haydon & Cobbett, 2007; Palmer & Guerinot, 2009), whose activities or pools may be impaired under low N status. High

protein amounts in plant tissues may represent a sink for Zn existing in soil, contributing to maintenance of high Zn absorption by roots (Cakmak et al., 2010). Under all conditions except the high Zn - high N, the grain Zn concentration decreased during grain development, which is in agreement with data from field experiments (Ozturk et al., 2006). However, at the high Zn - high N condition, the Zn concentration of the main stem grains remained constant throughout the grain-filling, while that of the tiller grains even increased markedly.

An antagonistic relation between Zn and Fe has been documented in both plant and mammalian systems (Warnock, 1970; Cakmak, 2000; Niles et al., 2008). The increased grain Fe concentration observed in low Zn plants may be explained by Zn deficiency-induced Fe accumulation (Cakmak, 2000; Table 2.7). Under low Zn condition, high N did not have a consistent effect on grain Fe concentration, but under high Zn condition high N both maintained and enhanced Fe concentration of both main stem and tiller grains. The change in grain N concentration during grain development was similar to that in grain Zn and Fe concentrations in the sense that the grain N concentration tended to decrease during the grain-filling under low N condition but remained constant or increased under high N condition (Table 2.8). The decrease in grain mineral nutrient concentrations during the grain-filling under field conditions is probably a result of nutrient dilution (Marschner, 1995). While the developing grain is growing, the mineral concentrations decrease if the mineral supply to the grain can not compensate for the dilution.

The apparent correlation of grain Zn and grain Fe with grain N is in agreement with the previous reports (Morgounov et al., 2007; Peleg et al., 2008; Zhao et al., 2009), and makes sense in the light of grain Zn and Fe localization and speciation data. Both grain Zn and Fe are known to be much more concentrated in the embryo and aleurone layer than the endosperm (Persson et al., 2009; Cakmak et al., 2010). These micronutrient-rich parts of wheat grain are at the same time rich in protein and phytate. Similar to Zn, which is mainly found in the form of phytate-rich globular crystals in protein bodies (Lott & Buttrose, 1978; Welch, 1986), Fe in the embryo and aleurone layer is localized to protein storage vacuoles as phytate complexes, while Fe in the endosperm may be associated with the Fe homeostasis protein ferritin within amyloplasts (Borg et al., 2009). In a recently published speciation experiment, it was shown that Fe is mainly associated with phytic acid, whereas Zn is mainly bound to

peptides in barley grains (Persson et al., 2009). Obviously, higher grain protein concentration can result in higher sink activity and storage capacity for Zn and Fe in the grain.

Both leaves and stems store significant amounts of Zn, but their relative contributions to the amount of total Zn stored in the straw depends on the Zn and N supply levels as well as the developmental stage of wheat (Fig. 2.2). Under low Zn regime, leaves and stems contain comparable amounts of Zn, whereas under high Zn condition, stems appear to store markedly higher amounts of Zn than leaves during the vegetative period. This result is in agreement with previous reports from the literature, which state that wheat stems can serve as important Zn stores that can be utilized during the grain-filling under Zn-sufficient conditions (Pearson & Rengel, 1994; Haslett et al., 2001). Under low Zn condition, there is probably no excess Zn to be stored in stems, which makes the leaf tissues relatively more important for Zn transport into grain.

Zinc is a relatively phloem mobile micronutrient, which is remobilized during grain development and senescence (Marschner, 1995). As expected, the vegetative parts exported net Zn, while the grain Zn yield (i.e. the total Zn content of grains) increased during the grain-filling period (Fig. 2.2). Consequently, the ratio of Zn stored in the straw to the total shoot Zn decreased, while that of grain Zn to the total shoot Zn increased during the generative phase (Fig. 2.3). Zinc remobilization from the vegetative parts to the grains was improved under high Zn condition than under low Zn condition both in terms of the amount of remobilized Zn and the straw remobilization ratio, i.e. the ratio of remobilized Zn to the highest straw Zn content reached during the development (Fig. 2.2; Table 2.9). There are probably both source- and sink-related reasons behind the impaired Zn remobilization from the straw under low Zn condition. When the Zn supply is low, the Zn concentration is also low in source tissues, and most of the Zn is, probably, strongly bound to cell constituents which cannot be remobilized easily, such as cell walls. Moreover, the grain yield and thus the sink activity are low due to impaired reproductive development caused by Zn deficiency (Cakmak & Engels, 1999), which may also limit Zn-remobilization capacity of the Zn deficient tissues.

The effect of N treatment on Zn remobilization depends on the Zn-nutritional status of wheat. In low Zn plants, high N reduced the Zn harvest index (Fig. 2.3), the straw Zn remobilization ratio as well as the amount of remobilized Zn from the straw (Fig. 2.2; Table 2.9). High N actually enhanced shoot Zn uptake even at low Zn supply

(Table 2.5); but, since it encouraged vegetative growth and tillering, the concentrations of leaves and stems were diluted (data not shown). Furthermore, the grain yield did not increase as much as the straw biomass (Tables 2.3 & 2.4), so that the harvest index was reduced by high N at low Zn supply, which in turn decreased the sink activity per gram of source tissue. When Zn supply was high, increasing N supply tremendously enhanced the Zn harvest index (Figs. 2.3 & 2.7) and Zn remobilization (Fig. 2.2; Table 2.9). Almost 80% of total shoot Zn was harvested with grains at high N level, while at low N, only 60% of the total shoot Zn was found in grain (Figs. 2.3 & 2.7). Moreover, the amount of Zn remobilized from pre-anthesis stores was almost quadrupled by high N treatment.

Zinc and Iron Partitioning among Shoot Parts at Maturity

Shoot Part	Zn			Fe	
	Low N	High N		Low N	High N
Grains	59%	78%		38%	60%
Husks	10%	6%		9%	7%
Stems	14%	7%		5%	6%
Leaves	17%	8%		48%	28%
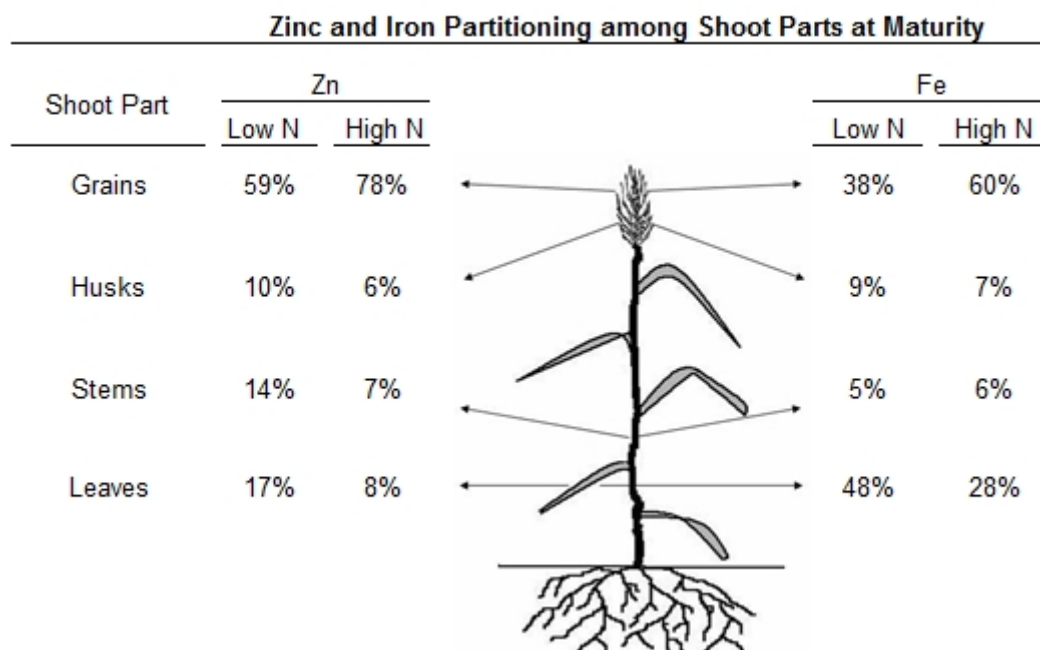
Fig. 2.7: Shoot Zn and Fe partitioning of mature durum wheat (*Triticum durum* cv. Balcali2000) grown at high Zn and low or high N supply

By increasing the Zn concentrations of source tissues, high N probably increased the availability of Zn that can be loaded into the phloem for retranslocation. In phloem transport of Zn to grains, the potential limitations include the phloem loading, the availability of organic chelates facilitating phloem loading and long distance transport as well as the phloem unloading (or grain loading) (Grusak et al., 1999; Garnett & Graham 2005; Cakmak et al., 2010). Members of the YSL group of transporters are thought to be responsible for the phloem loading and unloading of Zn (Curie et al.,

2009). Nicotianamine (NA)-chelated Zn is probably a critical Zn species in phloem loading and unloading (Curie et al., 2009; Trampczynska et al., 2010). By enhancing the activities of transporters involved in the phloem loading and unloading of Zn and/or increasing the abundance of NA or other possible nitrogenous chelators like amino acids and peptides, high N status may improve Zn retranslocation if these are limiting factors in low N plants. Finally, high N may also contribute to Zn remobilization under high Zn condition, as the demand of grains for Zn is increased due to higher grain yield and/or higher grain protein content (e.g., increased grain sink strength for Zn).

Any Zn ion that reaches the grain must have been either remobilized from source tissues or taken up by the shoot during the grain-filling period and directly destined to the grain. In any case, Zn has to pass through the phloem for reaching the grain because of the xylem discontinuity in the grain stalk (O'Brien et al., 1985). Zinc entering the shoot during the grain development must be transferred from the xylem to the phloem in order to be destined to the grain. Xylem-to-phloem transfer can take place in various tissues including the leaf, stem, peduncle and rachis (Herren & Feller, 1994; Pearson et al., 1995; Grusak et al., 1999). Since YSL proteins are possibly implicated in the xylem-to-phloem transfer of Zn (Curie et al., 2009; Palmer & Guerinot, 2009), high N might improve the xylem-to-phloem transfer by increasing the YSL activity.

In a sand culture experiment with bread wheat (*Triticum aestivum*), most of the grain Zn at maturity was accounted by Zn entering the shoot after anthesis (Garnett & Graham, 2005). Continued shoot micronutrient uptake during the generative development was also shown to be at least as important as remobilization of previously stored micronutrients in seed micronutrient accumulation of *Arabidopsis thaliana* and wheat growing at high nutrient availability (Waters & Grusak, 2008; Waters et al., 2009). The results of our study indicate that the relative contributions of retranslocation of the pre-anthesis stores and the post-anthesis shoot uptake to the grain Zn deposition are affected distinctly by Zn- and N-nutritional status of wheat (Table 2.9). When interpreting the results in Table 2.9, it should be noted that Zn or any other mineral taken up by shoot during the grain-filling can either be directly translocated to the grain or first reach a source tissue, from where it is retranslocated to the grain. So, the post-anthesis shoot mineral uptake may also contribute to grain mineral content by remobilization of the post-anthesis stores, which is in fact evident in cases, where mineral contents of some source tissues continue to increase at the beginning of the

grain-filling and then start decreasing at later stages (Figs. 2.2 & 2.4). Table 2.9 shows that high N supply more than doubled the amount of grain Zn provided by the post-anthesis shoot uptake under low Zn condition and increased it by over one order of magnitude under high Zn condition. Although high N also enhanced straw Zn remobilization ratio and the remobilization of pre-anthesis stores significantly under high Zn condition, the relative importance of post-anthesis shoot Zn uptake was increased by high N under both low and high Zn conditions. High N supply may increase the contribution of the post-anthesis shoot Zn uptake to grain Zn accumulation not only by increasing the activities of root Zn uptake transporters and/or the abundance Zn chelates facilitating root-to-shoot translocation of Zn but also by delaying senescence and thereby extending the grain-filling period (Yang & Zhang, 2006), where Zn uptake can continue.

Under field conditions, wheat experiences various degrees of drought stress in most growing regions during the grain-filling, because the grain-filling often occurs while temperatures are rising and soil moisture content is falling, especially in Mediterranean climates (Blum, 1998). It is well-known that limited soil moisture greatly reduces Zn diffusion in soils and root Zn uptake (Marschner, 1993). Consequently, plants under drought stress conditions are highly susceptible to soil Zn deficiency, leading to spike sterility and low grain yield (Bagci et al., 2007). Water deficit or any other biotic or abiotic stress condition limiting the Zn uptake during the reproductive development of wheat can render the remobilization of pre-anthesis stores relatively more important under field conditions than under irrigated greenhouse conditions. In that case, high N supply may increase the grain Zn accumulation mainly by enhancing pre-anthesis uptake of Zn and its remobilization.

In contrast to Zn, which was mostly stored in stems among vegetative tissues under high Zn condition, Fe was mostly allocated to leaves at all harvest stages (Figs. 2.4 & 2.7), possibly due to large amounts of heme and non-heme proteins as well as Fe-storage proteins in leaf cells such as phytoferritin, which have high Fe binding capacity (Marschner, 1995; Briat et al., 1995). In field-grown wheat, Fe remobilization to cereal grains has been shown to be very limited. In a study by Gupta (1991), foliar application of $FeSO_4$ and a Fe-chelate was not effective in increasing grain Fe concentration in oat and barley. One of the reasons for the inefficiency of foliar Fe fertilizers in increasing grain Fe concentration could be poor phloem mobility of Fe. Iron harvest indices as low

as 5% and 20% have been reported by Hocking (1994) and Miller et al., (1994), respectively, but in a sand culture experiment under growth chamber conditions, Garnett and Graham (2005) found a good Fe remobilization to grains and an Fe harvest index of 77%. In their experiment, wheat shoots did not take up any Fe after anthesis probably due to very low availability of Fe in the growth medium, and just remobilized Fe could account for the grain Fe yield. The results presented in this study indicate that Fe remobilization and Fe harvest index, like Zn remobilization and Zn harvest index, strongly depend on the N supply. Both values are significantly improved by high N supply under Zn-sufficient condition (Figs. 2.4 & 2.7; Table 2.9), but both are significantly lower than the corresponding values for Zn (Fig. 2.3; Table 2.9), which is in agreement with the literature reporting a lower phloem mobility and grain allocation for Fe than for Zn (Marschner, 1995; Grusak et al., 1999). In the case of high N supply, 60% of the total shoot Fe was transported into grain, while this value was 38% at low Fe supply (Figs. 2.4 & 2.7). As in the case of Zn, the relative contribution of post-anthesis uptake to grain accumulation of Fe is enhanced by high N supply so that remobilization of the pre-anthesis Fe stores can account only for 1/6 of the grain Fe under high Zn condition. This suggests that the improvement of the Fe harvest index by high N under high Zn condition is mainly due to enhanced Fe uptake during the grain-filling.

