ROLE OF NITROGEN NUTRITION IN BIOFORTIFICATION OF DURUM WHEAT WITH IRON

by

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Abstract

Iron (Fe) deficiency is a global nutritional problem in human populations and associated with inadequate dietary intake, especially in developing countries. Increasing Fe concentration of food crops by using agricultural tools represents a realistic and cost-e effective strategy to contribute to dietary intake of Fe and human health. Published data indicates that nitrogen (N) nutritional status of plants has positive impacts on shoot and grain zinc concentrations. The main goal of this PhD thesis was to study the role of N nutrition in root absorption, shoot transportation and grain accumulation of Fe in durum wheat (*Triticum durum*) plants grown under greenhouse and growth chamber conditions. Application of various soil or foliar Fe fertilizers had either a little effect or remained ineffective on shoot and grain Fe. By contrast, at a given Fe treatment, raising N supply to plants substantially enhanced shoot and grain concentrations of Fe. Inclusion of urea in foliar Fe fertilizers had also a positive impact on grain Fe. In the experiments using the radiolabelled Fe fertilizer (e.g.,^{59}FeEDTA), urea found to facilitate cuticular penetration of the foliarly-sprayed Fe and to improve its transportation into sink organs such as seeds. Root release of phytosiderophores (PS) is an important adaptive mechanism in acquisition of Fe by cereals. Improving plant N status had also a significant impact on release of PS release and root uptake and shoot translocation of PS-complexed. It is concluded that improving N nutritional status of plants represents an important agronomic practice for increasing grain Fe and improving human health.

ÖZET

Demir (Fe) noksanlığı, özellikle gelişmekte olan ülkelerde yaygın bir küresel bir beslenme problemi olup, ana nedeni düşük Fe içerikli beslenmeye dayanmaktadır. Bitkisel gıda ürünlerinin Fe bakımından iyileştirilmesini hedefleyen tarımsal uygulamalar, beslenmeyle Fe alımına ve insan sağlığına katkıda bulunan gerçekçi ve ekonomik bir strateji olarak görünmektedir. Yayınlanmış bazı sonuçlar, bitkilerin azot (N) beslenme statüsünün yeşil aksam ve tane çinko miktarına pozitif bir etki yaptığını göstermektedir. Bu Doktora tez çalışmasının ana amacı, sera ve yetiştirme odalarında yetiştirilen makarnalık buğdayda (*Triticum durum*) N beslenmesinin Fe'in kök alımı, yeşil akama taşınması ve tanede birikmesi üzerine etkisini araştırmaktır.Toprak veya yapraktan uygulanan Fe gübreleri, yeşil aksam ve tane Fe miktarı üzerine ya çok az etkili olmuş ya da etkisiz kalmıştır. Ancak, herhangi bir Fe gübrelemesinde artan şekilde uygulanan N yeşil aksam ve tane Fe miktarını kuvvetli biçimde arttırmıştır. Yaprak Fe uygulamasında üre kullanımı, tane Fe birikimi üzerinde pozitif bir etki göstermiştir. Radyoaktif Fe etiketli Fe'in (^{59}FeEDTA) kullanıldığı bir denemede, ürenin ^{59}Fe'in yapraktan kutiküler penetrasyonunu kolaylaştırdığı ve tane (tohum) gibi sink organlarına taşınmasını iyileştirdiği bulunmuştur. Köklerden fitosideroforların (PS) salgılanması, tahılların topraklardan Fe alımında önemli olan bir kök adaptasyon mekanizmasıdır. Bitkilerin N beslenmesinin iyileştirilmesinin, köklerin PS salgılaması üzerine de önemli bir etki göstermiştir. Fitosiderofor ile şelatlanmış Fe'in kökler tarafından alınması ve yeşil aksama taşınmasının artan N beslenmesiyle iyileştiği bulunmuştur. Elde olunan sonuçlar, gübreleme yoluyla bitkilerin N beslenme statüsünün iyileştirilmesinin, tane Fe miktarının arttırılması ve insan sağlığının iyileştirilmesinde önemli bir tarımsal uygulama olduğunu ortaya koymaktadır.

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This thesis is dedicated to those

who have something to believe in,

better yet, anything.

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A. GENERAL INTRODUCTION

A.1. Iron Deficiency Represents A Global Nutritional Problem in Human Populations

Micronutrient malnutrition is a growing health concern affects more than 2 billion people worldwide, mainly in the developing countries (Cartner et al. 2010; Bouis and Welch 2010). Among the micronutrient deficiencies, iron (Fe) deficiency is a well-documented problem and responsible for diverse of health complications. It may cause learning disabilities among children and lower worker productivity, decrease resistance to infection, increases morbidity and mortality rates, consequently causes high health care costs (Welch and Graham 2004; Beard, 2008). Inadequate Fe absorption is also responsible for anemia which weakens the body as a result of insufficient oxygen transport and reduction of red blood cells (Cartner et al. 2010; Welch and Graham 2000). Iron deficiency together with Zn deficiency is responsible for death of 500.000 children under 5-years-old annually. Micronutrient deficiencies have been ranked as the top priority global problem facing the world. This conclusion has been made in 2008 by a panel of eight economists (including five Nobel Laureates) at the Copenhagen Consensus (www. copenhagenconsensus.com).

Iron deficiency problem in children was seen not only in developing countries, but also in well-developed countries such as in United Kingdom and Switzerland (Cakmak, 2008 and Poletti et al. 2004). Micronutrient deficiencies also result in severe problems with social and economic development of countries. It is estimated that the loss in economic productivity due to micronutrient deficiencies in China is more than 3.6% of the gross national product (Ma et al. 2007).

Major reason for the widespread occurrence of Fe deficiency problem in human populations is high consumption of cereal based foods which are inherently very low in Fe concentrations. Cereal-based foods are the major source of daily calorie intake in developing worlds. In many rural areas of the developing countries, cereals contribute up to 75 % of the daily calorie intake. Cereal crops are of great importance and provide a major source of minerals and protein in developing world (Poletti et al. 2004). For instance, in most of Central and West Asian countries, wheat provides nearly 50% of the daily calorie intake on average and this amount increases up to 75 % in the rural regions (Cakmak, 2008). Besides low amounts of Fe, bioavailability of Fe is also very low in cereals due to high amounts of phytate and fibers (Gibso et al. 2010; Cakmak, 2008). The most common range of Fe concentrations found in wheat is between 25 to 35 mg kg^{-1} (Rengel et al. 1999; Cakmak et al. 2010a). These values are too low to meet daily Fe requirement of human populations. According to Graham et al (2007), in order to achieve measurable health effects, grain Fe concentrations should be over 50 mg kg^{-1}. Nearly 50 % of the cereal-grown areas globally contain low plant availability of Fe and Zn due to adverse soil chemical conditions such as high pH, low organic matter and low soil moisture (Graham and Welch 1996; Cakmak, 2002). When grown on soils with low chemically soluble Fe, grain Fe concentrations show further decline, worsening nutritional quality of cereal-based foods. Increasing concentration of Fe in food crops is, therefore, an important global agronomic target and humanitarian challenge.

The Food and Agriculture Organization and the World Health Organization (WHO) have estimated the daily requirements of the various micronutrients in the human diet. Individuals between 25 and 50 years of age require 10–15 mg Fe per day. In the case of Zn, people require between 12 and 15 mg per day (Welch and Graham 2004; Ghandilyana et al. 2006). Moreover, in the milling process the micronutrient rich parts of the grain including alleurone and scutellum layer of the embryo is removed and the rest part of grain (endosperm) containing low concentration of Fe is consumed. Consequently, heavy consumption of high proportion of milled wheat and other cereal products result in reduced intake of Fe and Zn (Borg et al. 2009 and Hao et al. 2007).

A.2. Strategies to Alleviate Iron Deficiency Problems in Human Populations

A.2.1. Supplementation and Fortification Programs

There are four most widely recognized strategies for reducing micronutrient malnutrition in human populations as following: i) supplementation with pharmaceutical preparations, ii) fortification of foods with the target micronutrients, iii) agronomic biofortification (e.g., application of fertilizers) and iv) plant breeding and genetic engineering (Welch and Graham 2004; Pfeiffer and McClafferty 2007; Cakmak et al. 2010a).

Supplementation and fortification of foods with Fe have been successfully practiced in industrialized countries (Frossard et al. 2000, Poletti et al. 2004, Welch and Graham 2004). Dietary diversity might be also a solution to minimize Fe deficiency related problems. Although supplementation and fortification approaches are highly effective interventions against Fe deficiency, but these approaches are impractical and expensive strategies to sustain in some countries where poverty is widespread. According to the calculations, a food fortification program in country with 50 million people suffering from micronutrient malnutrition especially from Zn and Fe requires US$ 25 million annually to eliminate these deficiencies (Bouis and Welch 2010). In addition, public acceptance and implementation of such approaches is a big concern, especially in the rural areas of the developing countries (Frossard et al. 2000; Bouis and Welch 2010).

A.2.2. Agricultural Approaches: Plant Breeding

Alternatively, agricultural strategies (e.g., plant breeding and fertilization) aiming at improving micronutrient concentrations of stable food crops seem to be sustainable and cost-effective approaches and easily applicable in the rural areas of the developing countries (Bouis and Welch 2010; Graham et al. 2007; Cakmak, 2008). As discussed below, there are excellent examples showing that soil and or foliar application of

micronutrient fertilizers are highly effective in increasing grain micronutrient concentrations, especially Zn. It is well-documented that plant genotypes are different in utilization of poorly-soluble sources of micronutrients in soils and translocation of micronutrients into grain (Cakmak, 2002; White and Broadley 2009). Consequently, there is a substantial genotypic variation in grain Fe and Zn (Cakmak et al. 2004; Zhao et al. 2009) which can be exploited in breeding programs to develop new plant genotypes with high Zn and Fe. Genotypic variation for grain Fe is pronounced in wild wheats. For example, wild emmer wheat (*Triticum dicoccoides*) contains high concentrations of Fe and exhibits a substantial genetic variation. In screening of a large wild emmer germplasm (*T. dicoccoides)* grain Fe concentrations ranged from 15 to 109 mg/kg (Cakmak et al. 2004). In the case of *Triticum spelta*, results revealed the existence of a wide and promising genetic diversity for grain Fe concentrations (e.g., Fe: 19 - 99 mg kg^{-1}). This variation has been found in a spelt wheat germplasm with 760 genotypes after their growth on 3 locations over 3 years. By contrast, modern cultivars are, very low in concentrations of Fe and exhibit a narrow genetic variation for (common range: 25 to 35 mg/kg) (Rengel et al. 1999).

Currently, intensive different breeding programs are on-going to improve stable food crops with high concentrations of micronutrients by using selected lines from wild wheats and spelt wheats. A major breeding program is being carried out by the HarvestPlus program (www.harvestplus.org), which is established under the Consultative Group on International Agricultural Research (Bouis and Welch 2010; Pfeiffer and McClafferty 2007). Harvest Plus program uses plant breeding tools to improve stable food crops with Zn, Fe and vitamin A and to contribute to human health globally.

In different wild emmer and spelt germplasms it has been also found that protein concentrations in grain correlate very positively with Zn and Fe concentrations. Such positive correlations between Fe and protein have been found also in many other plant species (Cakmak et al. 2010a). It seems that the physiological and molecular mechanisms affecting grain accumulation of Fe and protein are very similar and probably synergitics. Kutman et al (2010) suggested that N (protein), Fe and Zn act synergistically in improving their concentrations in grain. As discussed by Cakmak et

al. (2010b) high levels of proteins in grain might be also important for better bioavailability of Fe in diet or human body. Diets high in both protein and certain amino acids such as metionine, cysteine and histidine have been shown improve bioavailability (Lonnerdal, 2000).

Genetic engineering could be an alternative option in increasing Fe concentrations of food crops. Increasing number of evidence is available showing that expression of various targeted proteins (such as ferritin) or Fe transporter proteins are associated with high accumulation of Fe in seeds (Haydon and Cobbett 2007; Borg et al. 2009; Curie et al. 2011).

A.2.3. Agricultural Approaches: Fertilizer Strategy

Enrichment of food crops with micronutrients by using breeding tools or by applying transgenic technologies is a long-term process. It involves long-term crossing/back-crossing programs, adaptation trials and GxE tests (Cakmak, 2008). In addition, the success of a plant breeding program depends on the available pools of targeted micronutrients in soil solution. Agronomic biofortification (e.g., fertilizer strategy) is, therefore a short-term and complementary strategy to the micronutrient malnutrition problem.