The positive effects of high N supply on Fe uptake, retranslocation and grain allocation may be explained by mechanisms similar to those possibly operating in the case of Zn as discussed above. High N increases phytosiderophore release (S. Bahar Aciksoz *et al.*, unpublished results) and/or the activities of transporter proteins thought to be involved in Fe uptake and remobilization, which include YSL, ZIP, FRD3 and natural resistance-associated macrophage protein (NRAMP) proteins (Haydon & Cobbett, 2007; Borg et al., 2009; Palmer & Guerinot, 2009). The abundance of the non-proteinogenic amino acid nicotianamine, which is also involved in the chelation of ferrous iron (Fe^{2+}) as well as its phloem loading and grain loading (Haydon & Cobbett, 2007; Borg et al., 2009; Curie et al., 2009), may be enhanced by high N supply. Also, the level of ferritin, which increases the sink activity for Fe in seeds (Goto et al., 1999), might be elevated by high N availability.

In the literature, it has been reported that at least 50-70% of the total grain N is taken up before anthesis and later retranslocated to grains (Barneix, 2007). Nitrogen is

highly mobile in the phloem and translocated to grains in the form of amino acids (Barneix, 2007). In agreement with these facts, the straw remobilization ratio for N was at least 70% except under the low Zn - high N condition, where the harvest index and therefore the demand for remobilized N was very low (Table 2.9). Although the relative importance of post-anthesis shoot uptake increased also for N under high N condition, the contribution of remobilization of pre-anthesis stores was always higher for N than for Zn or Fe.

The reliability of the remobilization and nutrient harvest index data of this study are further supported by the results obtained for other essential nutrients including B, which is known to be phloem-immobile in most species including wheat (Marschner, 1995), and P listed among highly phloem-mobile macronutrients (Marschner, 1995). In the case of B, no net remobilization from vegetative tissues was observed and the harvest index was below 2% under all conditions, whereas P was the most efficiently remobilized nutrient and had the highest harvest index, which was above 90% under high Zn-high N condition (data not shown).

2.5. Conclusions

The results presented in this chapter confirmed that a sufficiently high N supply is a critical factor in increasing grain concentrations of both Zn and Fe in wheat. Apparently, improved N nutrition contributes to both root uptake and remobilization of Zn and Fe from vegetative tissues, and increases the relative importance of continued uptake during the grain-filling under greenhouse conditions with sufficient irrigation and Zn supply. During vegetative growth, Zn is stored in both stems and leaves, whereas Fe is mainly allocated to leaves. Iron exhibits relatively lower remobilization than Zn, and therefore, remobilization has a smaller contribution to grain accumulation in the case of Fe. Under field conditions, when uptake is limited during grain development due to drought or any other stress condition, the enhancing effect of improved N nutrition on remobilization might be more relevant in terms of biofortification.

CHAPTER 3

EFFECT OF POST-ANTHESIS ZINC AVAILABILITY ON GRAIN ZINC ACCUMULATION OF WHEAT UNDER NITROGEN SUPPLIES

3.1. Introduction

There are two sources of Zn and Fe accumulated in the wheat grain: concurrent uptake during the grain-filling period and net remobilization from the pre-anthesis stores of source tissues (Chapter 2; Stomph et al., 2009; Waters et al., 2009). Both the straw and the roots can theoretically serve as source tissues for Zn and Fe remobilization, but the roots are usually not taken into account because they cannot be harvested and analyzed easily in soil experiments, especially due to soil contamination problems of root surface and apoplast.

In the literature, there is disagreement about the relative contributions of uptake during the generative development and net remobilization to the grain Zn accretion. Palmgren et al. (2008) suggested that in wheat and barley, Zn remobilization contributes more to the total zinc allocated to grains, whereas Stomph et al. (2009) argued that different cereal species may behave differently in this respect, and that concurrent uptake during the grain-filling is more important in rice. However, according to Wu et al. (2010), the Zn concentration in rice grains is closely associated with the extent of Zn retranslocation from source tissues to grains. Garnett and Graham (2005) stated that, in wheat, most of the Zn allocated to grains could be accounted for by Zn entering the shoot post-anthesis in their experiment, which seems to contradict Palmgren et al. (2008). In a study by Waters et al. (2009), ample Zn and Fe supply during the grain development could totally supersede the need for net remobilization in hydroponically-grown wheat, whereas all the grain Zn could be provided by net remobilization in case

the Zn supply was withheld post-anthesis. Waters and Grusak (2008) claimed that continued uptake and translocation of minerals during the seed-filling stage is at least as important as net remobilization in *Arabidopsis thaliana*.

Wheat is widely grown as a rain-fed crop in semi-arid environments, where the top soil often dries as a result of dry weather (Graham & Rengel, 1993; Elias & Manthey, 2005). In Mediterranean-type climates, the end of the wheat-growing season is characterized by hot weather and restricted water availability (Distelfeld et al., 2007). When the top soil is dry, the roots in the fertilizer zone are inactive. Then, the plants must rely on deeper roots for water and nutrient uptake, and because deeper soil layers are typically poor in nutrients, remobilization of nutrients becomes critical for nutrient supply (Graham & Rengel, 1993). Moreover, nearly half of the cereal-growing soils have low Zn availability (Alloway, 2004; Cakmak, 2008), and Zn fertilizers, applied to the top soil, have low mobility in the soil profile (Marschner, 1993). Drought may therefore often restrict concurrent Zn uptake during the grain filling stage of wheat under field conditions. Under such dry conditions, remobilization becomes particularly important. Efficient remobilization induced by accelerated senescence can improve the grain Zn and Fe accumulation as well as the grain protein content, suggesting that the remobilization of Zn and Fe is linked to the remobilization of N (Uauy et al., 2006a, b; Distelfeld et al., 2007).

The previous chapters (Chapters 1 & 2) have documented that improving the N nutritional status of wheat enhances the grain Zn accumulation at sufficiently high Zn availability. Apparently, although both the uptake and the remobilization of Zn are positively affected by N nutrition, the share of concurrent uptake during grain filling in grain Zn and Fe accumulation is increased by higher N supply (Chapter 2). In this experiment, by discontinuing the Zn supply to hydroponically-grown wheat plants at the anthesis stage, an extreme model environment was created, which mimicked the field conditions restricting Zn uptake during the grain development. This model experiment enabled the study of the effect of post-anthesis Zn availability to roots on grain Zn accumulation of wheat under different N supplies.

3.2. Materials and Methods

In this experiment, durum wheat plants grown in solution culture under controlled climatic conditions. The solution culture system and the climatic conditions in the growth chamber were described in "General Materials and Methods". Plants were grown at three N levels throughout the development: Low N pots were supplied with 0.5 mM N, medium N pots with 1.5 mM N, and high N pots with 4.5 mM N in the form of $Ca(NO_3)_2.4H_2O$. Until the main stems of the low N plants reached the Zadoks stage 65 (anthesis half-way), 0.5 µM Zn was added to the nutrient solutions in all pots. At this stage, the roots and shoots (straw) of one third of the plants were harvested. Zinc was continuously supplied to one half of the remaining plants until maturity, whereas the Zn supply to the other half was discontinued at this stage. When the grains matured and the plants completely senesced, the spikes, straw and roots were harvested. Grains were manually separated from husk, and the husk samples were combined with the corresponding straw samples.

Harvested roots were washed in 0.5 mM $CaSO_4$ solution for 10 min and then with deionized water. All samples were dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods".

For each N treatment in each harvest group, there were 4 pot replicates and 4 single plants in each pot. The straw and spikes of single plants were harvested separately. So, values reported for straw and grain samples are means of 16 replicates. Since the roots of the plants growing in the same pot could not be harvested individually, the root data are based on 4 replicates.

3.3. Results

At maturity, higher N supply resulted in higher root biomass production under both discontinued and continued Zn supply conditions; but at anthesis, medium N plants had the greatest root biomass (Table 3.1). Continued Zn supply increased the root dry weight only at the high N treatment, but did not have any significant effect at the other N levels. The straw biomass production was consistently and markedly enhanced by increasing N supply at both anthesis and maturity. The higher the N supply level, the greater was the post-anthesis increase in straw dry matter.

Table 3.1: The root and straw dry weight of durum wheat (*Triticum durum* cv. Balcali2000) at anthesis and maturity, when grown in solution culture at different N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) levels and with standard (0.5 μM) Zn before anthesis and discontinued or continued Zn supply after anthesis under growth chamber conditions

Root Dry Weight, g plant^{-1}				
Stage	Zn Supply	Low N	Medium N	High N
Anthesis	Std.	*3.5 a	4.3 b	3.2 a
Maturity	Discont.	4.2 Aa	4.9 Ab	5.3 Ac
	Cont.	4.3 Aa	5.1 Ab	7.7 Bc
Straw Dry Weight, g plant^{-1}				
Stage	Zn Supply	Low N	Medium N	High N
Anthesis	Std.	6.4 a	12.3 b	16.4 c
Maturity	Discont.	8.4 a	20.9 b	43.0 c
	Cont.	8.1 a	19.8 b	38.2 c

* The statistical analysis is independent for anthesis and maturity. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p<0.05$). Lacking lettering indicates an insignificant effect according to ANOVA.

As shown in Fig. 3.1, higher N application did not only enhance tillering and thus biomass production, but also delayed senescence under continued Zn regime. However, the effect of high N on senescence was clearly impaired when Zn supply was discontinued after anthesis.

Fig. 3.1: Effect of N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) and Zn (continued vs. discontinued after anthesis) regimes on 86-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown in solution culture under growth chamber conditions

When the N supply was increased from low to medium, the grain yield was more than doubled under both discontinued and continued Zn regimes (Table 3.2). Increasing the N level from medium to high again almost doubled the grain yield at continued Zn treatment, whereas it significantly reduced the grain yield at discontinued Zn treatment. When the N level was low or medium, the grain yield was not affected by the Zn regime, but at the high N treatment, continued Zn supply increased the grain yield tremendously.

Table 3.2: The grain yield, spike number, grain yield / spike dry weight ratio and harvest index of durum wheat (*Triticum durum* cv. Balcali2000) at maturity, when grown in solution culture at different N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) levels and with discontinued or continued Zn supply after anthesis under growth chamber conditions

Grain Yield, g Plant^{-1}			
Zn Supply	Low N	Medium N	High N
Discont.	*6.4 Aa	15.6 Ac	12.8 Ab
Cont.	6.6 Aa	15.4 Ab	27.6 Bc
Spike Number, Plant^{-1}			
Zn Supply	Low N	Medium N	High N
Discont.	5.6 a	11.7 b	18.2 c
Cont.	5.2 a	10.9 b	19.9 c
Grain Yield / Spike Dry Weight, %			
Zn Supply	Low N	Medium N	High N
Discont.	77 Ab	77 Ab	52 Aa
Cont.	77 Ab	77 Ab	72 Ba
Harvest Index, %			
Zn Supply	Low N	Medium N	High N
Discont.	43 Ab	42 Ab	24 Aa
Cont.	45 Aa	43 Aa	42 Ba

* Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05). Lacking lettering indicates an insignificant effect according to ANOVA.

The number of spikes per plant was significantly increased by higher N supply, but unaffected by the Zn regime (Table 3.2). The discontinuation of Zn supply after anthesis resulted in a markedly reduced the ratio of the grain yield to spike dry weight at the high N level, whereas it did not affect this ratio at the other N levels. The harvest index fluctuated between 42% and 45%, except under the high N – discontinued Zn condition, where it was reduced to 24%.

At anthesis, the straw Zn content was significantly enhanced by increasing N supply, whereas the root Zn content was reduced, although the effect did not appear to be significant (Table 3.3). Consequently, the root-to-shoot translocation index for Zn, i.e. the ratio of the shoot Zn content to the total plant Zn content, was increased from 73% to 84%. When compared to anthesis, the root Zn content was markedly reduced at maturity under low or medium N conditions. However, at the high N treatment, there was a net increase in the root Zn content after anthesis, especially under the continued Zn condition. The straw Zn content was also improved by both increasing N and continued Zn treatments. A net increase in the straw Zn content was after anthesis only observed in the case of high N and continued Zn. Under all other conditions, there was a net export of Zn from straw pre-anthesis stores, which was more pronounced at discontinued Zn supply than at continued Zn supply.

In the case of Fe, both the root and straw contents at anthesis were distinctly improved by increasing N applications (Table 3.3). But as the positive effect of N on the straw Fe content was stronger than its effect on the root Fe content, the root-to-shoot translocation index for Fe was increased from 28% to 49%. At maturity, the root Fe content was reduced significantly when the N level was increased from low to medium, but then enhanced tremendously when the N level was increased from medium to high. The higher the N supply level, the higher was the straw Fe content at maturity. There was no net export from pre-anthesis Fe stores of the straw or root under any condition.

As expected, the N contents of both the root and straw at both anthesis and maturity were enhanced by increasing N levels (Table 3.3). Under all N regimes, the root N content at maturity was higher than the root N content at anthesis. Similarly, the straw gained net N after anthesis when the N level was high, but it exported nearly half of its pre-anthesis N store when the N supply was low or medium.