As indicated above, levels of Fe in cereal grains are further aggravated by growing cereal crops on Fe- deficient soils. It is estimated that nearly 50 % of the cereal cultivated soils contain low amount of plant available Fe and Zn concentration which results in further decline in grain concentrations of micronutrients (Cakmak, 2008). It is, therefore, not surprising that the well-documented micronutrient deficiency problems in human populations occurs mainly in the regions where soils are low with plant available concentrations of micronutrients. Most of the cereal cultivated soils have diverse of adverse chemical problems which reduce both solubility and root uptake of micronutrients such as low organic matter, high $CaCO_3$, low soil moisture and high pH (Marschner and Romheld 1994; Cakmak, 2008). In soils with adverse chemical conditions and thus low amounts of plant available Fe, the genetic capacity of the newly

developed and released biofortified genotypes to accumulate Fe at levels required for better for human nutrition may not be expressed. Thus, providing readily available pools of Fe to plants through soil and or foliar applications would be an important rapid and complementary solution.

In case of Zn, there are well-documented examples indicating significant impact of Zn fertilization on grain Zn, especially with foliar application of Zn fertilizers. Field experiments conducted in Turkey and China showed that application of soluble Zn fertilizers to foliar increases grain Zn concentrations up to 3-folds (Cakmak, 2008; Zhang et al. 2010) while soil applications remain less effective (Cakmak et al. 2010b). Foliar Zn application is more effective when sprayed late in the growing season. In the field trials conducted in Central Anatolia it has been shown that late-season foliar spray of Zn (e.g., at heading and early milk stage) caused much greater increases in grain Zn concentration when compared to the applications realized before the flowering stage (Cakmak et al. 2010b).

Published data indicates that in contrast to Zn, Fe seems to be difficult to biofortify food crops by using fertilizer stragey (Rengel et al. 1999). Inorganic Fe fertilisers applied to soil are rapidly converted into poorly soluble Fe (III) forms or precipitated (Rengel et al. 1999; Frossard et al. 2000). In order to achieve an important impact on grain Fe accumulation, Fe should be applied in chelated forms, but chelated-Fe sources are usually very expensive. Foliar application of $FeSO_4$ has been found to result in some positive effects on grain Fe, but the impact is not sufficiently high when compared to the effects achieved by application of foliar Zn fertilizer (Rengel et al. 1999). In China, field tests showed that applying inorganic or chelated forms of foliar Fe fertilizers to wheat can increase grain Fe concentrations only up to 36% (Zhang et al. 2010). For Fe, new application approaches or forms are needed to achieve better impact with Fe fertilization strategy on grain Fe accumulation.

Urea is known to be a facilitator and penetration enhancer of several nutrients into leaf cells through the leaf cuticula (Swietlik and Faust 1984; Weinbaum, 1988; Bowman and Paul 1992). There are published reports showing that urea also stimulates cuticular penetration Fe in different plants (Kannan and Wittwer 1965; Wittwer et al. 1967).

Spraying foliar Fe fertilizers together with urea results in quick regreening of chlorotic leaves. It seems that urea has a positive impact on leaf absorption from the foliarly sprayed Fe fertilizers. There is however, no published data about the impact of leaf applied urea on translocation (partitioning) of the leaf- absorbed (penetrated) Fe in the whole plant.

A.2.4. Impact of Nitrogen Nutrition on Grain Fe Accumulation

Recently published data shows that N nutritional status of plants may influence Fe acquisition by roots and transport within the plant. There are several steps or check-points in the plants which contribute to Fe accumulation in shoot and grains such as i) solubilization and mobilization of Fe in soils, ii) absorption by roots, iii) chelation and transportation through xylem, iv) re-translocation via phloem and v) seed deposition of Fe (Cakmak et al. 2010a). According to Grusak et al. (1999) plants have developed a number of transport mechanisms to control the acquisition, partitioning and deposition of Fe in tissues in order to obtain adequate levels of this essential nutrient for both vegetative and reproductive tissues. It seems all these steps are under direct influence of N through several transporter proteins and nitrogenous compounds (such as nicotianamine and amino acids) (Haydon and Cobbett 2007).

As discussed in more detail below, root release of phytosiderophores (PS) is an important adaptive response of cereals to low Fe soils (Takagi et al. 1988; Marschhner and Romheld 2004). Phytosiderophores are excellent Fe-mobilizing compounds in soils and contribute greatly to solubilisation and root transport of Fe in soils (Treeby et al. 1989; Romheld and Marschner 1986). In the literature several transporter proteins were identified which regulate root uptake, xylem loading and transport and remobilization within vegetative tissue of Fe (Borg et al. 2009; Curie et al. 2009). For example, YSL proteins contribute greatly to uptake of metals that are complexed with plant-derived phytosiderophores (PS) or nicotianamine (NA) (Conte and Walker 2011; Curie et al. 2009). ZIP and IRT1 proteins also play critical role in root absorption and transfer within the roots to the xylem pathway (Bauer and Bereczky 2003; Conte and Walker, 2011; Curie et al. 2009). In addition, nicotianamine functions as a precursor for

biosynthesis of phytosiderophores and is thought to play a primary role in long distance transport of Fe (Mori and Nishizawa 1987; Haydon and Cobbett 2007). It is very obvious that the pools and activity of those transporter proteins and nitrogenous compounds chelating Fe in plants are affected from the N nutritional status of plants. To our knowledge, in literature there is no published data about how N nutritional status of plants influences the activity/expression of transporter proteins affecting uptake and transport of Fe.

Probably, increasing grain N concentration may also affect Fe accumulation by creating a binding/storage capacity for Fe. Staining and localization studies on seeds showed that Fe is predominantly concentrated and localized in seed parts which are rich in proteins, indicating that seed proteins represents an important sink for Fe (Cakmak et al. 2010a). Existence of a close positive correlation between seed protein and Fe concentrations in diverse of plant species (Peterson et al. 1986; Zhao et al. 2009; Cakmak et al. 2010a) support the idea that seed proteins play an important role in Fe accumulation. A special attention should be paid, therefore, to N nutritional status of plants in Fe biofortification studies.

A.3. Root Mechanisms Contributing to Iron Acquisition in Cereals

Although Fe is present in very high amounts in cultivated soils, plant iron acquisition is often impaired due to several soil chemical and physical factors (Marschner and Romheld 1994). Iron is the fourth most abundant element in the Earth's crust; but it is extremely insoluble, not readily available for plants, and mainly present as oxihydrates with low availability in oxic environments (Kim and Guerinot 2007 and Schmidt, 2003). About 30% of the arable land worldwide consists of calcareous and alkaline soils in which chemical solubility of Fe is too low (Hell and Stephan 2003).

Cereal crops develop highly effective adaptation mechanisms when grown on calcareous soils. Root release of Fe-mobilizing phytosiderophores (PS) is a well-documented root response of cereals to Fe deficiency in calcareous soils. Insoluble Fe

sources are easily solubilized and mobilized by the secretion of PS. It is believed that differences between plant species in tolerance to Fe deficiency correlate well with the amount of PS release from roots (Marschner et al. 1986). Because of high Fe-chelating capacity of PSs and high stability of Fe(III) complexed-phytosiderophores in soils with high pH (Mori, 1994), genotypes releasing effectively PSs have high advantage to grow better in calcareous soils.

Phytosiderohores are synthesized from L-methionine that is used for the biosynthesis of via nicotianamine (Mori et al. 1987; Ma et al. 1995). Nicotianamine has dual role in Fe nutrition of plants. It affects the biosynthesis of PSs and also regulated Fe transport/delivery within plants by chelating Fe (Takahashi et al. 2003; Haydon and Cobbett 2007).

The phytosiderophores released from roots are able to form soluble Fe(III)–PS complexes which are then absorbed by roots through an effective Fe(III)–PS uptake system localized on plasma membranes of root cells (Romheld and Marschner 1986, von Wiren et al. 1996). Later, it has been shown that the root uptake of Fe(III)–PS is maintained by a highly inducible specific transporter protein, which called yellow stripe 1 (YS1) (Curie et al. 2001, Murata et al. 2008).

A.4. Objectives

The main goal of this PhD thesis was to study the role of N nutrition of durum wheat plants in root absorption, shoot transportation and grain accumulation of Fe. Based on the literature review above, it is seems very likely that N nutritional status of plants should have a positive impact on root absorption and shoot accumulation of Fe through affecting root release of Fe-mobilizing phytosiderophores, amounts of Fe-chelating nitrogenous substrates (e.g., amino acids) and the activity of transporter proteins which contributes to root uptake and transportation of Fe, and finally by increasing density of Fe-binding/storing proteins in seeds. To our knowledge, there is

no or very limited data about these topics in literature. This thesis consists of three chapters focusing on the following topics:

i) CHAPTER I: Effect of nitrogen on root release of phytosiderophores and root uptake of Fe(III) phytosiderophore in Fe-deficient wheat plants

ii) CHAPTER II: Biofortification of wheat with iron through soil and foliar application of nitrogen and iron fertilizers

iii) CHAPTER III: Inclusion of urea in the foliar ^{59}FeEDTA treatment solution stimulated leaf penetration and translocation of ^{59}Fe within wheat plants

Main aim of the **Chapter I** is to study the role of the N nutritional status of wheat plant on i) the root release of PS and ii) mobilization and root uptake and translocation of Fe from ^{59}Fe labeled Fe-hydroxide. Additionally, the amount of methionine (a precursor of PS synthesis) was also studied in leaves and roots of the plants with different N nutritional status. This chapter provides first scientific evidence about the positive impact of N nutrition of root release of PS and root uptake of Fe-complexed PS.

There is very limited data on the role of soil and foliarly applied Fe fertilizers on grain Fe concentrations in literature. Most of the Fe fertilizer studies conducted in the past focused on correction of Fe deficiency problem; but not investigated grain concentrations of Fe. **Chapter II** is dealing with role of various Fe fertilizers on shoot and grain accumulation of Fe under different nitrogen applications and provides highly valuable knowledge requited in bioforification of cereals with Fe.

Chapter III investigated role of urea inclusion in the foliar Fe fertilizers on translocation (partitioning) of the Fe in the whole plant. Role of urea in leaf penetration of Fe is a well-known issue. But it is not known how translocation (partitioning) of the leaf- absorbed (penetrated) Fe is affected within plants when urea is added in the Fe fertilizers. Results obtained under this chapter indicated that urea inclusion into foliar Fe treatment solutions represents a useful agronomic practice for an effective biofortification of cereal grains

B. GENERAL MATERIALS AND METHODS

B.1. Plant Material

All experiments have been conducted under either growth chamber (solution culture experiments) or greenhouse (soil culture experiments) conditions by using a Turkish durum wheat cultivar (*Triticum durum* cv. Balcali 2000) as described below.

B.2. Plant Growth Conditions

B.2.1. Soil Culture Experiments in Greenhouse

The soil culture experiments were realized in a greenhouse at the Sabancı University campus (40° 53' 24.5" N and 029° 22' 46.7" E) under natural daylight with an evaporate cooling system. The greenhouse is equipped with a heating system that keeps the temperature between 15-25°C depending on the season and weather conditions.

Durum wheat seeds (*Triticum durum* cv. Balcali 2000) were sown in plastic pots containing 3 kg soil from a Zn-deficient region in Central Anatolia (Cakmak et al. 1996). The soil used in the experiments had a clay-loam texture and low organic matter (15 g/kg), high $CaCO_3$ (180 g kg^{-1}) and high pH (8 in dH_2O). The diethylenetriamine pentaacetic acid (DTPA)-extractable Zn and Fe concentrations were 0.1 and 2.1 mg kg^{-1} soil, respectively, measured by using the method described by Lindsay and Norvell (1978). Before potting, experimental soil was supplied with the following nutrients (in

mg kg^{-1} soil): 100 mg phosphorus (P) as KH_2PO_4, 25 mg sulfur (S) as K_2SO_4 and 2 mg Zn as $ZnSO_4 .7H_2O$, and different rates N in the form of $Ca(NO_3)_2.4H_2O$ as mentioned in the related experiments. Depending on the experiments, Fe has been applied to soil and foliar in the forms of FeEDTA and Fe sulfate (soil experiments) and Fe-EDTA, FeEDDTA and Fe citrate (foliar spray experiments).

Twelve seeds were sown in each pot. The seedlings were thinned to 4, 5, or 6 per pot, depending on the experiment, shortly after emergence. The pots were watered daily with deionized water and randomized every 4 or 5 days interval.

B.2.2. Solution Culture Experiments in Growth Chamber

Solution culture experiments were conducted in a growth chamber under controlled climatic conditions (e.g. light/dark regimes of 16/8 h at 22/18°C, 60/70% relative humidity and a photosynthetic photon flux of 400 µmol m^{-2} $s^{-1)}$.

Seeds of durum wheat (*Triticum durum* cv. Balcali 2000) were germinated in perlite moistened with saturated $CaSO_4$ solution at room temperature. After 5-6 days, the seedlings were transferred to 3 L black plastic containing the following continuously aerated nutrient solution: 0.9 mM K_2SO_4, 0.2mM KH_2PO_4, 1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM KCl, 1 µM $ZnSO_4$, 1 µM H_3BO_3, 0.5 µM $MnSO_4 \cdot H_2O$, 0.2 µM $CuSO_4 \cdot 5H_2O$and 0.14 µM $(NH_4)6Mo7O_{24} \cdot 4H_2O$.

Iron was supplied in the form of FeEDTA at concentrations of 2 µM for the Fe deficient plants and 100 µM for the Fe-adequate plants. Depending on the experimental design, different concentrations of N were used in the nutrient solution in the form of $Ca(NO_3)_2 \cdot 4H_2O$. The nutrient solutions of the very low, low and medium N plants were supplied with additional Ca in form of $CaCl_2 \cdot 2H_2O$ to complement missing Ca. Nutrient solutions were changed every 3 or 4 days.

B.3. Harvest

In greenhouse experiments, shoot and grains are harvested separately. Shoot parts were washed with deionized water and dried at 60°C for determination of shoot dry weight. Grains were manually separated from husk and weighed to determine grain yield. In the case of the solution culture experiments the root and shoot parts were separately harvested for the determination of root and shoot dry weight and the concentrations of mineral elements. The roots were washed twice in deionized water and then in 0.5 mM $CaSO_4$ solution.

B.4. Element Analysis

Dried and ground plant samples (shoots, roots and grains) were subjected to acid-digestion [ca. 0.2 g sample in a mixture containing 2 mL of 30% (v/v) H_2O_2 and 5 mL of 65% (v/v) HNO_3] in a closed-vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). Determination of mineral nutrients other than N was done by using inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia). Nitrogen concentration in the samples was determined after dry combustion (950°C) using a LECO Tru-Spec C/N Analyzer (Leco Corp., St Joseph, MI, USA). Measurement of mineral nutrients was checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). To check for Fe contamination, aluminum (Al) concentration in the grain samples was measured and found to be less than 2 mg kg^{-1}, suggesting an absence of Fe contamination via soil dust (Pfeiffer and McClafferty 2007).

B.5. Measurement of ^{59}Fe Activity

In part of the experiments under greenhouse and growth chamber conditions, radiolabelled Fe (^{59}FeEDTA) has been used to study i) the role of root release of phytosiderophores (PS) in root uptake and transport of ^{59}Fe-complexed PS and ii) the impact of urea in foliar absorption and translocation of the foliar-treated ^{59}FeEDTA. The radioactivity of ^{59}Fe has been determined in roots, shoots and seeds by using a Perkin Elmer 2480 WIZARD2 Automatic Gamma Counter (PerkinElmer, Waltham, MA).

B.6. Statistical Analysis

All experiments were set up in a randomized complete block design with different number of replications according to the experimental design. Data analysis was conducted by JPM software (JMP, SAS Institute, Cary, North Carolina, USA), and comparison of means was performed by using the Student's test, whenever ANOVA (using general linear model) indicated significant effect of treatments.

CHAPTER 1

EFFECT OF NITROGEN ON ROOT RELEASE OF PHYTOSIDEROPHORES AND ROOT UPTAKE OF Fe (III)-PHYTOSIDEROPHORE IN Fe-DEFICIENT WHEAT PLANTS

1.1. Abstract

Root release of phytosiderophores (PSs) is an important step in iron (Fe) acquisition of grasses, and this adaptive reaction of plants is affected by various plant and environmental factors. The objectives of this study were to study the effects of varied nitrogen (N) supply on (1) root and leaf concentrations of methionine, a precursor in the PS biosynthesis, (2) PS release from roots, (3) mobilization and uptake of Fe from ^{59}Fe-labeled Fe(III)-hydroxide [$^{59}Fe(OH)_3$] and (4) root uptake of 59Fe-labeled Fe(III)–deoxymugineic acid (DMA) by durum wheat (*Triticum durum* cv. Balcali 2000) plants grown in a nutrient solution. Enhanced N supply from 0.5 to 6 mM in a nutrient solution significantly increased the root release of PS under Fe deficiency. High N supply was also highly effective in increasing mobilization and root uptake of Fe from ^{59}Fe-hydroxide under low Fe supply. With adequate Fe, N nutrition did not affect mobilization and uptake of Fe from $^{59}Fe(OH)_3$. Root uptake and shoot translocation of Fe supplied as ^{59}Fe(III)–DMA were also stimulated- by increasing N supply. Leaf concentration of methionine was reduced by low Fe supply, and this decline was pronounced in high N plants. The results show that the root release of PS, mobilization of Fe from $^{59}Fe(OH)_3$and root uptake and shoot translocation of Fe(III)–PS by durum wheat are markedly affected by N nutritional status of plants. These positive

N effects may have important implications for Fe nutrition of human populations and should be considered in biofortification of food crops with Fe.

1.2. Introduction

Iron (Fe) deficiency is a common micronutrient deficiency problem in crop plants grown on calcareous soils which have very low chemical solubility of Fe. Most of the grasses are well adapted to calcareous soils by releasing Fe-mobilizing compounds [so-called mugineicacid family phytosiderophores (PSs)] from their roots (Marschner et al. 1986, Takagi et al. 1984). PSs are highly effective in chelation and mobilization of Fe from sparingly soluble Fe compounds, such as Fe(III)-hydroxide (Treeby et al. 1989). The Fe(III)–PS complex formed is then taken up by an effective Fe(III)–PS uptake system localized on the root plasma membranes of the grasses (Romheld and Marschner 1986; von Wiren et al. 1996). Later, it has been shown that the root uptake of Fe(III)–PS is maintained by a highly inducible specific transporter protein, which was identified in maize and barley roots and called yellow stripe 1 (YS1) PS dependent transporter proteins (Curie et al. 2001; Murata et al. 2006). The genes encoding the YS1 transporter for Fe(III)–PS were shown to be specifically expressed in the epidermal cells of roots, and the expression was strongly enhanced in response to Fe deficiency in barley (Murata et al. 2006).

The root exudation of PS is influenced by various plant and environmental factors. There is a large genetic variation in the release of PS among the graminaceous species and also among the genotypes of a given species (Kawai et al. 1988; Ma et al. 2003, Marschner et al. 1986). For example, Fe deficiency-resistant species like barley, wheat and rye release very high amounts of PS, whereas in sensitive species such as rice and sorghum, PS release is very low (Romheld, 1991). Among the environmental factors affecting PS secretion from roots, the time of day and the level of light intensity play an important role. Generally, the PS release shows a peak during morning hours, nearly 2 h after sunrise, and then declines rapidly and remains at very low or at not-measurable levels in the afternoon and night periods (Cakmak et al. 1994; Nagasaka et

al. 2009; Takagi et al. 1984; Zhang et al. 1991). Increasing light intensity also promotes PS release from roots as shown in wheat and barley (Cakmak et al. 1998). According to Ueno and Ma (2009), root zone temperature rather than light intensity under growth conditions has greater impact on PS release from roots.

Release of PS from roots is not only affected by the Fe nutritional status of plants but also by the Zn nutritional status of plants. Different cereal species such as wheat, barley and wild wheats responded to Zn deficiency by inducing release of PS from roots (Cakmak et al. 1994; Suzuki et al. 2006; Tolay et al. 2001; Zhang et al. 1989).In contrast to Fe and Zn deficiencies, sulfur (S) deficiency reduced PS release from barley roots (Astolfi et al. 2006), most probably by causing impairments in PS biosynthesis pathway (Astolfi et al. 2010). Among the substrates contributing primarily to PS biosynthesis,the S-containing amino acid methionine and also the S-adenosyl methionine (SAM) play a key role, and probably their level is adversely affected by S deficiency that might be one major cause for the reduced biosynthesis of PS in S-deficient plants (Astolfi et al. 2010).

It is likely that N nutrition may also affect PS release from roots by reducing the amount of various nitrogenous substrates and the activity of enzymes contributing to PS biosynthesis such as the substrates nicotianamine (NA) and methionine, and the enzymes NA-synthase and NA aminotransferase (NAAT) (Haydon and Cobbett 2007; Mori and Nishizawa 1987; Shojima et al. 1990).The level of N nutrition may also affect the pool and activity of the transporter proteins mediating root uptake of Fe(III)–PS across the plasma membranes. To our knowledge, there are no publications on the effect of varied N nutrition on the PS release and root uptake and translocation of Fe supplied as Fe(III)–PS. The objective of this study was, therefore, to examine the role of the Nnutritional status on the root release of PS, mobilization and root uptake and translocation of Fe from Fe labeled Fe-hydroxide in wheat. Additionally, the amount of methionine was also studied in leaves and roots of the plants with different N nutritional status.

1.3. Materials and Methods

1.3.1. Plant Growth

Seeds of durum wheat (*Triticum durum cv.* Balcali 2000) were germinated in perlite moistened with saturated $CaSO_4$ solution at room temperature. After 5 days, theseedlings were transferred to 3 l plastic pots (25 seedlings per pot) containing the following continuously aerated nutrient solution: 0.9 mM K_2SO_4, 0.2mM KH_2PO_4,1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM KCl, 1 µM $ZnSO_4$, 1 µMH_3BO_3, 0.5 µM MnSO4·H_2O, 0.2 µM $CuSO_4 \cdot 5H_2O$ and 0.14 µM $(NH_4)6Mo_7O_{24} \cdot 4H_2O$. Iron was supplied in the form of FeEDTA at concentrations of 2 µM for the Fe deficient plants and 100 µM for the Fe-adequate plants. Depending on the experiment, different concentrations of N were used in the nutrient solution in the form of $Ca(NO_3)_2 \cdot 4H_2O$ including 0.5 mM (very low), 1 mM (low) 3 mM (medium) and 6 mM (high) N supplies. The nutrient solutions of the very low, low and medium N plants were supplied with additional Ca in form of $CaCl_2 \cdot 2H_2O$ to complement missing Ca. Nutrient solutions were changed every 3 or 4 days; before refreshing of the nutrient solutions, pH values ranged between 7.2 (for very low and low N plants) and 7.6 (for highN plants).

Plants were grown 14 days in a growth chamber under controlled climatic conditions (e.g. light/dark regimesof 16/8 h at 22/18 °C, 60/70% relative humidity and aphotosynthetic photon flux of 400 µmol m^{-2} s^{-1}).

1.3.2. Dry Matter Production and Analysis of Mineral Nutrients

When plants were 14 days old, their root and shoot parts were separately harvested for the determination of root and shoot dry weight and the concentrations of N and Fe. At harvest, the roots were washed twice in deionized water and then in 0.5 mM $CaSO_4$ solution. After drying at 70 °C and measuring the shoot and root dry weights, plant samples were subjected to acid digestion in a closed microwave system (MarsExpress; CEM Corp., Matthews, NC) by using 1 ml of 30% $H_2O_2$and 5 ml of 65%

HNO_3. Iron concentrations of the digested samples were measured by inductively coupled plasma optical emission spectrometry (ICP–OES) (Vista-Pro Axial; Varian Pty Ltd., Mulgrave, Australia). Nitrogen concentrations of dried and ground plant samples were determined by dry combustion (950 °C) using a LECOTru-Spec C/N Analyzer (Leco Corp., St Joseph, MI). The measurements of Fe and N were checked by using certified standard reference materials from the National Institute of Standards and Technology (NIST; Gaithersburg, MD). Chlorophyll concentrations (SPAD values) of leaves were measured on the newly expanded young leaves at harvest using a SPAD-502 chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan).

1.3.3. Determination of Methionine Concentration

Non-protein methionine concentration was determined in newly expanded young leaves and roots by analyzing methionine sulfone following performic acid oxidationas described by Spindler et al. (1984). About 1 (for leaves) or 2 (for roots) g of fresh plant sample was extracted in 12 ml of performic acid and incubated for 16 h at 4°C to complete the oxidation of methionineto methionine sulfone. At the end of the incubation period, samples were centrifuged at 5000 g for 15 min and 5 ml of supernatant was added with 0.84 g sodium metabisulfite to quench excess performic acid. The mixture was then stirred and 15 ml with 200 mM sodium citrate loading buffer added. Samples of 1000 µl were then aliquoted into new test tubes and 5475 µl of loading buffer and 75 µl of 32% NaOH for the adjustment of pH to about 2.2 were added. Finally, the samples were forced through syringe-tip PES filters into 2 ml glass HPLC vials and stored at 4°C until analysis. All samples and standards were analyzed using an automated aminoacid analyzer (Biochrom 32 Oxidised Hydrolysate System, Biochrom Ltd., Cambridge, UK) with post-columnninhydrin derivatization. The calibration standard was prepared in a sodium citrate loading buffer to yield a final concentration of 5 nmol methionine sulfone per 20 µl volume. A fixed injection volume of 20 µl was employed for all samples and the standards. The ninhydrincolor yields for methionine sulfone at 570 nm were used to calculate the tissue methionine concentration (i.e. mg of methionine sulfone kg^{-1} of fresh weight).