Table 3.3: The Zn, Fe and N contents of the root and straw of durum wheat (*Triticum durum* cv. Balcali2000) at anthesis and maturity, when grown in solution culture at different N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) levels and with standard (0.5 µM) Zn before anthesis and discontinued or continued Zn supply after anthesis under growth chamber conditions

Stage	Zn	Root Zn Content, µg Plant^{-1}			Straw Zn Content, µg Plant^{-1}		
		Low N	Medium N	High N	Low N	Medium N	High N
Ant.	Std.	*64	56	51	175 a	226 b	267 c
Mat.	Discont.	17 Aa	21 Aa	59 Ab	77 Aa	116 Ab	201 Ac
	Cont.	29 Ba	27 Aa	101 Bb	167 Ba	164 Ba	357 Bb

Stage	Zn	Root Fe Content, µg Plant^{-1}			Straw Fe Content, µg Plant^{-1}		
		Low N	Medium N	High N	Low N	Medium N	High N
Ant.	Std.	899 a	995 a	4263 b	355 a	728 b	4085 c
Mat.	Discont.	2196 Aa	1487 Aa	4798 Ab	745 a	2960 b	6854 c
	Cont.	2781 Ab	1435 Aa	10624 Bc	424 a	2915 b	5574 c

Stage	Zn	Root N Content, mg Plant^{-1}			Straw N Content, mg Plant^{-1}		
		Low N	Medium N	High N	Low N	Medium N	High N
Ant.	Std.	34 a	54 b	64 c	79 a	227 b	533 c
Mat.	Discont.	37 Aa	61 Ab	116 Ac	37 a	111 b	641 c
	Cont.	38 Aa	58 Ab	141 Bc	37 a	111 b	595 c

* The statistical analysis is independent for anthesis and maturity. Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test ($p < 0.05$). Lacking lettering indicates an insignificant effect according to ANOVA.

On one hand, discontinued Zn supply after anthesis reduced the grain Zn concentration by 40-55%, depending on the N level (Table 3.4). On the other hand, increasing N supply resulted in marked reductions in grain Zn concentrations. At all N levels, the grain Zn yield under the discontinued Zn regime was significantly lower than that under the continued Zn regime. When the Zn supply was withheld post-anthesis, the grain Zn yield was severely decreased by the high N treatment, whereas increasing the N level from low to medium or high led to slightly improved grain Zn yields.

Table 3.4: The grain Zn, Fe and N concentrations and yields of durum wheat (*Triticum durum* cv. Balcali2000) at maturity, when grown in solution culture at different N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) levels and with discontinued or continued Zn supply after anthesis under growth chamber conditions

	Grain Zn Concentration, mg kg^{-1}			Grain Zn Yield, µg Zn plant^{-1}		
Zn Supply	Low N	Medium N	High N	Low N	Medium N	High N
Discont.	*28.2 Ac	10.8 Ab	6.3 Aa	160 Ab	162 Ab	70 Aa
Cont.	46.9 Bc	23.8 Bb	12.9 Ba	310 Ba	354 Bb	352 Bb

	Grain Fe Concentration, mg kg^{-1}			Grain Fe Yield, µg Fe plant^{-1}		
Zn Supply	Low N	Medium N	High N	Low N	Medium N	High N
Discont.	67.3 a	84.0 b	91.5 c	426 Aa	1292 Ab	1178 Ab
Cont.	57.0 a	96.9 b	94.1 b	373 Aa	1464 Bb	2582 Bc

	Grain N Concentration, %			Grain N Yield, mg N plant^{-1}		
Zn Supply	Low N	Medium N	High N	Low N	Medium N	High N
Discont.	2.43 b	2.25 a	2.79 c	156 Aa	350 Ab	353 Ab
Cont.	2.20 a	2.23 a	2.90 b	147 Aa	345 Ab	806 Bc

* Means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different according to Fisher's protected LSD test (p<0.05). Lacking lettering indicates an insignificant effect according to ANOVA.

The Zn supply regime did not have a consistent effect on the grain Fe concentration (Table 3.4), but higher N supply enhanced it significantly. The positive effect of increasing N supply on the grain Fe yield was even more pronounced. When the N level was increased from low to high, the grain Fe yield was increased by nearly 200% under the discontinued Zn and 600% under the continued Zn regime. The grain N concentration reached its maximum at the high N treatment and was not significantly affected by the Zn supply. When the N level was increased from low to medium, the grain N yield was more than doubled, irrespective of the Zn regime; but when the N level was further increased to high, the grain N yield was unaffected at the discontinued Zn treatment while it was again more than doubled at the continued Zn treatment.

In the low N case, about 60% of the total Zn in the plant was allocated to the grains, irrespective of the post-anthesis Zn regime (Fig. 3.2). When the N level was increased from low to medium, the Zn harvest index tended to decrease at the discontinued Zn treatment but increase at the continued Zn treatment. The high N treatment resulted in markedly reduced Zn harvest indices, especially in the discontinued Zn case.
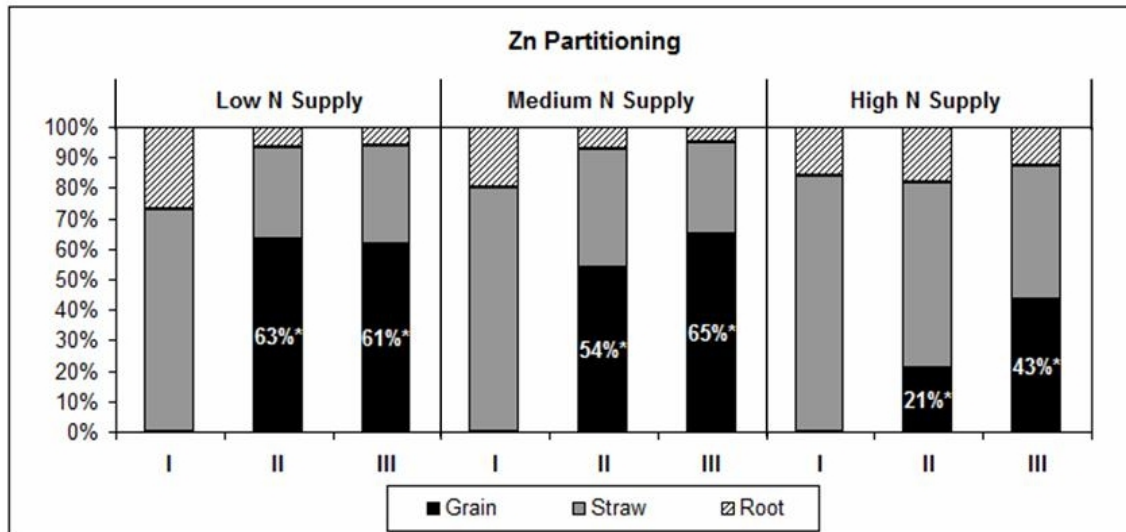
Fig. 3.2: Effect of N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) supply on the Zn partitioning and Zn harvest index of durum wheat (*Triticum durum* cv. Balcali2000) plants (**I**) at anthesis, (**II**) at maturity with discontinued Zn supply after anthesis, and (**III**) at maturity with continued Zn supply after anthesis.

Fig. 3.3 shows how the shares of post-anthesis Zn uptake and remobilization of Zn from pre-anthesis stores of the root and straw on the grain Zn yield are affected by the N and Zn nutrition. At the discontinued Zn treatment, the share of Zn remobilization from root pre-anthesis stores in grain Zn decreased, while the share of remobilization from straw pre-anthesis stores increased, when the N supply was increased. The apparent small share of continued Zn uptake under the discontinued Zn regime indicates a minor contamination. In contrast, under the continued Zn supply regime, post-anthesis Zn uptake contributed to grain Zn much more than remobilization from pre-anthesis stores. When the N supply was high, 100% of the grain Zn could be explained by post-anthesis Zn uptake.
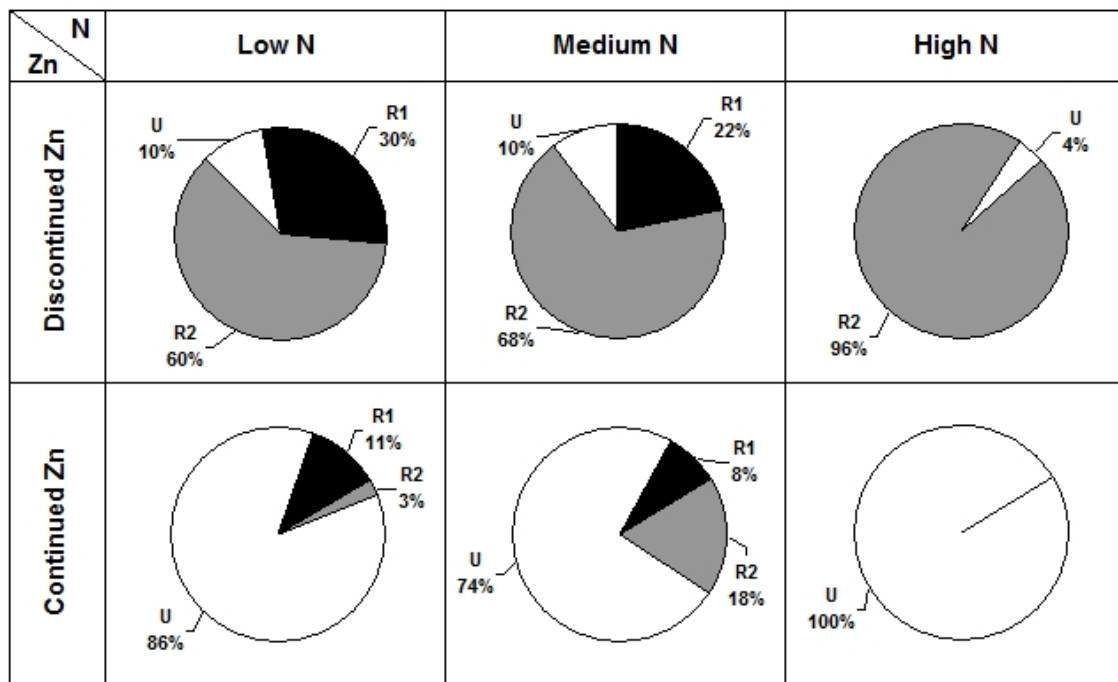
Fig. 3.3: Effect of N (low: 0.5 mM; medium: 1.5 mM; high: 4.5 mM) supply and post-anthesis Zn regime (discontinued vs. continued) on the shares of (**U**) post-anthesis uptake, (**R1**) remobilization from root pre-anthesis stores, and (**R2**) remobilization from straw pre-anthesis stores in the grain Zn accumulation of durum wheat (*Triticum durum* cv. Balcali2000) plants grown in solution culture under growth chamber conditions

3.4. Discussion

The N supply level affected the biomass of vegetative tissues much more strongly than the Zn regime (Table 3.1). By markedly stimulating the straw dry matter production and tillering (Fig. 3.1), as documented in previous chapters (Chapters 1 & 2) and in the literature (Ewert & Honermeier, 1999; Salvagiotti & Miralles, 2007), but only slightly affecting the root biomass, higher N supply reduced the root-to-shoot ratio from 55% to 20% at anthesis (Table 3.1). The positive response of the root-to-shoot ratio to low N availability is well documented in the literature (Levin et al., 1989; Marschner, 1995). Increasing N supply also had a clear effect on senescence. In agreement with the results reported in previous chapters (Chapters 1 & 2), higher N plants remained green longer than lower N plants and had longer grain-filling periods, as reported by Yang and Zhang (2006). However, this effect of high N almost

disappeared under the discontinued Zn regime, probably because of the retranslocation of all of the mobile Zn out of the source tissues (Table 3.3), which triggered senescence.

The increases in the grain yield due to improved N nutrition were parallel to the increases in the spike number per plant under all conditions except the high N – discontinued Zn condition (Table 3.2), which indicates that the grain yield was mainly a function of the number of tillers that formed spikes. The reason behind the significant reduction in the grain yield by the high N treatment under the discontinued Zn regime was the severe Zn dilution due to enhanced vegetative growth (Table 3.1). Dilution of Zn may have affected the grain yield by two mechanisms: Firstly, Zn deficiency may have impaired the reproductive development as documented in the literature (Sharma et al., 1990; Cakmak & Engels, 1999). The Zn supply was discontinued when the main stems were at the anthesis stage. However, at the high N treatment, most of the tillers that contributed to the grain yield were formed much later than the discontinuation of the Zn supply. Therefore, it is very likely that the reproductive development in later spikes was adversely affected by Zn deficiency. Secondly, since the senescence-delaying effect of high N was totally negated by Zn deficiency (Fig. 3.1), the grains had not yet completed their development, when these plants completely senesced. Consequently, most of the grains harvested from these plants were shriveled.

Higher N treatment increased the total plant Zn content, which can be calculated from the data in the Tables 3.3 and 3.4, by up to 33% at anthesis and by up to 60% at maturity under the continued Zn regime. This, in agreement with Chapters 1 and 2, indicates that improved N nutrition contributes significantly to Zn uptake, probably because the abundance of Zn uptake transporters is enhanced in the roots (Cakmak et al. 2010), and Zn uptake can continue for a longer time due to delayed senescence. Radioisotope studies conducted with ^{65}Zn revealed that improved N nutritional status is associated with significantly higher Zn uptake rates, but under static solution culture conditions, where the nutrient solutions are refreshed every 3-4 days, even low N plants can consume all of the Zn in the nutrient solution before its refreshment, so that the positive effect of N on the Zn uptake rate may not be reflected in the total plant Zn content (Erenoglu et al., unpublished results). Therefore, the positive effect of high N treatment on the Zn uptake may be more pronounced in soil culture with sufficient Zn availability throughout the development (Chapters 1 & 2).

Similar to the Zn uptake, the root-to-shoot translocation of Zn was also improved by higher N, as revealed by the increased Zn translocation index at anthesis, which can be calculated from the data in Table 3.3. The mechanism behind might be related to increased activities of transporter proteins involved in xylem loading of Zn and increased abundance of nitrogenous compounds facilitating Zn transport such as nicotianamine and deoxymugineic acid (Suzuki *et al.*, 2008; Curie *et al.*, 2009; Palmer & Guerinot, 2009).

In all cases, where the straw Zn content at maturity was lower than that at anthesis, the root Zn content at maturity was either also lower than or almost the same as the root Zn content at anthesis, indicating that 100% of the Zn remobilized from pre-anthesis stores is targeted to the grains (Table 3.3). The net remobilization of Zn from the root pre-anthesis stores at the low and medium N levels shows that part of the Zn entering the shoot post-anthesis is in fact Zn remobilized from the roots, as speculated by Garnett and Graham (2005). Waters et al. (2009) also demonstrated that net remobilization from the root stores can occur, depending on the nutritional status of the plants.

In the discontinued Zn group, the net amount of Zn remobilized from the straw pre-anthesis stores was slightly increased when the N level was increased from low to medium, but significantly decreased when the N level was further increased to high (Table 3.3). From the values in Tables 3.1 and 3.3, it can be calculated that the straw Zn concentration at maturity was reduced to ca. 5 mg·kg^{-1} at the medium and high N levels, when the Zn supply was withheld after anthesis. A small amount of Zn is probably incorporated into structural molecules such as cell walls and thus becomes unavailable for remobilization (Chapter 2; Marschner, 1995; Waters & Grusak, 2008). Then, remobilization is only possible if the amount of Zn stored in source tissues is higher than this minimal amount (Waters & Grusak, 2008). It seems very likely that this minimal concentration for Zn is ca. 5 mg·kg^{-1} for wheat straw. As the straw Zn concentration approaches this minimal value due to net export of Zn to the grains, the remobilization may cease. Because of the severe Zn dilution in the high N plants, the straw of these plants could most probably export less Zn to the grains before reaching the critical value, and therefore, net remobilization of Zn from straw pre-anthesis stores seemed to be impaired by higher N.

In plants continuously supplied with Zn until maturity, the amount of Zn remobilized from the straw pre-anthesis stores was significantly increased by the medium N as compared to the low N level, whereas no net remobilization from the straw was observed at the high N level (Table 3.3). This contradictory effect of increasing N supply on Zn remobilization may be explained by the well documented association of Zn remobilization with senescence. Zinc remobilization to developing grains is known to be enhanced by senescence (Longnecker & Robson, 1993; Marschner, 1995; Uauy et al., 2006b; Distelfeld et al., 2007), although Zn can also be retranslocated from non-senescent leaves (Hajiboland et al., 2001; Erenoglu et al., 2002). In contrast to the high N – discontinued Zn condition, where the source tissues senesced before the grain could complete their development, the source tissues were still green under the high N – continued Zn condition, when the grains matured (Fig. 3.1). This distinct delay in senescence under the high N – continued Zn condition may have severely reduced the amount of Zn remobilized from the straw to the grains and thus resulted in the apparent zero net remobilization from the pre-anthesis stores (Table 3.3). As revealed by genetic studies on the wheat *Gpc-B1* locus associated with accelerated senescence, micronutrient remobilization to developing grains is linked to nitrogen remobilization (Uauy et al., 2006b; Distelfeld et al., 2007). In agreement with this finding, the straw N concentration of high N plants was at maturity ca. 3 times higher than that of low or medium N plants under the continued Zn supply regime (Tables 3.1 & 3.3). So, not only the Zn but also N remobilization was impaired by delayed senescence.

In the case of Fe, the positive effect of increasing N on uptake was much more distinct then in the case of Zn, probably because the standard Fe concentration in the nutrient solution was high (Table 3.3). In fact, the reported root contents in Table 3.3 cannot be directly used for calculating the amounts taken up, because apoplastic Zn and Fe were not removed. However, the substantial differences in the root Fe contents caused by varied N supply cannot be explained by apoplastic Fe, as they do not correlate with root biomass (Tables 3.1 & 3.3). Moreover, the root-to-shoot translocation of Fe was so strongly enhanced that the straw Fe contents of medium N plants were much higher than their root contents, whereas exactly the opposite was true for low N plants.

Increasing N supply consistently decreased the grain Zn concentration but increased the grain Fe concentration (Table 3.4). The reason behind the reduction of the grain Zn concentration by higher N application was, probably, the low Zn availability even under the continued Zn regime, in conjunction with the N-induced enhancements in vegetative biomass and grain yield (Tables 3.1 & 3.2). Under these conditions, the dilution effect overshadowed the positive effects of N on Zn uptake, translocation and remobilization. Iron availability was, in contrast to Zn availability, high throughout the development. These results confirmed the previous finding that the contribution of N fertilization to grain Zn accumulation is dependent on Zn availability (Chapters 1 & 2).

It is a long-lasting debate whether uptake during the seed development or remobilization from the source tissues contributes more to seed accretion of minerals (Waters & Grusak, 2008; Stomph et al., 2009; Waters et al. 2009; Wu et al., 2010). The results of this experiment demonstrated that the answer to this question depends very much on the Zn and N nutrition regimes and that both extremes are possible: Zinc remobilized from the straw can supply almost 100% of the grain Zn if the plants cannot take up Zn by roots during the grain development, whereas up to 100% of the grain Zn can be attributed to Zn uptake during the seed-filling stage if the Zn uptake continues and the conditions do not favor Zn retranslocation (Fig. 3.3).

3.5. Conclusions

Continued Zn and Fe uptake during later developmental stages contributes greatly to the grain accretion of these micronutrients in wheat. Both the uptake and the root-to-shoot translocation of these minerals are enhanced when the N nutritional status of plants is improved. In field-grown wheat, however, the remobilization may be a more important issue if the uptake is restricted due to drought during the seed development. Micronutrient remobilization is a complex phenomenon affected by N nutrition, micronutrient availability in source tissues and the timing of senescence. Although improved N status may favor Zn remobilization if Zn is abundant in source tissues, it can also impair Zn retranslocation by resulting in Zn dilution due to enhanced biomass production under low Zn availability. No matter if the conditions favor the contribution of uptake or that of remobilization to the grain micronutrient accumulation, a sufficiently high micronutrient availability is a prerequisite for an efficient utilization of N fertilization for biofortification purposes.

CHAPTER 4

IMPROVED NITROGEN STATUS ENHANCES
ZINC AND IRON CONCENTRATIONS NOT ONLY IN THE WHOLE GRAIN
BUT ALSO THE ENDOSPERM OF WHEAT

4.1. Introduction

The wheat grain is composed of three major parts, which are the embryo (germ), the bran and the endosperm (Fig. 4.1). On average, the wheat embryo (germ) constitutes about 2-4% and the true bran layers about 7-8% of the grain weight (Posner, 2000). However, the bran fraction of milled wheat grain also includes the aleurone layer, which typically constitutes 5-8% of the kernel weight and breaks away together with the bran layers (Nelson, 1985), although it is in fact part of the endosperm. The starchy endosperm, surrounded by the aleurone layer, is the main constituent of white flour, makes up about 81-84% of the wheat grain weight (Posner, 2000). These values are affected by genotypic and several agronomic factors including the grain size (Chaurand et al., 1999). The average grain size is a rough measure for the endosperm-to-bran ratio: A greater grain size means a greater volume-to-surface ratio, which implies a greater endosperm-to-bran ratio.

In literature related to the enrichment of cereal grains with micronutrients, the reported data are mostly based on whole grain analyses. However, it is well documented that the concentrations of micronutrients, protein and the antinutrient phytate show big differences among the grain fractions, which may have a great impact on the dietary intake and bioavailability of micronutrients (Tang et al., 2008). As compared to whole grain, bran and embryo, the endosperm part (white flour) contains markedly lower concentrations of protein, Zn and Fe (Ehret, 1985; Lott et al., 1995; Ozturk et al., 2006;

Persson et al., 2009), but also much lower concentrations of phytate (Pomeranz, 1978; Lehrfeld & Wu, 1991; Joyce et al., 2005). In many countries, both common wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*) products including bread, pasta, flat bread, pizza and couscous are most often made of white flour. During the milling process for white flour production, the protein-, Zn- and Fe-rich parts of the grain are removed, which results in reduced Zn intake (Nelson, 1985; Ozturk et al., 2006; Cakmak, 2008; Dewettinck et al., 2008). However, bioavailability is as important as intake in nutrition.
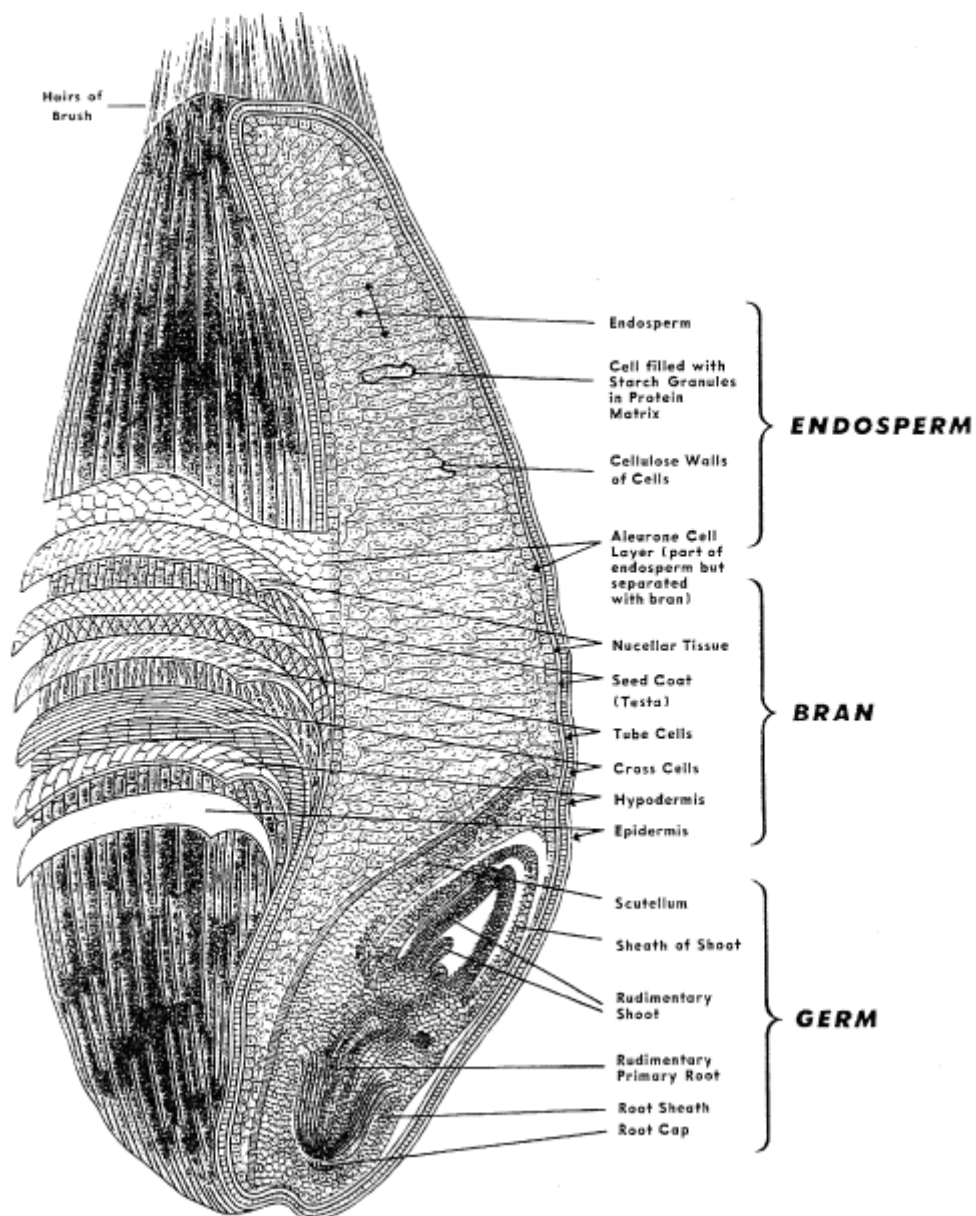
Fig. 4.1: Parts of the wheat grain; longitudinal section; enlarged ca. 70 times (Slavin et al., 2001)

Bioavailability is a complex phenomenon affected by various factors including the individual's characteristics, the food composition (promoters and antinutrients), and the processing and preparation of food (Welch & Graham, 2004). Due to its high polyvalent cation-chelating activity and the lack of phytase enzyme in monogastric animals and humans, phytate reduces the absorption of dietary Zn and Fe (Hotz & Gibson, 2007). The molar ratio of phytate to Zn or Fe is often used as an indicator for the bioavailability of these minerals (Ryan et al., 2008). As there is a very close positive correlation between the phytate and total P concentrations in seeds, and 70-80% of the seed P is found in the form of phytate in seeds (Raboy et al., 1984; Erdal et al., 2002), the molar ratio of seed P to Zn or Fe can also be directly used as a bioavailability indicator (Simic et al., 2009).

It has been demonstrated by both greenhouse and field experiments that N management is a critical tool for agronomic biofortification of wheat grain with Zn and Fe (Chapters 1 & 2; Cakmak et al., 2010; Shi et al., 2010). Combining high N fertilization with soil and/or foliar Zn applications has a synergistic positive effect on grain Zn concentration (Chapters 1 & 2). Moreover, the positive effect of high N fertilization on the grain protein concentration may also be relevant in terms of Zn and Fe bioavailability, because proteins and amino acids in the grain may act as promoters, and high grain protein content may contribute to higher bioavailability of micronutrients in diet (House et al., 1997; Lonnerdal, 2000).

In the literature, there is no publication which investigated the effects of N fertilization on the concentrations of Zn and Fe in different fractions of wheat grain. Since the consumption of whole grain products is far less common than that of white flour, the endosperm tissue is more relevant than the whole wheat grain in terms of biofortification. In this study, the effects of increasing N fertilization on the concentrations of Zn, Fe, P and protein have been examined in grain fractions, including the endosperm, embryo and bran, of wheat grown on a Zn-deficient soil with different Zn treatments.

4.2. Materials and Methods

The experiment presented in this chapter had a factorial design and eight pot replicates in each treatment group.