1.3.4. Collection and Measurement of Phytosiderophore

Collection and measurement of PSs were realized according to Cakmak et al. (1996) and Gries et al. (1998). Intact plants (25 seedlings) were removed from nutrient solution 1.5 h after the onset of the light period in the growth chamber, and transferred to 500 ml aerated deionized water for 3 h in the growth chamber. The root exudate solutions collected were stored at $-20\,^{o}C$ until the start of the PS assay that was based on the mobilization of Fe from freshly precipitated Fe(III)-hydroxide (Takagi, 1976). Iron hydroxide [$Fe(OH)_3$] solution was prepared by precipitating 4 mM $FeCl_3$ in 10 mM MES buffer with a pH of 6. For the PS assay, 8 ml root exudates and 2 ml $Fe(OH)_3$ solution were mixed and agitated for 45 min at 150 rpm, and after filtration the aliquots were subjected to Fe measurement by ICP–OES as described above. The amount of PS in the root exudates was calculated as mobilized Fe equivalents per plant or per gram of root dry weight. The capacity of roots to release PS was determined also by measurement of copper (Cu) mobilized from a Cu-loaded resin (Chelite-N, Serva, Heidelberg, Germany) according to Cakmak et al. (1996). The results obtained with Cu assay were very similar to the results obtained with the Fe assay (not reported).

1.3.5. Mobilization and Uptake of Fe from Fe(III)-Hydroxide

The capacity of plants to mobilize and absorb Fe fromFe-labeled Fe-hydroxide was measured according to Romheld and Marschner (1986) with modifications. First, roots of intact plants were rinsed in micronutrient-freenutrient solution for 1 h and then transferred to glassbeakers containing 150 ml of aerated micronutrient freenutrient solution. One milliliter of the fine suspended 2.5 mM Fe-labeled $Fe(OH)_3$ solution was delivered into dialysis tubes (Serva Servapor ø16 mm,Serva Feinbiochemica GmbH, Heidelberg, Germany) containing 5 ml of micronutrient-free nutrient solution. The dialysis tubes were then inserted into the beakers and continuously aerated by bubbling of air as illustrated in Fig. 4.1. The $Fe(OH)_3$ solution used was prepared by precipitating $Fe(NO_3)_3$ at alkaline pH and washing the precipitate until the wash solution was free

from Fe and of neutral pH. The specific activity of Fe added with the Fe-hydroxide solution was 308 GBq mol^{-1} Fe.

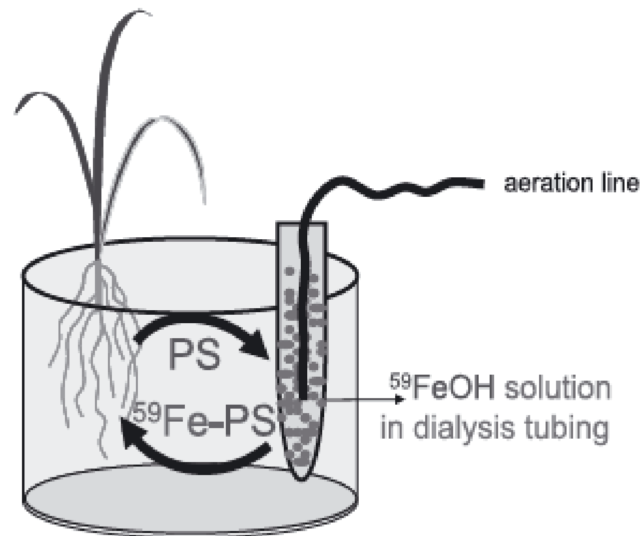

Fig. 1.1. Ilustration of the experimental setup testing mobilization and root uptake of Fe from $Fe(OH)_3$ that was supplied in aerated dialysis tube.

The mobilization and root uptake of Fe from the $Fe(OH)_3$ in the dialysis tube was measured both during the morning (2 h after the start of the light period) and evening (8 h after the start of the light period) taking into account the diurnal rhythm of PS release from roots that is very high in the morning and very low or not measurable during the afternoon and evening hours (Takagi et al. 1984; Zhang et al. 1989). The uptake experiments lasted for 6 h and were performed within the same day. The extracellular (apoplastic) Fe of roots was removed by treating the roots with 1.5 mM bipyridyl and 7.5 mM sodium dithionite for 10 min under continuous supply of N_2 in tightly closed plastic boxes with 500 ml volume as described by Bienfait et al. (1985). Then, the roots were washed in aerated 10 mM $CaSO_4$ solutionfor 10 min. The activity of Fe in the plant samples was measured separately in root and shoot samples after drying the samples at 70°C by using a Perkin Elmer 2480 WIZARD2 Automatic Gamma Counter (PerkinElmer, Waltham, MA). There were nine replications for each treatment with two plants per replicate sample. The Fe translocation from roots to shoots was calculated by dividing the Fe activity in the shoot by the root dryweight and expressed as nmol Fe g^{-1} root dry weight.

1.3.6. Root Uptake of Fe-Labeled PS

Measurement of root uptake of ^{59}Fe-labeled PS was carried out according to Yehuda et al. (1996). ThePS released from the experimental plants under Fedeficiency was used to prepare the ^{59}Fe-labeled PSfor the root uptake experiments. First, the root exudates collected were filtered through a 0.45 μm filter and the filtrates concentrated with a Buchi rotary evaporator (BUCHI Labortechnik AG, Flawil 1, Switzerland) undervacuum at 45°C. On the basis of our previous tests and published data, the only identified PS in the root exudates of different hexaploid or tetraploid wheat plants under Fe or Zn deficiency is deoxymugineic acid (DMA) (Cakmak et al. 1996; Romheld and Marschner 1990; Tolay et al., 2001). This finding has also been confirmed by others (Bashir et al. 2006; Ma and Nomoto 1994). Therefore, we assumed that the PS released from the roots of durum wheat plants was DMA, and the prepared Fe-labeled PS in this study was designated as FeDMA.

FeDMA was prepared by mixing $FeCl_3$ with 10% excess molar concentration of DMA prepared as above from the experimental plants and adjusted to pH 6.0.Before the start of the uptake experiment, roots were washed in an aerated micronutrient-free nutrient solution for 1 h. Then, plants were transferred to glass beakers containing 150 ml aerated micronutrient-free nutrient solution. The Fe-labeled DMA was added into the uptake solution at a concentration of 1.2 μM with a specific activity of 106 GBq mol^{-1} Fe. The uptake tests lasted for 2 h and were conducted independently both during the morning (2 h after the start of the light period) and evening (8 h after the start of the light period) within the same day. After a 2 h uptake period, plants were transferred to 1 mM $CaSO_4$ solution for 10 min, and then transferred to a new micronutrient-free nutrientsolution without Fe for 2 h in order to obtain adequate root-to-shoot translocation of Fe. The measurement of the apoplastic Fe of the roots and the Fe radioactivity in the harvested shoot and root samples were conducted as described above. For each treatment, there were nine replications with three plants per replicate sample.

1.3.7. Statistical Analysis

Data were analyzed by using Fisher's protected least significant difference (LSD) test at the 5% probability level following ANOVA using JMP® software (SAS Institute,Cary, NC).

1.4. Results

1.4.1. Growth and Concentrations of Fe and N

When compared with the low Fe supply, adequate Fe supply significantly enhanced both shoot and root growth at each N supply (Table 1.1). Increasing N application from low to high resulted in significantly increased shoot growth at adequate Fe supply, but had little effect at low Fe supply. However, increasing N tended to decrease the root growth under both Fe treatments, resulting in a greater shoot to root ratio (Table 4.1). The interaction between N and Fe treatments was significantin the case of shoot growth. Chlorophyll concentrations estimated by measurement of the SPAD values were much lower in the Fe-deficient than the Fe-adequate plants. Nitrogen supply resulted in significant differences on chlorophyll concentrations, but the differences were very small at each Fe supply (Table 1.1).

Table 1.1. Effect of increasing N supply on shoot and root dry weight, shoot-to-root dry weight ratio and SPAD (chlorophyll) levels of durum wheat plants grown for 14 days in nutrient solution with low (2 µ*M*) and adequate (100 µ*M*) Fe supply. Values are means of four replications. ns, not significant at the 0.05 level.

Fe supply	NO_3^- supply	Dry matter production ($mg\ plant^{-1}$)			
(µM)	(mM)	Shoot	Root	Shoot/root	SPAD
2	1	112	67	1.7	28
	3	122	56	2.2	25
	6	112	53	2.1	24
100	1	152	94	1.6	45
	3	180	87	2.1	45
	6	197	89	2.2	44
$LSD_{0.05}$	(Fe,NO_3^-, Fe X NO_3^-)	(10, 12, 18)	(10, ns, ns)	-	(1, 2, 2)

As expected, Fe-adequate plants had higher levels of Fe in shoot and roots. Varied N supply had no significant effect on Fe concentrations of roots and shoots at the low Fe supply, but significantly increased shoot Fe concentrations for the adequate Fe treatment (Table 1.2). The nutrient solution pH was slightly affected by the N treatments, and ranged generally between 7.2 for the low N plants and 7.6 for the high N plants (pH measured at the time of nutrient solution changes).

Table 1.2. Effect of increasing N supply on shoot and root concentrations of Fe and N of durum wheat plants grown for 14 days in nutrient solution with low (2 µ*M*) and adequate (100 µ*M*) Fe supply. Values are means of four replications. ns, not significant at the 0.05 level.

Fe supply	NO_3^- supply	Fe concentration (mg kg^{-1})		N concentration (%)	
(µM)	(mM)	Shoot	Root	Shoot	Root
2	1	37	49	4.4	2.7
	3	33	53	6.0	3.8
	6	34	48	6.5	4.4
100	1	82	412	4.7	3.2
	3	125	393	5.4	4.1
	6	138	557	5.3	4.3
$LSD_{0.05}$	(Fe,NO_3^-, Fe X NO_3^-)	(3, 4, 5)	(90, ns, ns)	(0.3, 0.3, 0.4)	(ns, 0.3, ns)

1.4.2. Methionine Concentrations

Compared with roots, methionine concentration was consistently higher in young leaves in all treatments (Table 1.3). Increasing N supply enhanced methionine concentration of young leaves by about 3-fold with adequate Fe supply, but this increase was only 1.7-fold higher under Fe deficiency. The N by Fe interaction was significant for shoot methionine concentration. Nitrogen treatments had little effect on the root concentrations of methionine under both Fe treatments. Root concentrations of methionine for the low Fe plants were significantly higher than in the adequate Fe plants forboth N treatments. At high N supply, the methionine concentration of leaves was substantially reduced by Fe deficiency, whereas this reduction was much less in the case of low N supply (Table 1.3).

Table 1.3. Effect of low (2 µ*M* FeEDTA) and sufficient (100 µ*M* FeEDTA) Fe supply on concentrations of methionine (analyzed as methionine sulfone) in expanded young leaves and roots of 14-day-old durum wheat plants grown in nutrient solution supplied with 1 and 6 m*M* N. The values are means of three replications. ns, not significant at the 0.05 level.

Fe supply (µM)	NO_3^- supply (mM)	Methionine sulfone concentration (mg kg^{-1} FW) Leaves	Root
2	1	179	162
	6	310	171
100	1	398	118
	6	1165	127
LSD 0.05	(Fe,NO_3^-, Fe X NO_3^-)	(42, 42, 59)	(7,7, ns)

1.4.3. Root Release of PSs

Iron deficiency increased the PS release from roots during the 2 weeks of growth, especially with higher N supply Fig. 1.2). Increased N supply significantly increased PS release of Fe-deficient roots. When compared with the lowest N supply, the highest N supply enhanced PS release up to fivefold (Fig. 1.2).

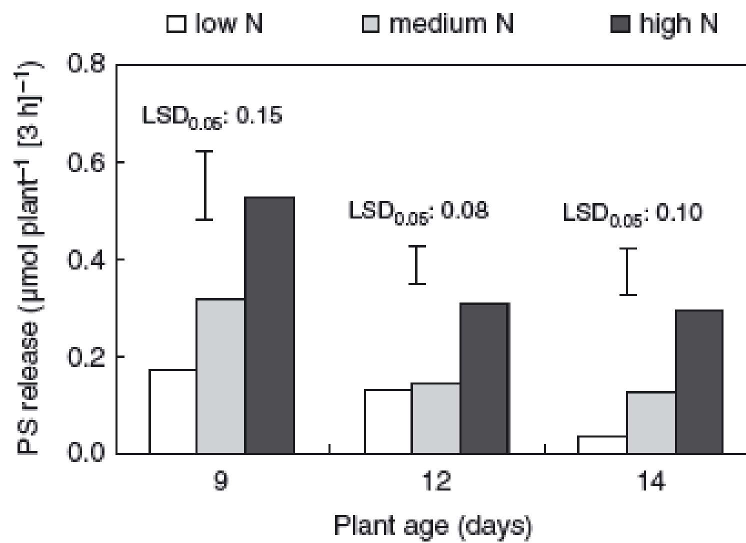

Fig. 1.2. Effect of increasing N supply on root release of PSs from roots of Fe-deficient wheat plants over 14 days. Nitrogen rates applied were 1 m*M* NO_{-3} (low N), 3 m*M* NO_{-3} (medium N) and 6 m*M* NO_{-3} (high N). Collection of PS was started 1.5 h after the onset of the light period in the growth chamber and continued for 3 h. The values are means of five replications. Vertical bars show LSD0.05 at $P < 0.05$ probability level.

This increase in PS release by high Nsupplywas much greater (up to ninefold) when the N supply was 0.5 mM (Fig. 4.3).

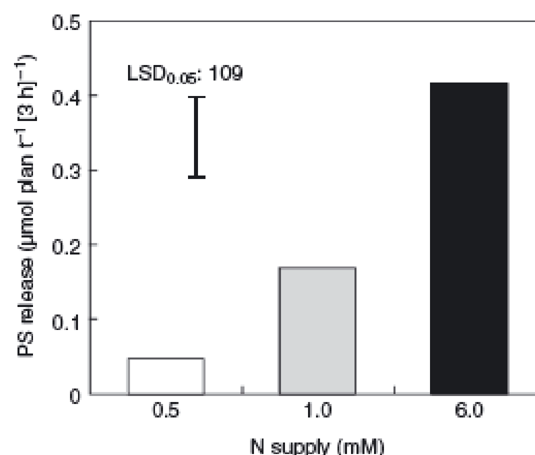

Fig. 1.3. Effect of increasing N supply on root release of PSs from root of 12-day-old wheat plants grown at low Fe supply (2 µ*M*). Nitrogen rates applied were 0.5 m*M*NO_{-3} (very low N), 1 m*M*NO_{-3} (low N) and 6 m*M*NO_{-3} (high N). Collection of PS was started 1.5 h after the onset of the light period in the growth chamber and continued for 3 h. The values are means of three replications. Vertical bars show LSD0.05) at *P* <0.05 probability level.

In the case of the adequate Fe supply, the PS release was not measurable during the 2 weeks of growth, and not affected by the varied N supply (data not shown). There was a decline in the PS release with the age of the plants (Fig. 1.2). When the root exudates were collected in the evening, PS release was not detected. Because the root dry matter production was decreased by increasing N supply under given conditions (Table 1.1), the N effect on PS release was even slightly stronger when expressed per unit root dry weight.

1.4.4. Mobilization and Uptake of Fe From Fe(III)-Hydroxide

Increasing N supply was also highly effective in increasing Fe mobilization from Fe-labeled Fe(OH), supplied in dialysis tubes. As presented in Fig. 1.4. mobilization (solubilization) and root uptake of Fe from $Fe(OH)_3$was significantly increased by increasing N supply during the morning hours. In the case of the test conducted in the evening, there was very little (or undetectable) root uptake of Fe from $Fe(OH)_3$ (Fig. 1.4). When plants were supplied with adequate Fe, no mobilization and thus no root uptake of Fe from the $Fe(OH)_3$could be measured at each N supply either in the morning

or evening (data not shown). The effect of varied N supply on the root-to-shoot translocation rate of Fe mobilized from $Fe(OH)_3$ followed a pattern similar to the root uptake rate of Fe (Fig. 1.4.), but was even more clearly affected by the increasing N supply than the root uptake rate of Fe. Also, the percentage of radioactivity in shoot to the sum of the radioactivity in shoot and root was higher in plants with high N supply (up to 57%) compared with the plants with very low N supply (data not shown).

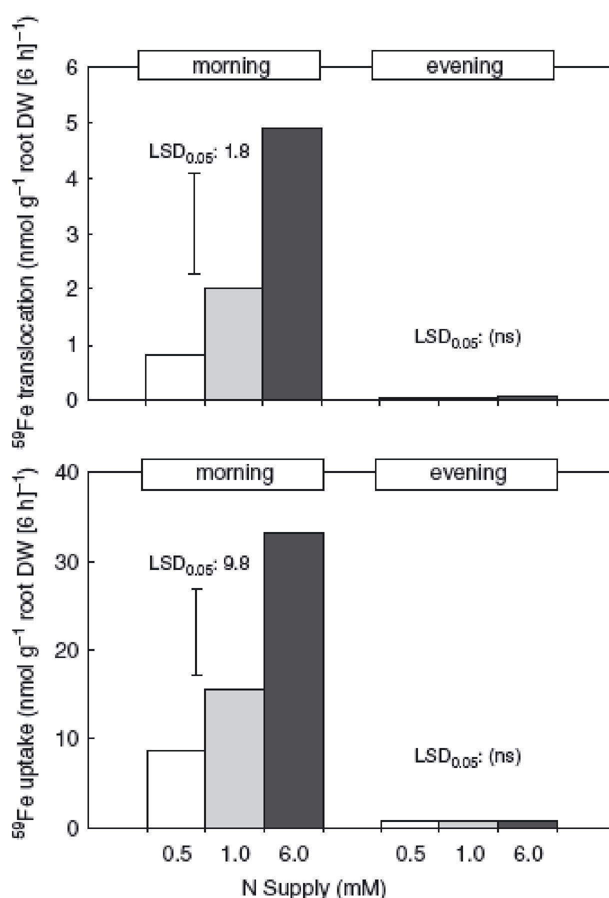

Fig.1.4. Effect of increasing N supply on root uptake and root-to shoot translocation of Fe from $Fe(OH)_3$ supplied in a dialysis tube. Plants were 14 days old and grown under low Fe supply (2 μ*M*). The mobilization and root uptake of ^{59}Fe from the $Fe(OH)_3$ in the dialysis tube was measured during the morning (2 h after the start of the light period) and evening (8 h after the start of the light period) and lasted for 6 h. Uptake of Fe includes radioactivity in roots and shoots. Nitrogen rates applied were 0.5 m*M* NO_{-3} (very low N), 1 m*M* NO_{-3} (low N) and 6 m*M* NO_{-3} (high N). The values are means of nine replications. Vertical bars show LSD0.05 at $P < 0.05$ probability level.

1.4.5. Root Uptake of Fe-Labeled PS

In the subsequent experiment, the effect of varied N supply on the root uptake of Fe–DMA was measured during the morning and evening hours. Increasing Nsupply resulted in a significant enhancement in the root Fe uptake from Fe–DMA both in the morning and evening (Fig. 1.5). In the morning uptake experiment, the N treatments of 1 and 6 mM were not significantly different in their effect on Fe uptake, whereas in the

evening experiment, there was a statistically significant, progressive increase in Fe uptake by increasing N supply (Fig. 1.5). The effect of increasing N supply on the root-to shoot translocation of Fe–DMA was very similar to its effect on the Fe uptake both in the morning and evening experiments (Fig. 1.5). However, there was a tendency that the effect of increased N supply was slightly more pronounced on the root-to-shoot translocation of Fe than the root uptake of Fe.

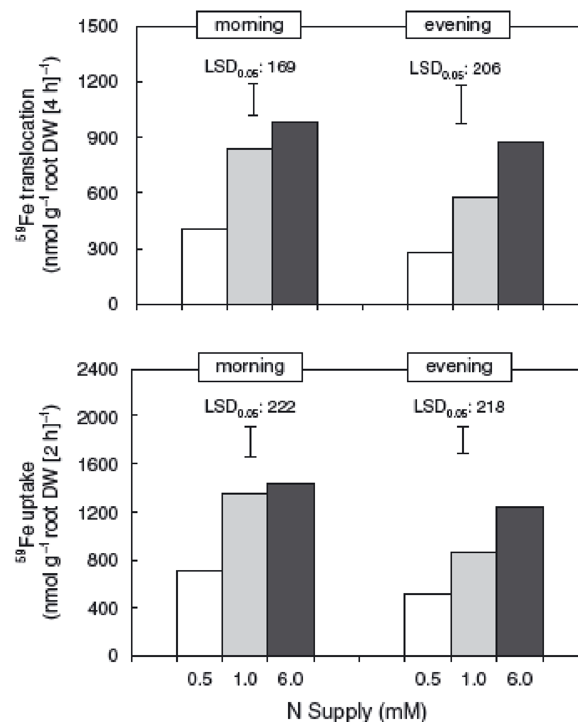

Fig. 1.5. Effect of increasing N supply on root uptake and root-to-shoot translocation of Fe from Fe-labeled PS in 14-day-old wheat plantsgrown under low Fe supply (2 µ*M*). The experiment has been conductedduring the morning (2 h after the start of the light period) and evening (8 h after the start of the light period) and lasted for 2 h for the Fe uptake and 4 h for the shoot translocation. Nitrogen rates applied were 0.5 m*M* NO_{-3} (very low N), 1 m*M* NO_{-3} (low N) and 6 m*M* NO_{-3} (high N). The values are means of nine replications. Vertical bars show LSD0.05 atv$P$ <0.05 probability level.

1.5. Discussion

Irrespective of Fe supply, low N treatment (e.g. 1 mM nitrate supply) tended to increase root dry weight, especially at low Fe supply and resulted in higher shoot to-root dry weight ratio (Table 1.1). Increase in root growth as a response to low N supply is a well-known phenomena (Marschner, 1995; Rufty et al. 1988). As expected, the low N precultured plants had lower N concentrations than the plants grown under higher N supplies (Table 1.2). These results confirm that the lower N supply experimental plants of this study had a varied N nutritional status.

Iron deficiency-dependent increase in root release of PS is a well-known phenomenon (Marschner et al. 1986; Takagi et al. 1984), and has been confirmed for this study conditions (Figs 1.2 and 1.3). This article also showed that the PS release from Fe-deficient roots is strongly affected by the level of N nutrition of plants. To our knowledge, this is the first study showing that N nutritional status of plants significantly affected the PS release from roots. The positive influence of high N on Fe mobilization from $Fe(OH)_3$ was not related to the release of any other compounds other than PS, because the Fe-sufficient plants with different N supplies did not show any effect on solubilization of Fe from $Fe(OH)_3$. In addition, it is well known that the release of PS from roots follows a distinct diurnal rhythm, exhibits a maximum rate nearly 2 h after the onset of light period and ceases within a few hours (Takagi et al. 1984; Zhang et al. 1989). In this study, variation in N nutrition did not affect Fe mobilization from $Fe(OH)_3$ when the root exudates were collected in the evening. These observations clearly indicate that the enhanced Fe mobilization by increased N supply in Fe-deficient plants is caused mainly by the PS released from roots. Enhanced PS release from roots is not specific for Fe-deficient plants and it can be also enhanced under Zn deficiency (Cakmak et al. 1994, Suzuki et al. 2006; Zhang et al. 1989). The experimental plants under low Fe supply contained sufficiently high concentrations of Zn in leaves (up to 150 mg kg^{-1}) confirming that they were not Zn deficient. Thus, any possible contribution of Zn deficiency in N-dependent changes in PS release can be excluded.

One plausible explanation for the enhanced PS release in Fe-deficient plants because of increasing N supply would be enhanced dry matter production and thus increased physiological demand for Fe as a result of higher N supply. However, under the conditions of thisstudy, varied N supply did not cause a marked change in biomass production (Table 1.1). Expression of the PS release per plant or per gram root dry weight did not change the results.