The soil was prepared as described in "General Materials and Methods". Plants were grown with four different soil N and two different soil Zn treatments, and with or without foliar Zn application. In order to establish the different N levels, the following amounts of N were applied per kg soil: 50 mg (low), 100 mg (medium), 200 mg (high), and 400 mg (very high). The low soil Zn condition was established by adding 0.5 mg.kg^{-1} Zn to the soil, and the high soil Zn condition by adding 5 mg.kg^{-1} Zn. Foliar Zn applications were started just before flowering, when the plants were eight weeks old. One half of the plants were left untreated, whereas the other half were sprayed with a 0.2% (w/v) $ZnSO_4 \cdot 7H_2O$ solution containing 0.01% (w/v) Tween20 to the point of run-off. The foliar application was repeated twice at two-week intervals. Throughout this paper, the Zn treatment groups are referred to as follows:

i. *Group 1*: Low soil Zn & no foliar Zn treatment

ii. *Group 2*: High soil Zn & no foliar Zn treatment

iii. *Group 3*: Low soil Zn & foliar Zn treatment

iv. *Group 4*: high soil Zn & foliar Zn treatment

When grains matured and the plants completely senesced, both spikes and straw were harvested. Grains were manually separated from husk.

Three grain samples were chosen randomly from each of the treatments for fractionation into embryo, bran and endosperm. Each sample contained nearly 120 grains. First, the embryo was manually separated from the rest of the grain by using a scalpel. In order to separate the bran from the endosperm, the remainders of the grains were ground in an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany) for 120 seconds. The milled samples were first sieved through a 1 mm sieve, and the particles retained on the sieve were collected as bran samples. Then, the particles that passed through the 1 mm sieve were sieved through a 100 µM sieve. The particles passing through the sieve were collected as endosperm samples. The particles retained

on the second sieve were discarded, because they contained both endosperm and bran parts.

All samples were dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods".

4.3. Results

As revealed by three-way ANOVA, all N and Zn treatments as well as their double and triple interactions affected the straw dry matter significantly (Table 4.1). Increasing the N supply increased the straw dry weight progressively in all Zn treatment groups (Table 4.2). In the absence of foliar Zn treatment, plants grown with high soil Zn supply had higher straw dry weights than the plants grown with low soil Zn supply. However, when plants were treated foliarly with Zn, the positive effect of high soil Zn supply on straw production disappeared at high N level. The straw dry matter production was, in general, negatively affected by foliar Zn application, especially when the soil Zn supply was high.

The grain yield was significantly affected by all treatments and their interactions, except the soil N x foliar Zn interaction (Table 4.1). Response of the grain yield to N treatments was highly similar to those of straw dry matter production (Table 4.2). Increasing N supply from low to very high more than doubled the grain yield under the low soil Zn condition and more than tripled it under the high soil Zn condition. High soil Zn application resulted in significantly higher yields at all N levels except the low level. In contrast, foliar Zn application improved the grain yield only at low soil Zn and very high soil N level (Table 4.2). At high soil Zn supply, foliar Zn application had an adverse effect on the yield, when the N level was high or very high. Otherwise, there was no significant effect of foliar Zn application on the grain yield. The harvest index fluctuated between 50% and 63% in all treatment groups.

Table 4.1: Three-way analysis of variance (ANOVA) of the effects of soil N, soil Zn and foliar Zn applications as well as their interactions on reported traits of durum wheat (*Triticum durum* cv. Balcali2000): Degrees of freedom, F value probabilities and Fisher's protected $LSD_{0.05}$ test scores

Source of Variation	DF	Straw Dry Weight		Grain Yield		Av. Grain Size	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.11	<0.001	0.14	<0.001	2.0
Soil Zn (B)	1	<0.001	0.07	<0.001	0.10	0.016	1.4
Foliar Zn (C)	1	<0.001	0.07	<0.001	0.10	<0.001	1.4
A x B	3	0.003	0.15	<0.001	0.20	0.090	*n.s.*
A x C	3	0.047	0.15	0.161	*n.s.*	0.169	*n.s.*
B x C	1	<0.001	0.11	<0.001	0.14	0.593	*n.s.*
A x B x C	3	<0.001	0.21	<0.001	0.28	0.620	*n.s.*

Source of Variation	DF	Whole Grain N		Whole Grain Zn		Whole Grain Fe	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.18	<0.001	3.7	<0.001	2.5
Soil Zn (B)	1	0.041	0.12	<0.001	2.6	0.007	1.8
Foliar Zn (C)	1	0.025	0.12	<0.001	2.6	0.040	1.8
A x B	3	0.210	*n.s.*	0.069	*n.s.*	0.249	*n.s.*
A x C	3	0.995	*n.s.*	<0.001	5.2	0.025	3.6
B x C	1	0.512	*n.s.*	<0.001	3.7	0.006	2.5
A x B x C	3	0.757	*n.s.*	0.345	*n.s.*	0.019	5.1

Source of Variation	DF	Endosperm N		Endosperm Zn		Endosperm Fe	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.17	<0.001	1.6	<0.001	1.7
Soil Zn (B)	1	0.008	0.12	<0.001	1.1	<0.001	1.2
Foliar Zn (C)	1	0.002	0.12	<0.001	1.1	0.687	*n.s.*
A x B	3	0.018	0.24	<0.001	2.3	0.157	*n.s.*
A x C	3	0.693	*n.s.*	<0.001	2.3	0.100	*n.s.*
B x C	1	0.677	*n.s.*	<0.001	1.6	0.001	1.7
A x B x C	3	0.898	*n.s.*	0.014	3.2	0.008	3.3

Source of Variation	DF	Embryo N		Embryo Zn		Embryo Fe	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.23	<0.001	6.7	<0.001	5.5
Soil Zn (B)	1	0.165	*n.s.*	<0.001	4.7	<0.001	3.9
Foliar Zn (C)	1	0.047	0.17	<0.001	4.7	<0.001	3.9
A x B	3	0.115	*n.s.*	<0.001	9.4	0.753	*n.s.*
A x C	3	0.487	*n.s.*	<0.001	9.4	0.007	7.7
B x C	1	0.140	*n.s.*	<0.001	6.7	0.362	*n.s.*
A x B x C	3	0.560	*n.s.*	0.072	*n.s.*	0.003	10.9

Source of Variation	DF	Bran N		Bran Zn		Bran Fe	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.19	<0.001	16.7	<0.001	6.3
Soil Zn (B)	1	<0.001	0.13	<0.001	11.8	<0.001	4.4
Foliar Zn (C)	1	0.060	*n.s.*	<0.001	11.8	0.002	4.4
A x B	3	0.001	0.26	0.064	*n.s.*	0.005	8.9
A x C	3	0.242	*n.s.*	<0.001	23.6	0.041	8.9
B x C	1	0.565	*n.s.*	<0.001	16.7	0.070	*n.s.*
A x B x C	3	0.889	*n.s.*	0.395	*n.s.*	<0.001	12.6

Table 4.2: Straw dry weight, grain yield and average grain size of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil with low (50 mg N per kg soil) or medium (100 mg N per kg soil) or high (200 mg N per kg soil) or very high (400 mg N per kg soil) N supply, low (0.5 mg Zn per kg soil) or high (5.0 mg Zn per kg soil) soil Zn supply, and with or without foliar Zn (3X 0.2% $ZnSO_4 \cdot 7H_2O$) application

	Straw Dry Weight (g plant^{-1})			
	No Foliar Zn Treatment		Foliar Zn Treatment	
N Level	Low Soil Zn	High Soil Zn	Low Soil Zn	High Soil Zn
Low	[a]1.58 Ab	1.65 Abc	1.42 Aa	1.53 Ab
Medium	2.06 Bb	2.31 Bc	1.91 Ba	2.07 Bb
High	3.02 Cb	3.29 Cc	2.77 Ca	2.77 Ca
Very High	3.56 Da	4.61 Dd	4.04 Dc	3.92 Db
	Grain Yield (g plant^{-1})			
	No Foliar Zn Treatment		Foliar Zn Treatment	
N Level	Low Soil Zn	High Soil Zn	Low Soil Zn	High Soil Zn
Low	1.76 Aa	1.71 Aa	1.63 Aa	1.64 Aa
Medium	2.70 Ba	2.93 Bb	2.64 Ba	2.84 Bb
High	3.58 Ca	5.20 Cc	3.71 Ca	4.66 Cb
Very High	3.90 Da	6.20 Dd	4.16 Db	5.20 Dc
	Average Grain Size (mg grain^{-1})			
	No Foliar Zn Treatment		Foliar Zn Treatment	
N Level	Low Soil Zn	High Soil Zn	Low Soil Zn	High Soil Zn
Low	47.8 A[b]	46.4 A	44.2 A	42.5 A
Medium	59.9 B	53.5 B	52.0 B	48.7 B
High	63.5 C	61.7 C	60.4 D	60.9 D
Very High	60.0 B	60.9 C	58.0 C	57.1 C

[a] Values are means of eight independent replicates. For each trait, means in columns followed by different uppercase letters and means in rows followed by different lowercase letters are significantly different from each other according to Fisher's protected LSD test (p<0.05).

[b] Lacking lowercase lettering indicates an insignificant effect according to ANOVA.

The average grain size was not consistently affected by the soil Zn level under given conditions (Tables 4.1 & 4.2). Foliar Zn application decreased the average grain size to a small extent, but increasing the N supply up to the high level enhanced the average grain size significantly. The N-dependent relative increases in the average grain size were between 33% and 43%. Increasing the N level from high to very high, however, did not further improve the grain size, but tended to reduce it (Table 4.2).

The N concentrations of whole grains and grain fractions were progressively and substantially enhanced by increasing soil N treatments in all Zn application groups (Fig. 4.2; Table 4.1). In the endosperm, the N concentration was almost doubled when the N supply was increased from low to very high. The endosperm N concentration slightly decreased by high soil Zn supply at higher N levels, but foliar Zn application enhanced the endosperm N concentration significantly in all groups. Soil or foliar applications of Zn did not have marked effects on the N concentration of the embryo. High soil Zn had a decreasing effect on the bran N concentration at high or very high N level, but foliar Zn treatment did not affect the bran N. In all grain samples, irrespective of the N, soil Zn and foliar Zn treatments and without any exception, the N concentration of the embryo was higher than that of the bran, which was in turn higher than that of the endosperm.
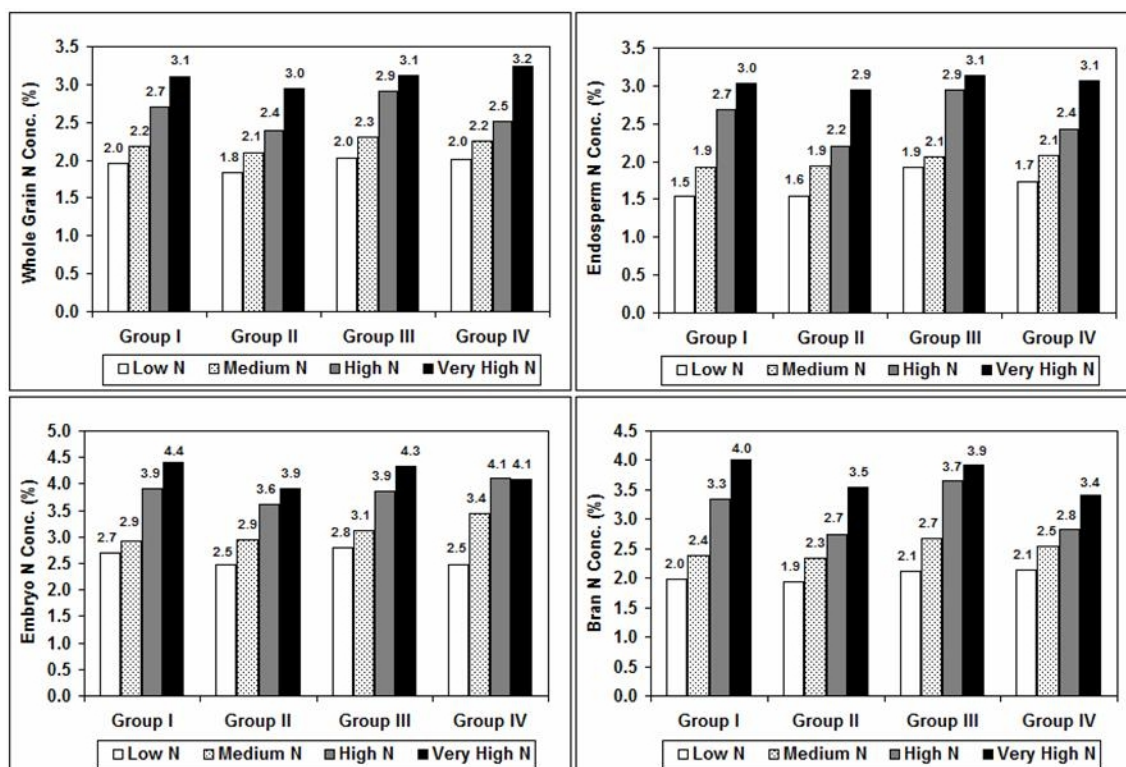
Fig. 4.2: Nitrogen concentrations of whole grain, endosperm, embryo and bran samples of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels in four different Zn treatment groups: *Group 1*: low soil Zn & no foliar Zn; *Group 2*: high soil Zn & no foliar Zn; *Group 3*: low soil Zn & foliar Zn; *Group 4*: high soil Zn & foliar Zn. For LSD$_{0.05}$ scores, please refer to Table 1.