A plausible alternative explanation for the low release of PS from N-deficient roots would be a limited biosynthesis of PS. There are many proteins and nitrogenous compounds which are required for biosynthesis of PS, such as methionine, NA, NA synthase and NAAT (Ma et al. 1995; Mori and Nishizawa 1987; Shojima et al. 1990). It is possible that the level of at least some of these N compounds or enzymes was

adversel affected because of low supply of N in wheat plants. In good agreement with this argument, this study showed that leaf concentration of methionine was significantly affected by the N nutritional status of plants (Table 1.3). The changes in methionine concentrations in relation to N supply were much greater in the leaf than the root tissues (Table 1.3). By reducing N supply, leaf concentrationof methionine was significantly decreased, and this decrease was pronounced in Fe-deficient plants (Table 1.3). The greater decreases in the amount of methionine in Fe-deficient leaves under high N supply might be ascribed to increased utilization of methionine, e.g.in PS biosynthesis to maintain high PS release caused by Fe deficiency. Evidence is available showing that PS biosynthesis also takes place in leaf tissue as in root tissue under Fe deficiency (Inoue et al. 2008; Ma et al. 1995; Mori and Nishizawa 1987; Shojima et al. 1990). It is known that PS concentrations might be very high in Fe deficient leaf tissue (Mori et al. 1987). An enhanced use of methionine can be also expected in Fe-deficient plants because of Fe-deficiency-induced ethylene production (Nakanishi et al. 1999; Romera et al. 1999). In contrast to leaf tissue, low Fe supply slightly increased root concentration of methionine compared with the Fe-adequate plants at both N treatments. This may suggest that Fe deficiency possibly increases physiological demand for methionine to maintain high rate of PS biosynthesis in Fe-deficient roots (Ma et al. 1995; Nagasaka et al. 2009; Negishi et al. 2002). In contrast to root release of PS (only morning hours), PS biosynthesis takes place continuously throughout the day, and the PS synthesized accumulate in roots to be secreted during the morning hours (Nagasaka et al. 2009). Root concentration of PS in Fe-deficient roots is extremely high that constitute upto 2% of the root dry weight. Possibly, because of intensive PS biosynthesis and existence of large amounts of PS in roots, there is a high cellular demand for methionine to maintain PS biosynthesis in roots. In order to meet the high methionine demand in Fe-deficient roots, there is, probably, a continuous transport of methionine from shoots into roots. This might be one plausible reason for higher methionine concentration in Fe-deficient roots. Relatively high concentrations of methionine in phloem fluid of wheat plants (Hayashi and Chino 1986) may indicate that methionine is possibly transported into roots through phloem to be involved in PS biosynthesis in the root cells. For better understanding the role of substrate limitation in PS release from Fe-deficient roots, future studies should investigate the role of

methionine (and other related nitrogenous compounds) in PS biosynthesis in root and also leaf tissue under varied N supply.

In a previous study, Romheld (1991) showed thatwhen Fe was supplied in the form of $Fe(OH)_3$ in a dialysis tube, Fe-deficient barley plants were able to mobilize and take up Fe from $Fe(OH)_3$ when the test was performed during morning hours. The findings of this study with wheat are in good agreement with the results obtained in Fe-deficient barley plants, and show that this process is strongly affected by the N nutritional status of plants. The results in Figs 1.4 and 1.5 suggest that besides its effect on mobilization of Fe from $Fe(OH)_3$, increased N status also has also a positive impact on the root uptake and root-to-shoot transport ofthe resulting Fe–PS complex. It is widely believed that the Fe(III)–PS complexes formed after root exudation of PS are transported into root cells by the YS1 transporter proteins (Curie et al. 2001; Murata et al. 2008; Schaaf et al. 2004). When this protein is not active in roots as shown in mutant lines, plants quickly developed Fe deficiency chlorosis because of impaired root uptake of Fe(III)–PS complex (Curie et al. 2001; von Wiren et al. 1994). Possibly, the activity and/or abundance of the YS1 transporters might be also adversely affected by reducing N supply to plants, leading to a clear decline in root uptake of Fe–PS (Figs 1.2 –1.4). This issue opens new research topics for future investigations.

When radiolabeled Fe (^{59}Fe) was supplied to the Fe-deficient plants as Fe–PS in the morning and evening, the root uptake and root-to-shoot translocationof Fe–PS at a given N supply were very similar in the morning and evening (Fig. 1.5). This result confirms that in contrast to the root release of PS, root uptake of Fe–PS takes place at comparable rates in the morning and evening. The enhancing effect of N nutrition on uptake and transport to shoots of Fe–PS did not change when the assay was conducted in the morning or evening. Operation of root absorption of Fe–PS during the evening was also shown by Yehuda et al. (1996) in Fe-deficient barley plants. However, in contrast to these results with wheat, Yehuda et al. (1996) showed that the root uptake of Fe–PS by Fe-deficient barley roots is greater during the morning than the evening hours. This difference between both the experiments is not clear and could be related to use of different plant species or experimental conditions.

The positive effects of N nutrition on the PS releaseand the root uptake of Fe–PS may have important consequences regarding improving Fe concentrations in edible parts of food crops (e.g. seeds/grains). Recent studies conducted in both greenhouse and field show that increases in the rate of N fertilization significantly improve grain Fe concentrations of wheat plants (Cakmak et al. 2010; Kutman et al. 2010). Nitrogen promoted release of PSs from roots and root uptake of Fe–PS might be one contributing factor to improving grain Fe concentrations of food crops. Currently, Fe deficiency anemia caused by reduced dietary intake represents a global nutritional problem and affects nearly half of the world population (Cakmak et al. 2010; Welch and Graham 2004). Enhancement in concentration of Fe in staple food crops is an important challenge in order to minimize Fe-deficiency-related health problems in human populations. The results of this study indicate that in efforts for enrichment of food crops with Fe, attention should be paid to N nutritional status of these crops.

CHAPTER 2

BIOFORTIFICATION OF WHEAT WITH IRON THROUGH SOIL AND FOLIAR APPLICATION OF NITROGEN AND IRON FERTILIZERS

2.1. Abstract

Increasing iron (Fe) concentration in food crops is an important global challenge due to high incidence of Fe deficiency in human populations. Evidence is available showing that nitrogen (N) fertilization increases Fe concentration in wheat grain. This positive impact of N on grain Fe was, however, not studied under varied soil and foliar applications of Fe. Greenhouse experiments were conducted to investigate a role of soil- and foliar-applied Fe fertilizers in improving shoot and grain Fe concentration in durum wheat (*Triticum durum*) grown under increasing N supply as Ca-nitrate. Additionally, an effect of foliar Fe fertilizers on grain Fe was tested with and without urea in the spray solution. Application of various soil or foliar Fe fertilizers had either a little positive effect or remained ineffective on shoot or grain Fe. By contrast, at a given Fe treatment, raising N supply substantially enhanced shoot and grain concentrations of Fe and Zn. Improving N status of plants from low to sufficient resulted in a 3-fold increase in shoot Fe content (e.g., total Fe accumulated), whereas this increase was only 42% for total shoot dry weight. Inclusion of urea in foliar Fe fertilizers had a positive impact on grain Fe concentration. Nitrogen fertilization represents an important agronomic practice in increasing grain Fe. Therefore, the plant N status deserves special attention in biofortification of food crops with Fe.

2.2. Introduction

Low dietary diversity and inadequate daily intake are the main reasons for the widespread occurrence of Fe deficiency in human populations, especially among children and women living in developing world. Impairments of cognitive function, immune system and work capacity and increases in infant and maternal mortality represent major health complications associated with Fe deficiency (Hunt, 2005; Carter et al. 2010). Iron deficiency is the most common cause of anemia globally. According to arecent report based on the WHO Database, anemia affects nearly 1.6 billion people, and pre-school children and pregnant women are under greatest risk of Fe deficiency anemia (McLeon et al. 2009).

In the developing world, cereal-based foods are the major dietary component, but have low concentration and bioavailability of Fe (Hurrell et al. 2004; Cakmak, 2008; Gibson et al. 2010). Improving both concentration and bioavailability of Fe in cereal grains is, therefore, an important challenge and a high-priority research area (Bouis and Welch 2010; Cakmak et al. 2010a).

Among the strategies applied for reducing the prevalence of Fe deficiency problem in human populations, enrichment (biofortification) of food crops with Fe through agricultural approaches is a widely applied strategy (Pfeiffer and McClafferty 2007; Borg et al. 2009; Cakmak et al. 2010a). Agronomic biofortification (e.g., fertilizer applications) and plant breeding (e.g., genetic biofortification) represent complementary and cost-effective agricultural approaches to the problem (Cakmak, 2008; White and Broadley 2009). In case of Zn, published data provide convincing evidence that soil and especially foliar applications of Zn fertilizers are effective in improving grain concentration of Zn (Yilmaz et al. 1998; Peck et al. 2008; Cakmak et al. 2010b; Zhang et al. 2010). In contrast to Zn, a role of Fe fertilizers and their application methods in improving Fe concentration in cereal grains is rarely studied. Most of the studies dealing with soil and foliar application of Fe fertilizers focused on correction of Fe deficiency chlorosis and improving yield (Tagliavini et al. 2000; Abadia et al. 2002; Fernandez and Ebert 2005). Due to rapid conversion of Fe into unavailable forms when

applied to calcareous soils and poor mobility of Fe in phloem, soil and/or foliar Fe fertilization appears to be less effective than Zn fertilization in enrichment of cereal grains (Rengel et al. 1999; Cakmak, 2008). For example in wheat, foliar application of Zn fertilizers improved grain Zn concentration by up to 2- or 3-fold depending on the plant availability of Zn in soils (Cakmak, 2008; Cakmak et al. 2010b), whereas increases in grain Fe concentration by foliar spray of $FeSO_4$ or Fe chelates did not exceed 36% (Zhang et al. 2010). Moreover, some work has showed that plants did not respond to foliar Fe fertilization in terms of grain Fe concentration (Gupta, 1991).

In recent studies it has been shown that the plant N status is an important factor in enrichment of cereal grains with Fe. Increasing molecular evidence is available showing that remobilization from vegetative tissue and translocation into seed of N and Fe (as well as Zn) is maintained by the similar genetic mechanisms (Uauy et al. 2006a; Waters et al. 2009), resulting in a positive correlation between grain Fe and N concentrations (Cakmak et al. 2004; Distelfeld et al. 2007). Studies under both field and greenhouse conditions demonstrated that increasing soil N application significantly improved shoot and grain Fe concentrations (Cakmak et al. 2010b; Kutman et al. 2010; Shi et al. 2010). Similarly, foliar spray of urea enhanced grain Fe (Kutman et al. 2010). However, in the above greenhouse and field experiments, plants were grown under different N treatments, but no treatments with either soil or foliar applications of Fe fertilizers were tested.

There are various inorganic and chelated forms of Fe fertilizers that are used and tested for correction of Fe deficiency chlorosis in crop plants, such as $FeSO_4$, FeEDTA, FeDTPA, FeEDDHA, Fe-citrate and FeIDHA (iminodisuccinic acid) However, the effectiveness of those Fe compounds in overcoming Fe deficiency chlorosis is highly variable depending on their stability, penetration ability through leaf cuticle and mobility/translocation following diffusion into leaf tissue (Schönherr et al. 2005; Fernandez et al. 2006, 2009; Rodriguez-Lucena et al. 2010a). Inclusion of urea in the spray solution of Fe compounds has been shown to stimulate penetration of Fe into the leaf tissue (Swietlik and Faust 1984; Rodriguez-Lucena et al. 2010b). It is not known whether use of urea together with Fe fertilizers can improve effectiveness of foliar Fe fertilization in increasing grain Fe concentration.

This study was conducted under greenhouse conditions to investigate the role of soil and foliar applied Fe fertilizers in improving grain Fe concentration of plants grown under increasing soil N supply. Additionally, effectiveness of various foliar Fe fertilizers in increasing grain Fe concentration was tested with and without inclusion of urea in the foliar spray solution.

2.3. Materials and Methods

Fifteen seeds of durum wheat (*Triticum durum* L. cv. Balcali 2000) were sown in each plastic pot containing 3 kg soil from a Zn-deficient region in Central Anatolia (Cakmak et al. 1996). The soil used in the experiments had a clay-loam texture and low organic matter (15 g/kg), abundant $CaCO_3$ (180 g $kg^{-1)}$ and high pH (8 in dH_2O). The diethylenetriamine pentaacetic acid (DTPA)-extractable Zn and Fe concentrations were 0.1 and 2.1 mg kg^{-1} soil, respectively, measured by using the method described by Lindsay and Norvell (1978). Plants were grown under greenhouse conditions at the Sabanci University campus (40° 53′ 24.5″ N; 029° 22′ 46.7″ E). Before potting, soil in all experiments was supplied with the following nutrients (in mg kg^{-1} soil): 100 phosphorus (P) as KH_2PO_4, 25 sulfur (S) as K_2SO_4 and 2 Zn as $ZnSO_4$ $.7H_2O$. Different amounts of N and Fe fertilizers were incorporated in the soil, depending on the experimental treatments described below.

In the first experiment, pots contained five plants. Nitrogen treatments were 100 (low), 200 (medium) or 400 mg (high N) N kg^{-1} dry soil applied as Ca $(NO_3)_2$. Iron was applied at the rate of 10 mg Fe kg^{-1} soil in forms of FeEDTA or $FeSO_4$. Soil application of low and medium rates of N was done before potting. High N supply was made up of two portions: first half was incorporated into soil before potting and the second half during the stem elongation stage, after dissolving in deionized water. Plants were harvested at the early flowering stage (e.g., Feekes stage 10.5), when they were 52 days old. At harvest, whole shoots (all above-ground plant parts) were harvested, washed with deionized water and dried at 70°C for determination of shoot dry weight.