The effects of N, soil Zn and foliar Zn treatments on the Zn concentrations of whole grains and grain fractions were all highly significant (Table 4.1). In group I plants, the Zn concentration was reduced in whole grains, the endosperm and the embryo by increasing N supply, but increased in the bran (Fig. 4.3). The Zn concentrations of whole grains and all grain fractions were significantly enhanced by increasing N level in all other Zn treatment groups. In all cases where N had a positive effect on Zn concentration, the relative enhancement of Zn concentration was stronger in the endosperm (also in the bran) than in the whole grain (Fig. 4.3). The group IV plants (high soil Zn and foliar Zn) showed the most dramatic responses to N treatment: When the N supply was increased from low to very high, the Zn concentration in the whole grain was increased by over 50%, while that in the endosperm was enhanced by over 80%.
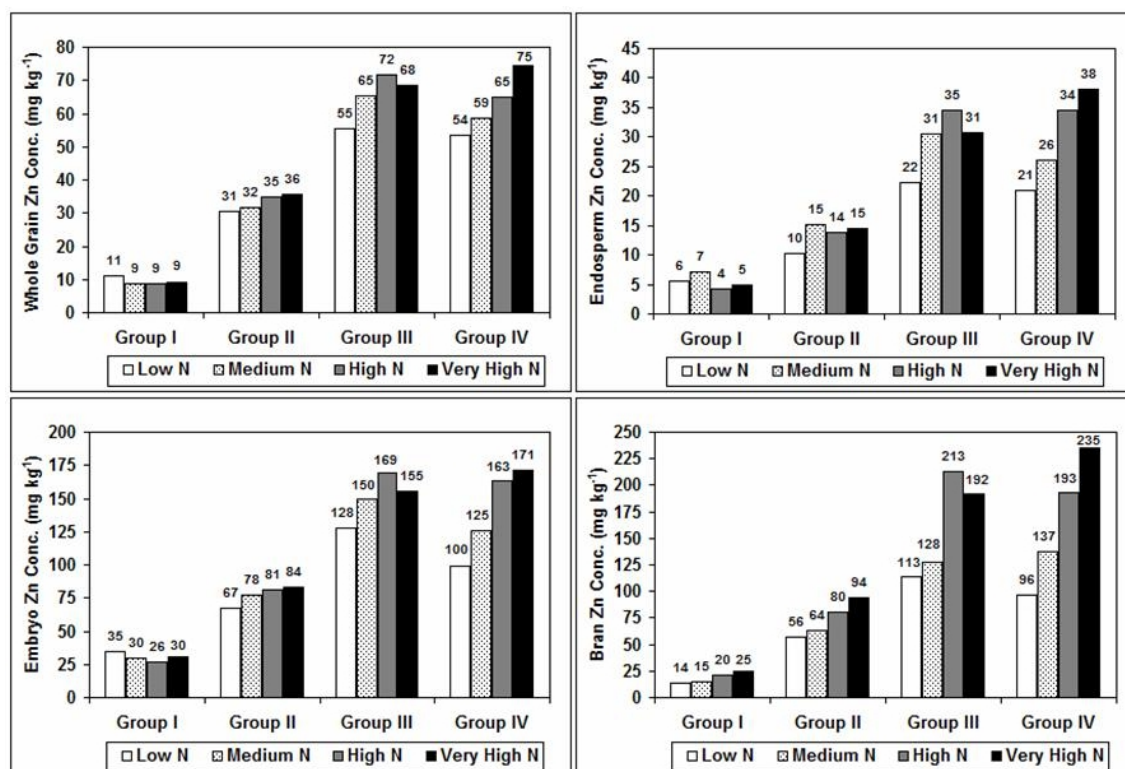
Fig. 4.3: Zinc concentrations of whole grain, endosperm, embryo and bran samples of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels in four different Zn treatment groups: *Group 1*: low soil Zn & no foliar Zn; *Group 2*: high soil Zn & no foliar Zn; *Group 3*: low soil Zn & foliar Zn; *Group 4*: high soil Zn & foliar Zn. For $LSD_{0.05}$ scores, please refer to Table 1.

High soil Zn supply increased the Zn concentration in whole grains and grain fractions tremendously in the absence of foliar Zn treatment (Fig. 4.3), but foliar Zn application overshadowed the positive effect of high soil Zn supply on the reported Zn concentrations. Among all treatment groups, the endosperm was the grain fraction with the lowest Zn concentration. Under low Zn supply (e.g. group I plants), the embryo had a higher Zn concentration than the bran; however, the bran could reach much higher Zn concentrations than the embryo, when plants were exposed to higher N levels and foliar Zn treatment.
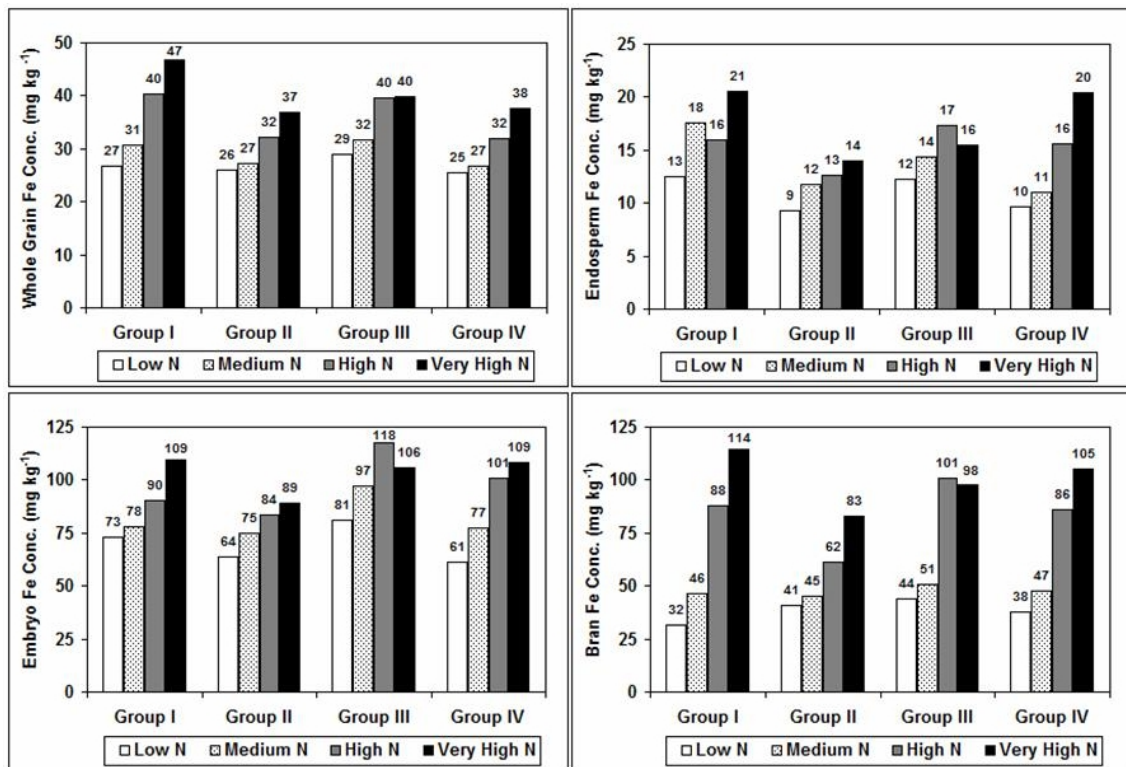
Fig. 4.4: Iron concentrations of whole grain, endosperm, embryo and bran samples of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels in four different Zn treatment groups: *Group 1*: low soil Zn & no foliar Zn; *Group 2*: high soil Zn & no foliar Zn; *Group 3*: low soil Zn & foliar Zn; *Group 4*: high soil Zn & foliar Zn. For LSD$_{0.05}$ scores, please refer to Table 1.

The Fe concentrations of whole grains and grain fractions were significantly affected by soil N and Zn applications (Table 4.1). Increasing N supply resulted in distinct positive effects on Fe concentrations in all Zn treatment groups (Fig. 4.4). The relative enhancements in the Fe concentrations of the endosperm and embryo by increasing N supply represented more or less the relative enhancement in the whole

grain Fe concentration, but the bran Fe concentration responded to N much more dramatically. For instance, when the N supply was increased from low to very high in group I plants, the Fe concentrations of the whole grain, endosperm and embryo were enhanced by up to 75%, while that of the bran was increased by over 250%. In the absence of foliar Zn application, correction of Zn deficiency by soil Zn application tended to reduce the Fe concentration in the reported fractions. The effects of foliar Zn treatment on the reported Fe concentrations were not consistent.
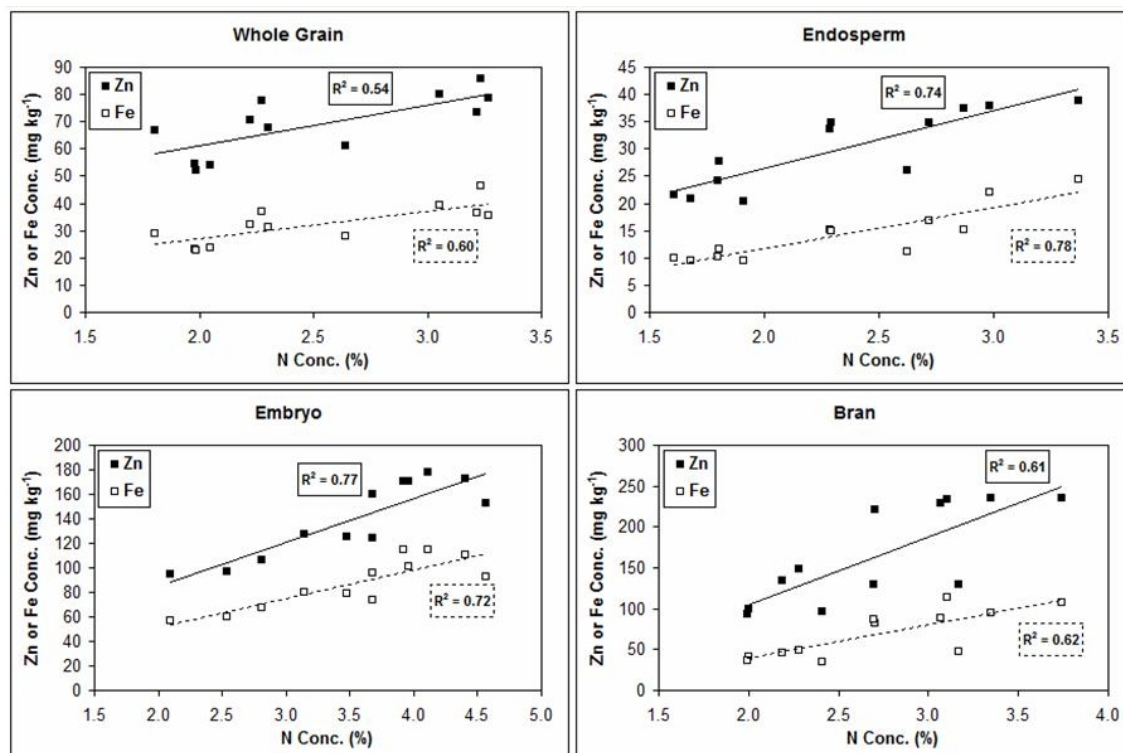
Fig. 4.5: Zinc-nitrogen and iron-nitrogen concentration correlations in whole grains and grain fractions of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels with high soil and foliar Zn supply (*Group 4*)

In both the whole grain and grain fractions of interest, the concentrations of Zn and Fe correlated positively with the N concentration. Fig. 4.5 shows these correlations for the group IV plants. The linear regression curves for both Zn-N and Fe-N value pairs have positive slopes, and the coefficients of determination (R^2 values) demonstrate that significant percentages of the variations in the Zn and Fe concentrations can be explained by the variations in the N concentration.

Fig. 4.6: Phosphorus concentrations of whole grain, endosperm, embryo and bran samples of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels in four different Zn treatment groups: *Group 1*: low soil Zn & no foliar Zn; *Group 2*: high soil Zn & no foliar Zn; *Group 3*: low soil Zn & foliar Zn; *Group 4*: high soil Zn & foliar Zn. For LSD$_{0.05}$ scores, please refer to Table 4.3.
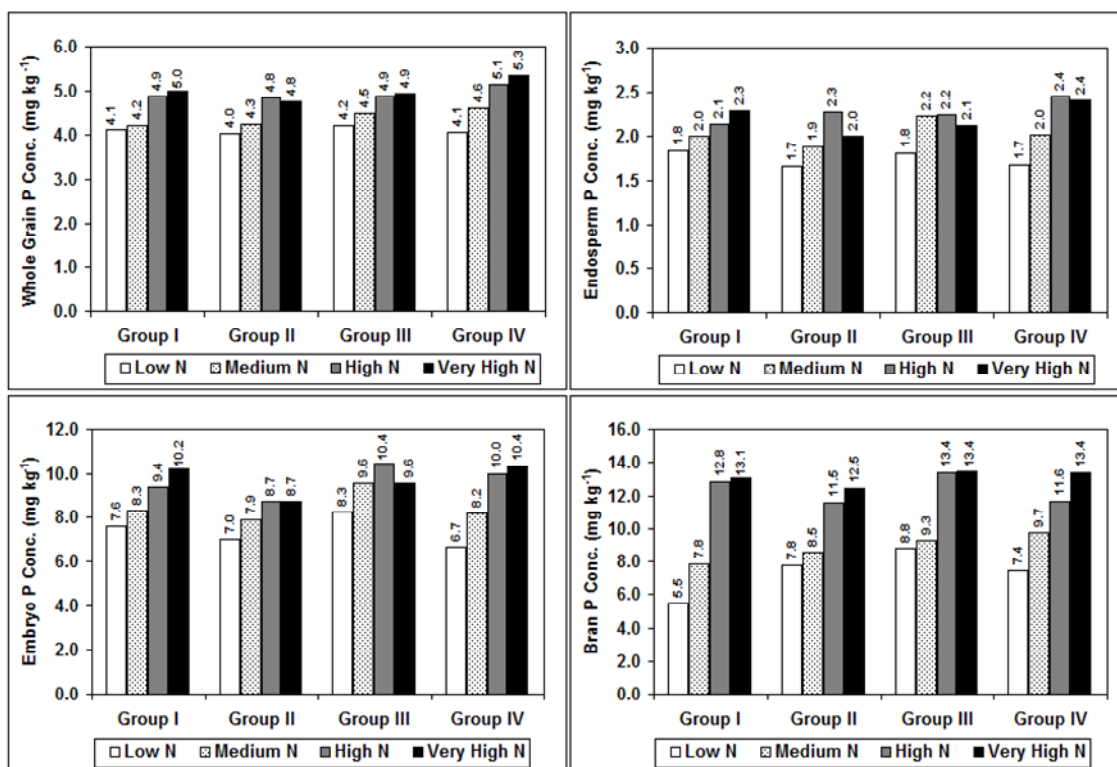
Table 4.3: Three-way analysis of variance (ANOVA) of the effects of soil N, soil Zn and foliar Zn applications as well as their interactions on the P concentration in the whole grain and grain fractions of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil under greenhouse conditions: Degrees of freedom, F value probabilities and Fisher's protected $LSD_{0.05}$ test scores