In the second experiment, an effect of foliar applied FeEDTA or $FeSO_4$ on grain Fe concentration was studied in plants grown at different soil N applications. Soil N applications were 75, 250 or 500 mg N per kg soil added as $Ca(NO_3)_2$. The N treatments with 75 and 250 mg N kg^{-1} were applied before potting. In the case of 500 mg N kg^{-1} soil application, first 250 mg N kg^{-1} was incorporated into soil before potting, and the remaining amount was applied in two equal portions at the stem elongation and flowering stages. Foliar treatment of FeEDTA was conducted at the rate of 0.25% (w/v). In the case of $FeSO_4$ application, foliar solution contained the same amount of Fe that was present in the 0.25% (w/v) FeEDTA. Tween has been used as a surfactant at 200 mg $L^{-1.}$ Foliar spraying with Fe fertilizers was continued until run-off by using a hand-sprayer. Spraying was conducted twice: at the booting and early milk stages. After full maturity, grains (manually threshed) and straw (shoot) were harvested separately and used for determination of dry weight (straw was dried at 70°C first).

In the third experiment, various Fe fertilizers were applied onto foliage with and without 1% (w/v) urea in the spray solution. The foliar Fe fertilizers tested were FeEDTA, $FeSO_4$, FeEDDHA and Fe-citrate, each applied twice as described above at the booting and early milk stages. Solutions of all Fe fertilizers contained the same amount of Fe that was present in the 0.25% (w/v) FeEDTA. At maturity, grains and straw parts were harvested separately and weighed as described above.

Dried and ground plant samples were subjected to acid-digestion [ca. 0.2 g sample in a mixture containing 2 mL of 30% (v/v) H_2O_2 and 5 mL of 65% (v/v) HNO_3] in a closed-vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). Determination of mineral nutrients other than N was done by using inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia). Nitrogen concentration in the samples was determined after dry combustion (950°C) using a LECO Tru-Spec C/N Analyzer (Leco Corp., St Joseph, MI, USA). Measurement of mineral nutrients was checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). To check for Fe contamination, aluminum (Al) concentration in the grain samples was measured and found to be less than 2 mg kg^{-1},

suggesting an absence of Fe contamination via soil dust (cf. Pfeiffer and McClafferty 2007).

All experiments were set up I n a randomized complete block design with four replications (first and second experiment) and three replications (third experiment). Data analysis was conducted by JPM software (JMP, SAS Institute, Cary, North Carolina, USA), and comparison of means was performed by using the Student's t test, whenever ANOVA (using general linear model) indicated significant effect of treatments.

2.4. Results

In the first experiment, plants were harvested at theearly flowering stage. At the low N (100 mg N kg^{-1} soil) supply, older leaves were light green, while the plants at medium (200 mg N kg^{-1} soil) and high N (400 mg N kg^{-1} soil) supplies looked normal without any leaf symptoms. This observation indicated that plants at low N supply were possibly under a low N status. Shoot dry matter production was significantly enhanced by increasing N supply at each Fe treatment, whereas at a given N supply shoot growth was not influenced by the Fe treatments, except the FeEDTA treatment at the medium N supply (Table 2.1). Shoot N concentrations were not affected by the Fe treatments (Table 2.1). However, increasing N supply significantly increased shoot N concentrations at each Fe treatment. High N supply also enhanced shoot concentration of other cationic macronutrients (Table 1.1). Averaged over all Fe treatments, increasing the N supply from 100 to 400 mg N kg^{-1} soil enhanced shoot concentrations of Ca, Mg and K by about 100%, 73% and 40%, respectively, whereas the increase in shoot P concentration was about 12%.

Table 2.1. Effect of increasing soil N supply on shoot dry weight and shoot concentrations of N, P, K, Ca and Mg in durum wheat (*Triticum durum* cv. Balcali 2000) under different soil Fe treatments. Plants were grown in soils with low (100 mg N kg^{-1} soil), medium (200 mg N kg^{-1} soil) and high (400 mg N kg^{-1} soil) N supply for 52 days (until flowering stage) under greenhouse conditions. Iron treatments were: no iron, FeEDTA or $FeSO_4$, applied at the rate of 10 mg Fe kg^{-1} soil. Values are means of four independent replicates.

Soil treatments		Shoot dry weight	Shoot concentration				
Fe	N		N	P	K	Ca	Mg
	(mg kg^{-1})	(g plant)			(g kg^{-1})		
no Fe	100	1.63	17.3	2.90	21.0	4.84	0.90
	200	1.88	31.0	2.87	28.3	6.62	1.33
	400	2.27	45.8	2.90	28.5	10.06	1.52
FeEDTA	100	1.66	15.8	2.69	20.3	4.75	0.82
	200	2.25	27.5	2.85	25.8	6.10	1.20
	400	2.37	46.0	3.26	29.8	9.32	1.51
$FeSO_4$	100	1.74	16.0	2.95	23.0	4.99	0.90
	200	1.85	31.5	3.01	30.3	6.49	1.28
	400	2.48	45.5	3.44	31.8	9.77	1.49
CV (%)		14.50	5.6	8.70	7.1	11.6	6.6
F test*		**	**	**	**	**	**
$LSD_{0.05}$		0.41	2.5	0.38	2.7	1.19	0.12

* F test: ** = Significant at (*P* =0.01) level, * = Significant at (*P* =0.05) level, n.s. = not significant at (*P* =0.05) level

Increases in soil N application had a distinctlypositive effect on shoot concentration of Fe and Zn ateach Fe treatment (Figs. 2.1 and 2.2). As an average of allFe treatments, increasing N supply from low to high enhanced shoot Fe concentration from 27 to 43 mg kg^{-1}. Shoot Zn concentrations were also similarly improved by N application (Fig. 2.2). When compared to the control plants (no Fe treatment), soil treatment with FeEDTA had increasing and with $FeSO_4$ decreasing effect on shoot Zn concentration (Fig. 2.2). Due to increases in shoot dry matter yield by increasing the N supply (Table 2.1), the total uptake of Fe and Zn per shoot (e.g., shoot content) was substantially increased by N supply at each Fe treatment (Figs. 2.1 and 2.2). Averaged over all Fe treatments, the relative increases in shoot content of Fe and Zn by high N supply were 127% and 137%, respectively, while the increase in shoot dry matter accumulation by increasing N was much less. In addition, shoot content of the cationic macronutrients also showed particular increases, especially in the case of Ca (data not shown).

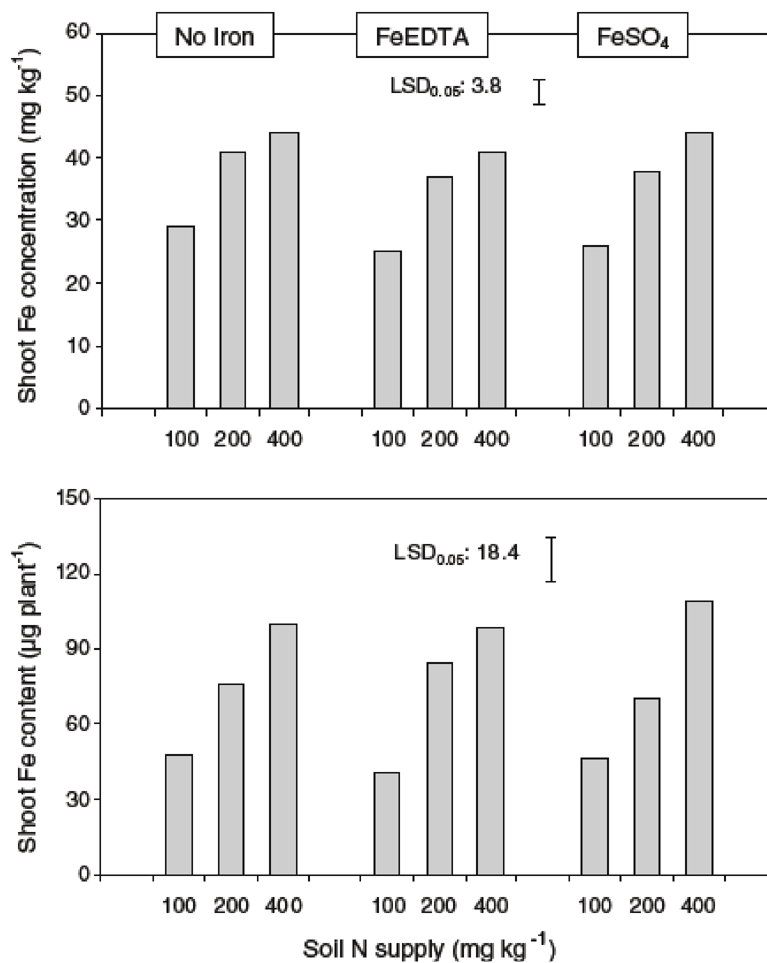

Fig. 2.1. Effect of increasing the soil N supply on shoot concentration and shoot content (e.g., total accumulation) of Fe in durum wheat (*Triticum durum* cv. Balcali 2000) under different soil Fe treatments. Plants were grown in soils with low (100 mg N kg^{-1} soil), medium (200 mg N kg^{-1} soil) or high (400 mg N kg^{-1} soil) N supply for 52 days (until flowering stage) under greenhouse conditions. Iron treatments were: no iron, FeEDTA or $FeSO_4$, applied at the rate of 10 mg Fe kg^{-1} soil. Values are means of four independent replicates.

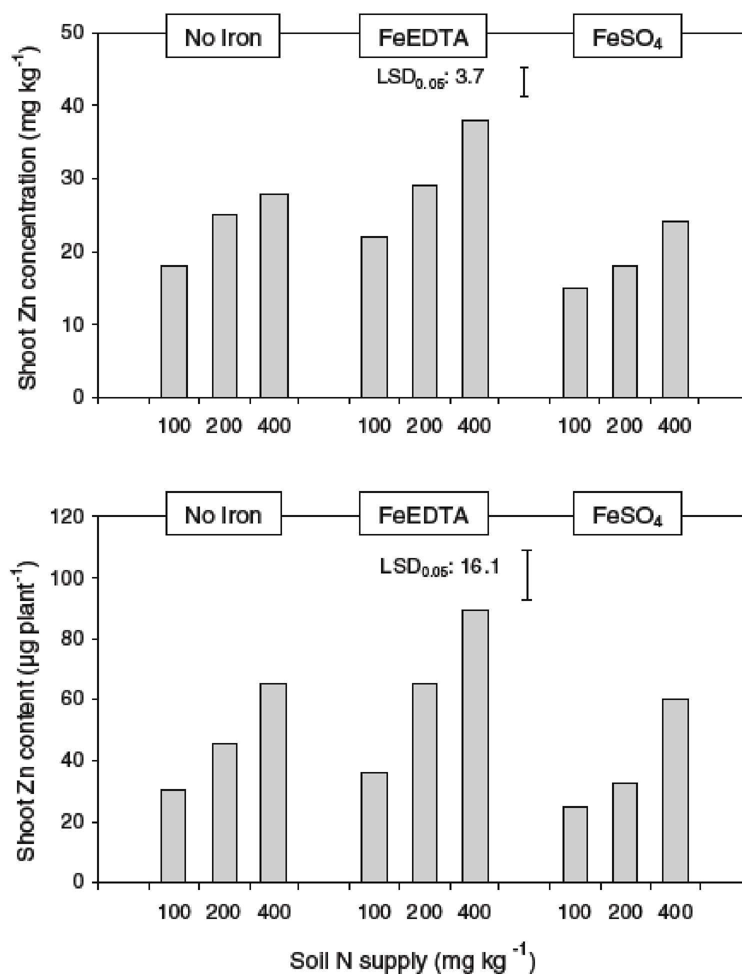

Fig. 2.2. Effect of increasing soil N supply on shoot concentration and shoot content (e.g., totalaccumulation) of Zn in durum wheat (*Triticum durum* cv. Balcali 2000) under different soil Fe treatments. Plants were grown in soils with low (100 mg N kg^{-1} soil), medium (200 mg N kg^{-1} soil) or high (400 mg N kg^{-1} soil) N supply for 52 days (until flowering stage) under greenhouse conditions. Iron treatments were: no iron, FeEDTA or $FeSO_4$, applied at the rate of 10 mg Fe kg^{-1} soil. Values are means of four independent replicates.