Source of Variation	DF	Whole Grain P		Endosperm P	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.12	0.027	0.33
Soil Zn (B)	1	0.372	*n.s.*	0.253	*n.s.*
Foliar Zn (C)	1	<0.001	0.08	0.931	*n.s.*
A x B	3	0.141	*n.s.*	0.388	*n.s.*
A x C	3	0.142	*n.s.*	0.481	*n.s.*
B x C	1	0.005	0.12	0.843	*n.s.*
A x B x C	3	0.024	0.24	0.962	*n.s.*
Source of Variation	DF	Embryo P		Bran P	
		F Pr.	$LSD_{0.05}$	F Pr.	$LSD_{0.05}$
Soil N (A)	3	<0.001	0.38	<0.001	0.98
Soil Zn (B)	1	<0.001	0.27	0.555	*n.s.*
Foliar Zn (C)	1	<0.001	0.27	0.010	0.69
A x B	3	0.203	*n.s.*	0.128	*n.s.*
A x C	3	0.065	*n.s.*	0.567	*n.s.*
B x C	1	0.588	*n.s.*	0.169	*n.s.*
A x B x C	3	<0.001	0.75	0.153	*n.s.*

The P concentrations in the whole grain and grain fractions were significantly increased by improved N nutritional status (Fig. 4.6; Table 4.3). Foliar Zn treatment also resulted in increased P accumulation in the whole grain, embryo and bran, whereas soil Zn treatment tended to reduce the P concentration in the embryo. The endosperm was under all conditions by far the P-poorest grain fraction. The P / Zn and P / Fe molar ratios are reported in Fig. 4.7 for the whole grain and endosperm samples. In the group I plants, the P / Zn molar ratio was very high and this high ratio was further enhanced by increasing N supply. Both soil and foliar Zn applications led to great reductions in the P / Zn ratios. Increasing N supply did not significantly affect the P / Zn ratio in the whole grains of group II, group III and group IV plants, but tended to decrease the P / Zn ratio in the endosperms of the plants sprayed with Zn. Improved N nutrition also tended to lower the P / Fe ratio in both whole grains and endosperms.
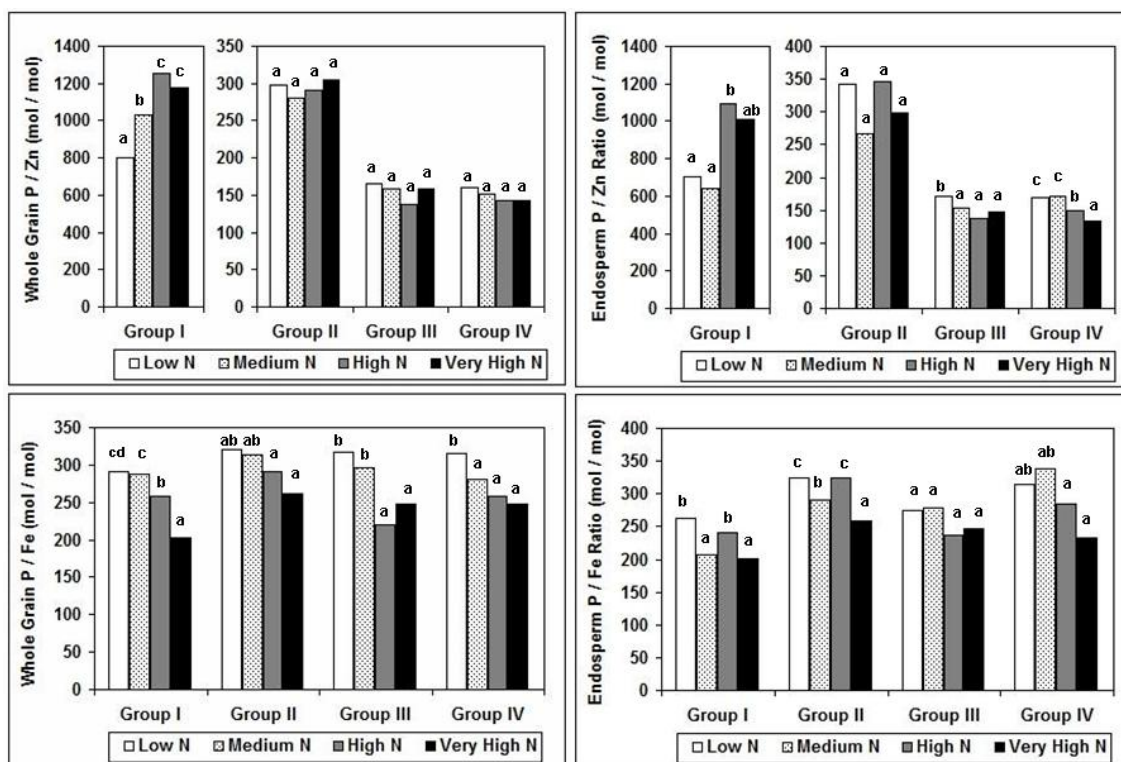
Fig. 4.7: Phosphorus / zinc and phosphors / iron molar ratios in whole grain and endosperm samples of durum wheat (*Triticum durum* cv. Balcali2000) grown on Zn-deficient soil at four different N levels in four different Zn treatment groups: *Group 1*: low soil Zn & no foliar Zn; *Group 2*: high soil Zn & no foliar Zn; *Group 3*: low soil Zn & foliar Zn; *Group 4*: high soil Zn & foliar Zn. Within each Zn treatment group, different lowercase letters indicate significant differences due to N level according to Fisher's protected LSD test (p<0.05).

4.4. Discussion

The progressive increases of both the straw dry weight and grain yield to increasing N supply in all Zn treatment groups demonstrated that the selected N doses were suitable to cause differences under the given experimental conditions (Table 4.2). Even the very high N level used in this study was not excessive. The extent of the effects of Zn deficiency at the low soil Zn level on the straw dry weight and grain yield was determined by the N level: When the soil N supply was low, Zn deficiency impaired neither the vegetative and nor the generative growth. A similar observation was also reported by Lonergan and Webb (1993). However, when the N level increased,

the positive effects of high soil Zn became more and more clear, probably because higher N stimulated straw biomass production and tillering (Salvagiotti & Miralles, 2007), resulting in greater Zn dilution. In terms of avoiding yield losses due to Zn deficiency, foliar Zn application could not substitute for soil Zn application (Table 4.2), probably because the foliar Zn spray was carried out at the reproductive stage. This observation indicates the importance of Zn nutrition at early growth stage of plants. In well agreement with this suggestion, field tests in Central Anatolia on Zn deficient soils showed that severe decreases in grain yield due to soil Zn deficiency could not be prevented by foliar Zn application (Yilmaz et al., 1997).

Soil Zn application was very effective in increasing the Zn concentration in both whole grains and grain fractions, but the effect of foliar Zn application was, in this respect, even stronger (Fig. 4.3). Based on these results, it can be suggested that combined soil and foliar Zn applications should be taken into consideration when both high grain yield and high grain Zn concentration are targeted.

Increases in concentrations of Zn in whole grain through soil and foliar applications of Zn were stimulated by increasing soil N application (Fig. 4.3), confirming our previous results (Chapters 1 & 2). The positive effect of increasing N supply on the Zn concentration of the whole grain was reflected in all grain fractions, even in greater extent. In the cases of the bran and embryo, N-dependent increases in Zn concentrations were much greater than the whole grain Zn concentration. Similarly, the endosperm Zn concentrations tended to be affected from N applications in greater extent than the whole grain. The positive effects of high N on the endosperm Zn concentration have important implications for human nutrition, because this part of grain is the most commonly eaten part in many countries. The observation that the whole grain Zn concentration was relatively less affected than all of its fractions can be explained by the size of endosperm and the grain size effect of high N supply. Higher N supply resulted in significantly bigger grains (Table 4.2) and thus in greater endosperm-to-whole grain ratios. Since the endosperm is, in absolute terms, by far the Zn-poorest part of the grain (Fig. 4.3; Ozturk et al., 2006; Persson et al., 2009), the increase of its share in the whole grain weight reduced the apparent increase in the whole grain Zn concentration. The results presented in Fig. 4.3 also suggest that whole grain analysis may underestimate the positive effects of Zn and N fertilization on concentrations of Zn in grain fractions.

Improved N nutritional status also enhanced the endosperm Fe concentration (Fig. 4.4), but in contrast to the case of Zn, not consistently more than the whole grain Fe concentration (Fig. 4.3). The bran fraction exhibited by far the most dramatic increases in Fe concentration through N fertilization. This may be related to the high phytate accumulation in the wheat bran and the very low phytate content of the wheat endosperm (Reddy et al., 1989). Recently, it has been shown that Fe is associated with phytate in protein storage vacuoles in the aleurone layer and embryo (Persson et al., 2009). The high phytate concentration in the bran may make the bran a much stronger sink for Fe than other grain fractions. Although Zn in the grain is reportedly also found in the form of phytate-rich globular crystals in protein bodies (Welch, 1986), a recently published speciation experiment showed that Zn is mainly bound to peptides in barley grains (Persson et al., 2009).

The positive whole grain Zn-N and Fe-N correlations illustrated in Fig. 4.5 are in well agreement with previously published data and with the hypothesis that protein is a sink for Zn and Fe in the grain (Chapter 1; Cakmak et al., 2010). These strong correlations also exist in the endosperm, embryo and bran fractions (Fig. 4.5). The strength of the Zn-N correlation depends on sufficiently high Zn availability to the plants. The only condition under which the positive Zn-N correlation is lost is the low soil Zn – no foliar Zn condition (data not shown). Under Zn-deficient conditions, not the sink activity but the Zn availability to the plant is the limiting factor regarding the enrichment of the grain with Zn. Because of the insufficient availability of Zn, the enhanced sink activity due to increased protein concentration cannot increase the Zn concentration in the whole grain or its fractions, and consequently, the Zn-N correlation is not observed.

The bioavailabilities of Zn and Fe in the food are as important as their concentrations (WHO, 2002; Welch & Graham, 2004). Both soil and foliar Zn applications drastically reduced P / Zn ratios in both the whole grain and endosperm, which may positively affect the bioavailability of Zn (Fig. 4.7; Simic et al., 2009). Severe Zn deficiency is known to stimulate P uptake and translocation (Cakmak & Marschner, 1986), and result in increased grain P accumulation (Erdal et al., 2002). However, under the given experimental conditions, the whole grain P concentrations were not higher under the low soil Zn than under the high soil Zn condition (Fig. 4.6), probably because the soil P supply was very high and the Zn deficiency was not very

severe. Under severely Zn-deficient field conditions, soil Zn applications are expected to reduce the P / Zn ratio not only by increasing the Zn concentration but also by decreasing the P concentration (Cakmak, 2008). Interestingly, foliar Zn treatment increased the P concentration in the whole grain as well as in some grain fractions (Fig. 4.6). A speculative explanation can be that high amounts of Zn in the plants treated with foliar Zn increased the demand for Zn-chelating compounds in the grain for the maintenance of Zn homeostasis. Because P was highly available, the plants possibly responded by increasing the phytate concentration in the bran and embryo. This may also explain why foliar Zn application had a significant positive effect on the Fe concentration in the bran and embryo (Table 4.1; Fig. 4.4): Increased phytate concentration in these fractions due to foliar Zn application might have resulted in increased sink activity for not only Zn but also Fe, because phytate has also high affinity for Fe (Reddy et al., 1989). The mechanism behind this observation needs to be investigated in future.

The improvement of the N nutritional status tended to reduce the P / Zn ratios in both the whole grain and endosperm, while it also lowered the P / Fe ratios in all treatments (Fig. 4.7). However, in many cases, the effects were not strong. The reason behind is the high correlations of phytate with P and protein in wheat grain (Raboy et al., 1991). Higher N supply resulted in higher protein concentrations (Fig. 4.2), which were accompanied not only by higher Zn and Fe concentrations (Figs. 4.3 & 4.4) but also by higher P concentrations (Fig. 4.6).

4.5. Conclusions

Nitrogen management is an effective strategy for the Zn and Fe biofortification of not only the whole grain but also all grain fractions including the embryo, bran and endosperm of wheat. The positive effects of increased N supply on the whole grain and endosperm Fe concentrations are comparable in extent, whereas the relative enhancement of the endosperm Zn by improved N nutrition is even more pronounced than that of the whole grain Zn under Zn-sufficient conditions. Optimized nitrogen application, particularly in conjunction with foliar Zn treatment, appears to be a very powerful tool for tackling micronutrient deficiency in societies which obtain most of the daily calories from white wheat flour products. In biofortification efforts targeting such societies, endosperm analysis is highly recommended in addition to whole grain analysis.

(C) GENERAL DISCUSSION AND CONCLUSIONS

Deficiencies of Zn and Fe are global nutritional problems affecting the health of at least two billion people, most of which are living in developing countries (Welch & Graham, 2004; Cakmak et al., 2010). Agronomic biofortification, i.e. the cultivation of staple crops with high concentrations of nutrients in their edible parts by the adoption of agronomic strategies, appears to be the most practical and rapid solution to this problem (Cakmak, 2008). In this approach, N management is a very powerful tool for the agronomic biofortification of wheat. Improved N nutritional status is associated with siginificantly enhanced grain Zn and Fe accumulation in wheat (Chapters 1, 2 & 4). The Zn and Fe concentrations are increased by N fertilization not only in the whole grain, but also in the endosperm, which is the most widely consumed part of the wheat grain (Chapter 4). This N effect is pronounced when Zn supply is suffienctly high either in the growth medium or within the plant tissue (Chapters 1, 2 & 4).

Wheat, as a strategy II plant with respect to Fe acquisition, releases MA family phytosiderophores to mobilize and take up Fe^{3+} from the rhizosphere (Marschner & Romheld, 1994). Phytosiderophores also contribute to mobilization and root uptake of Zn^{2+} (Marschner & Romheld, 1994). They are synthesized from the non-proteinogenic amino acid, NA, for which the proteinogenic amino acid methionine is the precursor (Mori & Nishizawa, 1987). The complexes they form with micronutrient cations are taken up into the root epidermal cells by transporters in the YSL family (Palmer & Guerinot, 2009). Alternatively, ZIP family transporters can take up Zn and Fe into the root cells as free divalent cations (Palmer & Guerinot, 2009). The latter mechanism probably contributes to Zn uptake significantly; however, the identities of the transporter proteins primarily involved in Zn uptake are still not unknown.

The next step after the root uptake is the translocation (long-distance transport) of Zn and Fe from the root to the shoot. The main pathway for root-to-shoot translocation is the xylem. In order to reach the xylem, the micronutrient cations must first travel

from the epidermal cells toward the stele of the root and then loaded from the xylem parenchyma cells into the xylem, which is part of the apoplast (Palmgren et al., 2008). Because xylem parenchyma cells have an inside-negative membrane potential, divalent cations must overcome a strong barrier for export. Therefore, xylem loading is an active transport phenomenon, which requires transporter proteins. In dicots, HMA family transporters are involved in the xylem loading of Zn (Hussain et al. 2004). Related proteins are also found in cereals; however, if they are involved in the xylem loading of Zn remains to be elucidated (Palmgren et al., 2008). YSL transporters are probably involved in Fe and Zn loading into xylem parenchyma cells (Curie et al., 2009).

Once inside the xylem vessels, free cations of Zn and Fe can be immobilized by the negatively charged carboxyl groups existing in the cell walls. Anions or organic ligands can facilitate their mobility within the xylem by neutralizing their positive charges (Kochian, 1991). At the slightly acidic xylem pH, NA is unlikely to serve as a ligand for Zn and Fe but can form stable complexes with Cu (Curie et al. 2009). Iron in the xylem is mainly found as a Fe^{3+}-citrate complex (Lopez-Millan et al., 2000), and citrate transporters like FRD3 are required for the efficient translocation of Fe in the xylem (Durrett et al., 2007). Another important form of Fe in the xylem may be Fe^{3+}-deoxymugineic acid (DMA) complex (Mori et al., 1991). Citrate and malate are also discussed as potential chelators of Zn in the xylem (White et al., 1981a, b), although evidence is still lacking. The unloading of Zn and Fe from the xylem into the mesophyll cells in the shoot is probably facilitated by transporters in the YSL and ZIP families (Curie et al., 2009; Palmer & Guerinot, 2009).

Although the main path of root-to-shoot translocation is the xylem, direct translocation of Fe from roots to young leaves via phloem was recently demonstrated in barley by Tsukamoto et al. (2009). Accordingly, the ions to be translocated are first loaded into the xylem and then transferred to the phloem either in the roots or the discrimination center. Suzuki et al. (2008) showed that DMA enhances the shoot translocation of Zn in Zn-deficient plants, but whether the Zn^{2+}-DMA complex reaches the shoot via the xylem or the phloem remains to be investigated. The xylem-to-phloem transfer may also take place in various tissues including the leaf, stem, peduncle and rachis (Herren & Feller, 1994; Pearson et al., 1995; Grusak et al., 1999) and facilitate direct translocation of Zn to developing grains. Due to the xylem discontinuity in the grain stalk (O'Brien et al., 1985), Zn ions must at some stage pass through the phloem

in order to reach developing grains. Possibly, YSL proteins are implicated in the xylem-to-phloem transfer of Zn and Fe (Curie et al., 2009; Palmer & Guerinot, 2009).

The phloem pathway is critical for the retranslocation of Zn and Fe from source tissues to developing grains. The pH value of the phloem sap is alkaline (ca. 7.8) (Marschner, 1995). Around this pH, Fe and Zn cannot exist as free soluble ions and thus must be chelated to be transported in the phloem. Evidence indicates that NA or MA-family compounds are required for the phloem loading, transport and unloading of Fe and Zn (Waters et al., 2006; Borg et al., 2009; Curie et al., 2009). Moreover, NA is also a critical chelating agent facilitating the intra- and intercellular mobility of metals (von Wirén et al., 1999; Takahashi et al., 2003; Haydon & Cobbett, 2007). Members of the YSL family transporters are most probably responsible for the phloem loading and unloading of Zn as a NA-chelate (Curie et al., 2009; Trampczynska et al., 2010).

Grain deposition is the final level in the journey of Zn and Fe. The metals unloaded from the phloem in chelated form travel from cell to cell and reach the testa and transfer cells, where they are pumped out of the maternal tissue into the endosperm cavity by unidentified proteins (Palmgren et al., 2008; Borg et al., 2009). From the endosperm cavity, they are taken up with the help of YSL and ZIP transporters into the cells of the aleurone layer, which is part of the filial tissue (Palmgren et al., 2008; Borg et al. 2009). From the aleurone cells, Zn and Fe can move via the symplast into the embryo and endosperm cells. The aleurone and embryo cells, most probably, store Zn and Fe in protein storage vacuoles, where they are found in the form of protein-metal-phytate complexes (Palmgren et al., 2008; Borg et al. 2009). In the endosperm, however, Fe may be associated with the Fe homeostasis protein ferritin within amyloplasts (Borg et al., 2009), while Zn is again destined to protein storage vacuoles, which are here largely devoid of phytate (Palmgren et al., 2008).

Apprarently, all the major steps on the route of Zn and Fe from the rhizosphere into the developing grain, including mobilization in the rhizosphere, root uptake, root-to-shoot translocation, remobilization and grain deposition, are positively affected by high N availability (Fig. C.1). Improved N nutritional status can bring about these positive effects by increasing the abundance and/or activities of at least some of the proteins and nitrogenous compounds mentioned above (Fig. C.1.). Further studies are needed to determine which of these critical components are responsive and most susceptible to N fertilization. The enhancement of phytosiderophore production and

release by higher N supply has already been demonstrated in Fe-deficient wheat (S. Bahar Aciksoz et al., unpublished results).
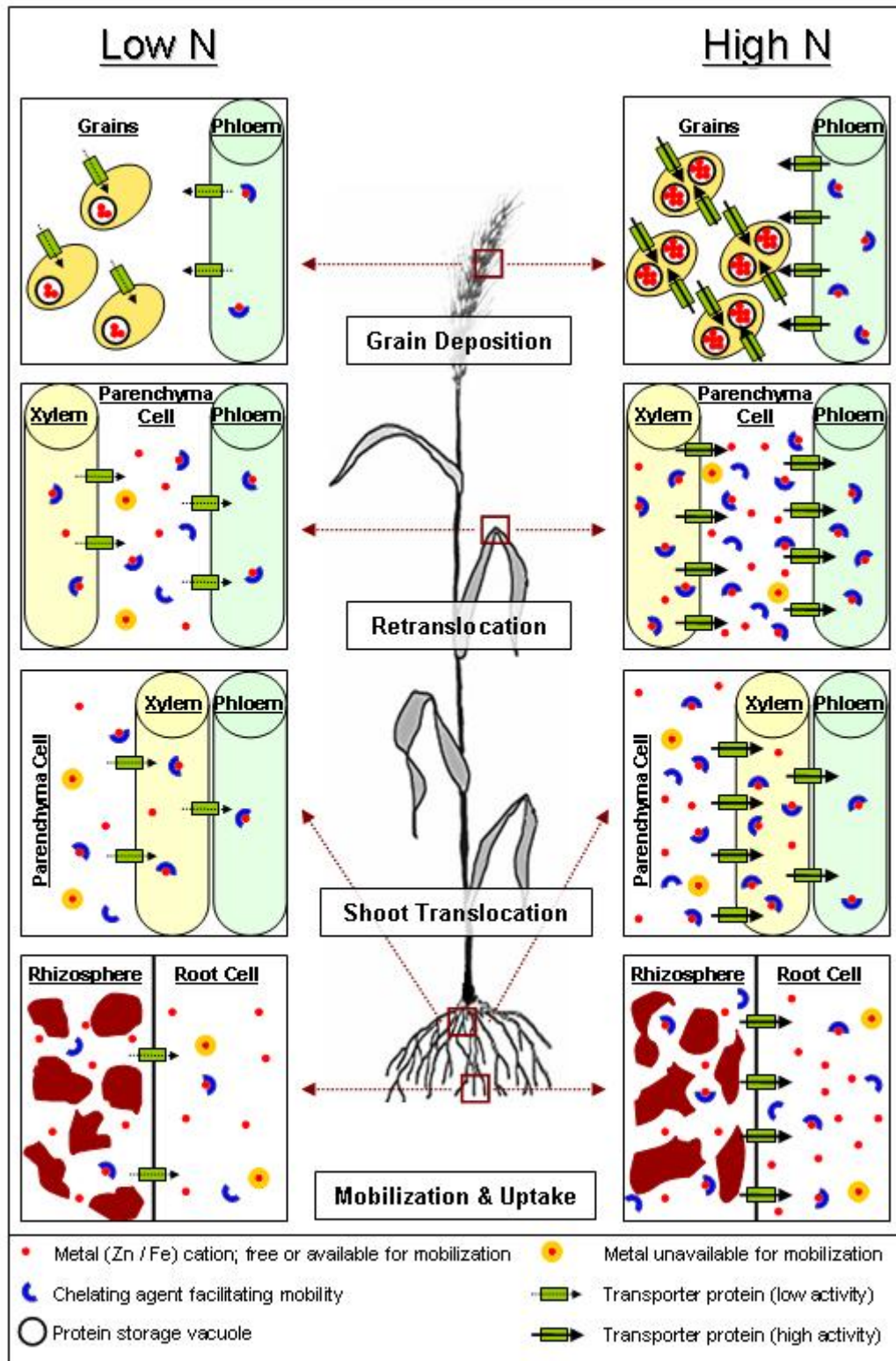
Fig. C.1: The possible effects of N nutritional status of wheat on the major steps on the route of Zn and Fe from the rhizosphere into the grain

The utilization of N fertilization for the biofortification of wheat grain with Zn requires a sufficiently high Zn availability (Chapters 1, 2, 3 & 4). Under low Zn availability, the dilution of Zn caused by the enhancement of vegetative growth and/or grain yield by high N treatment may overshadow the positive effects of N (Chapter 3). The higher the availability of Zn in the growth medium (soil) and source tissues, the stronger the impact of high N supply on the grain Zn accumulation. There is a synergistic interaction between Zn and N fertilization (Chapter 1).

Nitrogen fertilization is also very effective in the biofortification of wheat grain with Fe (Chapters 1, 2 & 4), whereas Fe fertilization may not contribute significantly to grain Fe accumulation. Soil applications of Fe in any form might be useless (S. Bahar Aciksoz et al., unpublished results), because wheat can efficiently mobilize the Fe^{3+} in the rhizosphere by releasing phytosiderophores, and the Fe uptake is a function of the efficiency of the phytosiderophore-based uptake system rather than a function of the very large Fe pool in the soil. Foliar Fe fertilization may also not be effective in increasing the grain Fe concentration, as shown by Gupta (1991) for oat and barley, possibly because the absorption of foliar Fe fertilizers is poor and/or Fe has relatively low phloem mobility.

While Fe is predominantly stored in leaves during the vegetative development, Zn is allocated to both leaves and stems (Chapter 2). Stems contribute significantly to Zn remobilization to developing grains, as suggested by Garnett and Graham (2005). Although substantial Fe remobilization is possible, the contribution of remobilization to grain mineral accretion is higher in the case of Zn than in the case of Fe (Chapter 2). If the mineral availability is sufficiently high, high N supply improves the efficiency of retranslocation; but by enhancing the uptake even more strongly, it may increase the share of concurrent uptake during grain filling in grain mineral accumulation (Chapter 2). In Chapter 2, it was reported that at low N, 22% of the grain Zn was provided by concurrent uptake during grain filling, while at high N, this ratio increased to %54. Whether remobilization or concurrent uptake during grain filling contributes more to grain mineral accumulation depends on the N supply, besides the Zn supply, and probably also other factors like water availability (Chapters 2 & 3). Generalizing statements should be avoided.

Senescence is a critical factor affecting the grain mineral accumulation. Both early and delayed senescence may have positive effects, depending on the conditions.

The *Gpc-B1* locus, which is associated with accelerated senescence, is responsible the for the remobilization of Zn, Fe and N (amino acids) from senescing leaf tissues into grain through the action of *NAM-B1* gene (Uauy et al., 2006a, b; Distelfeld et al., 2007; Waters et al., 2009). Under dry field conditions, where wheat is subject to terminal drought, accelerated senescence can result in increased grain protein, Zn and Fe content without reducing the grain yield significantly by improving the remobilization from source tissues to developing grains (Distelfeld et al., 2007). However, in case wheat is not subject to terminal drought and the mineral (eg. Zn) availability in the soil is sufficiently high, delayed senescence can be desirable, because it extends the grain filling period, where mineral uptake and translocation to grains can continue. Delayed senescence caused by high N supply (Chapters 1, 2, 3 & 4; Yang & Zhang, 2006) may contribute to the positive effects of high N on the grain Zn and Fe accumulation under appropriate conditions.

The widespread adoption of any practical method depends on its economic feasibility. Farmers cannot be expected to adopt new agronomic strategies unless these have an economic return. Nitrogen fertilization for biofortification is also from this economic perspective a very promising strategy, because a sufficiently high N fertilization is also required for maximizing the grain yield. Field experiments should be conducted in order to develop optimized N fertilization regimes, which will maximize the grain yield and the grain concentrations of Zn and Fe at the same time. Foliar Zn application, which is desirable to get the most out of the N application, may be combined with other foliar applications in order to minimize the cost of application. Whether foliar Zn fertilizers can be effectively combined with widely used insecticide or fungicide sprays should also be investigated.

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