In the second experiment, plants were harvested at grain maturity. The changes in grain concentrations of N, Fe and Zn were studied in plants treated by increasing soil N fertilization and foliar spray of FeEDTA or $FeSO_4$. Plants at the medium and high N supplies had a significantly greater grain yield thanthose at the low N supply at each Fe treatment (Table 2.2), indicating that plants at low N supply were in a low N status. Foliar Fe treatments did not result in consistent effects on grain yield.

Irrespective of the Fe treatments, raising soil N application from low to high rate significantly increased grain N concentrations (Table 2.2). On average, grain N

concentration was increased from 22 g kg^{-1} (low-N plants) to 36 g kg^{-1} (high-N plants). Improving N nutrition also tended to increase grain P concentration (Table 2.2). Grain concentrations of K, Ca and especially Mg were also positively affected by increasing N supply. However, the increases in grain concentration of cationic macronutrients resulting from the high N supply (Table 2.2) were less than the corresponding increases found in shoot tissue (Table 2.2). As an average of the Fe treatments, N dependent increases in grain concentrations of K, Ca and Mg were 8.7%, 6.5% and 34%, respectively (Table 2.2). At each N supply, foliar spray of FeEDTA or $FeSO_4$ had an insignificant positive effect on grain Fe concentrations. Generally, at a given N supply,foliar spray of Fe slightly (and inconsistently) decreased grain Zn concentrations (Table 2.2). The most obvious was the effect of increasing soil Napplication on the grain Fe and Zn concentrations. As shown in Table 2.2, high N nutrition significantly increased grain concentrations of Fe (about 38%) and especially Zn (104%).

Table 2.3 shows grain N and Fe concentrations forplants sprayed by different Fe fertilizers with and without 1% (w/v) urea. In this experiment, grain yield was not affected by the Fe and urea treatments. Similarly, Fe treatments did not affect grain N concentration. However, spraying Fe fertilizers together with 1% (w/v) urea improved grain N concentrations, but these increases were not statistically significant, except in the $FeSO_4$+urea treatment (Table 2.3). Spraying Fe fertilizers without inclusion of urea either slightly enhanced grain Fe concentrations or remained ineffective. By contrast, when Fe fertilizers were sprayed together with 1% (w/v) urea, there were consistent increases in grain Fe concentrations, and in the case of $FeSO_4$ this increase was statistically significant.

2.5. Discussion

Shoot growth, grain yield and Fe concentrations of durum wheat plants were only slightly affected by soil and foliar application of Fe fertilizers. Even the application of Fe-chelates, like FeEDTA and FeEDDHA, remained ineffective. Many graminaceoues species, like wheat, release Fe-mobilizing compounds (phytosiderophores, PS) to

solubilize and absorb Fe from calcareous soils with low chemical availability of Fe (Marschner et al. 1986; Takagi et al. 1988). According to calculations by Romheld (1991), in Fe chlorosis- resistant cereal species like wheat, the rate of PS release from roots is high enough to satisfy plant Fe demand and maintain adequate growth in calcareous soils without requirement for Fe fertilization. Most probably, because of this root adaptive reaction, growth (Tables 2.1 and 2.2) and shoot concentrations of Fe (Fig. 2.1) of durum wheat plants were not consistently affected by the soil application of Fe fertilizers. Similarly, ryegrass grown in an alkaline soil did not respond to application of various Fe chelates and $FeSO_4$ regarding growth and shoot concentrations of Fe (Ylivainio et al. 2004).

Table 2.2. Effect of increasing soil N supply and foliar application of Fe fertilizers on grain yield and grain concentrations of Fe, Zn, N, P, K, Ca and Mg in durum wheat (*Triticum durum* cv. Balcali 2000) under different soil Fe treatments. Plants were grown on soils with low (75 mg N kg^{-1} soil), medium (250 mg N kg^{-1} soil) and high (500 mg N kg^{-1} soil) N supply until full maturity under greenhouse conditions. Foliar Fe treatments were: no iron, 0.25% (w/v) FeEDTA and 0.25% (w/v) FeSO4. Foliar $FeSO_4$ fertilizer contained the same amount of Fe that was present in the FeEDTA solution. Values are means of four independent replicates.

Foliar Fe applications	Soil N applications	Grain Yield	Grain concentration						
			Fe	Zn	N	P	K	Ca	Mg
	(mg kg^{-1} soil)	(g $plant^{-1}$)	(mg kg^{-1})				(g kg^{-1})		
Control	75	1.24	25	27	22	3.81	4.39	0.26	1.16
	250	2.03	33	50	36	4.02	4.77	0.38	1.44
	500	1.94	34	51	36	3.93	4.69	0.38	1.51
FeEDTA	75	1.39	27	21	21	3.53	4.43	0.27	1.02
	250	2.07	37	40	35	3.93	4.68	0.38	1.37
	500	2.35	39	45	36	3.98	4.75	0.38	1.47
$FeSO_4$	75	1.39	30	25	22	3.77	4.33	0.27	1.12
	250	2.57	36	39	34	3.52	4.62	0.43	1.38
	500	1.86	39	51	35	4.16	4.57	0.34	1.45
CV (%)		21.3	11	13.1	6.4	6.4	3.3	7.9	5.0
F test*		**	**	**	*	*	**	**	**
LSD0.05		0.7	6.34	8.86	0.42	0.42	0.26	0.05	0.11

* F test: ** = Significant at (P =0.01) level, * = Significant at (P =0.05) level, n.s. = not significant at (P =0.05) level

In contrast to Fe fertilization, increasing soil N fertilization enhanced shoot and grain Fe concentrations.When the plant N status improved by soil N application, grain concentration of Fe increased by up to 47%, whereas the application of Fe fertilizers either in inorganic ($FeSO_4$) or chelated form (e.g., Fe-EDTA, Fe-EDDHA or Fe-citrate) had only a small positive impact. At a given Fe treatment, the high soil N supply enhanced shoot Fe concentrations by up to 70% (Fig. 1). There are controversial results

in the literature regarding the effectiveness of foliar Fe fertilizers in improving Fe concentration of cereal grains. In wheat, foliar spray of $FeSO_4$ improved grain Fe concentration by about 28% in China (Zhang et al. 2010) and 21% in Iran, (Pahlavan-Rad and Pessarakli 2009) whereas in Canada foliar Fe fertilizers did not influence grain Fe concentrations (Gupta, 1991). In the present study, application of Fe fertilizers increased grain Fe concentration by about 14% with FeEDTA and 10% with $FeSO_4$, averaged over the N treatments (Table 2.1). The plant N status might have been one reason for such differential effects of foliar Fe fertilization on grain Fe concentration. At a given N treatment, foliar Fe applications resulted in decreased grain Zn concentration, especially with the FeEDTA treatment (Table 2.2). Although the extent of the decreases in grain Zn concentration by foliar Fe spray was not large, this effect should be considered as undesirable influence of foliar Fe spray on grain Zn.

Given that the enhanced N supply also promoted dry matter production, high N nutrition resulted in substantial increases in total amount of Fe per shoot (e.g., shoot Fe content) (Fig. 2.1). Marked increases in shoot content of Fe and Zn by increased N supply have also been shown by Kutman et al. (2010) in wheat. It can be, therefore, suggested that enhancement in Fe and Zn tissue concentrations by increasing N supply could be, at least in part, due to a growthenhancement effect of N. Nitrogen nutrition may also have positive impacts on accumulation of Fe and Zn besides its effect on growth. In the study presented here, the relative increase in shoot Fe content by N was around 125% averaged over all Fe treatments (Fig. 2.1), whereas an increase in shoot dry matter production by N was only 42% (Table 2.1). Shoot Zn content was also similarly enhanced by N application. Considering much greater increases in shoot Fe accumulation (about 3-fold) than the shoot dry matter accumulation by N, it is suggested that increases in shoot growth caused by increased N application can not be a major reason for increased shoot content of Fe (and Zn). In addition, as discussed below, the positive effects of increasing N supply on Fe and Zn concentrations or contents were much less in the case of P and for some cationic nutrients. It appears likely that the mechanisms contributing to root uptake and root-to-shoot transport of Fe and Zn are positively affected by improving plant N status.

In this study, N was supplied as $Ca(NO_3)_2$. It has been well documented that increasing N supply in the form of nitrate stimulates root uptake and shoot transport of cationic nutrients in order to balance excess anionic charges from uptake and assimilation of nitrate (Kirkby and Knight 1977; Marschner, 1995). It is, therefore, likely that enhanced nitrate nutrition promoted root uptake and shoot transport of Fe to contribute to the charge balance in shoot tissues, even though the role of Fe, as a micronutrient, in charge balance should be proportionally much less than for cationic macronutrients. Indeed, an increase in nitrate nutrition in the present study greatly enhanced shoot concentration of cationic macronutrients, especially Ca (up to 100% increase). Part of this increase in shoot Ca by N can be ascribed to increasing Ca supply together with soil N application in the form of $Ca(NO_3)_2$. In a recent study, increases in shoot accumulation of Zn in a Zn-hyperaccumulator Noccaea caerulescens by nitrate nutrition has been ascribed to cellular balance of excess anionic charges resulting from nitrate nutrition (Monsant et al. 2010). In the present study, the positive impact of high N supply on concentration of cationic macronutrients in the grain tissue (Table 2.2) was, however, less pronounced than in the shoot tissue (Table 2.1), especially for Ca and K. Due to its poor phloem mobility, Ca is known to be predominantly deposited in vegetative tissues in maturing plants (Marschner, 1995). This might be one plausible reason for the less influence of N on grain Ca concentration when compared to the shoot tissue. Similarly, a positive impact of high N nutrition on tissue K concentration was lower in grain (e.g., 7% increases) than shoot (e.g., 40% increase). Little influence of N nutrition on grain K concentration might be related to preferential accumulation of K in leaf and stem tissues when compared to the grain tissue (Tables 2.1 and 2.2; Marschner 1995; Fageria et al. 2010).

It seems likely that there are mechanisms other than charge balance that may contribute to higher accumulation of Fe (and Zn) in shoot and grain as a consequence of the improved plant N status. Recently, nitrate-induced root uptake and root-to-shoot transport of Zn were shown in durum wheat plants, and this nitrate effect was ascribed to elevated pools and activity of transporter proteins and nitrogenous compounds (e.g., nicotianamine) maintaining root uptake and shoot translocation of Zn (Kutman et al. 2010; Erenoglu et al. 2011). Probably, high N nutrition increases activity and abundance of Fe transporter proteins such as yellow stripe 1 (YS1) in root cell membranes (Murata

et al. 2008; Curie et al. 2009), which positively affects root uptake and shoot transport of Fe. Improving the plant N status may have also a significant impact on biosynthesis and release of PS from roots by increasing the amount of nitrogenous substrates and activity of enzymes contributing to PS biosynthesis, such as nicotianamine (NA) and NA-synthase (Mori and Nishizawa 1987; Haydon and Cobbett 2007). Accordingly, in a recently published paper it has been shown that root release of PS, mobilization of Fe from Fe-hydroxide and root uptake and shoot translocation of Fe(III)-PS by the wheat genotype used in the present study were markedly enhanced by improving N nutritional status of plants (Aciksoz et al. 2011a).

Nitrogen nutritional status of wheat plants has also a significant impact on allocation of Fe into grain. When plants were supplied well with N, 60% of the total shoot Fe was allocated into grain, whereas this value was 38% in the plants with low N supply (Kutman et al. 2011). Similarly, Zn allocation into grain was promoted by the high N supply. Various nitrogenous compounds have been discussed in the literature in terms of contribution to phloem loading, long-distance transport and seed deposition of Fe, such as amino acids, nicotianamine and peptides (Grusak et al. 1999; Borg et al. 2009; Curie et al. 2009). Evidence is also available in the literature showing that raising grain protein concentration increases a storage capacity for Fe and Zn (Cakmak et al. 1994, 2010a; Kutman et al. 2010). Positive correlations between Fe, Zn and protein concentrations in grains of different wheat genotypes (Peterson et al. 1986; Zebarth et al. 1992; Zhao et al. 2009; Gomez-Becerra et al. 2010) support the idea that the size of grain capacity for accumulation of Fe and Zn is largely influenced by the amount of grain protein. These results and observations support the suggestion that increase in plant growth by N supply cannot be a major reason for the increased concentration and content of Fe (and Zn) of plants found in this study.

The results presented in Table 1.3 showed that grain Fe concentrations might be increased due to foliar Fe fertilization when Fe fertilizers were sprayed together with 1% (w/v) urea. To our knowledge, this is the first report showing a positive impact of urea on grain Fe concentration when sprayed onto foliage together with Fe fertilizers. The mechanism behind this positive impact of urea on allocation of Fe into grain is not well understood. In a previous study, it was shown that leaf absorption of Fe from Fe-

labelled $FeSO_4$ was strongly stimulated in tomato plants when $FeSO_4$ and urea had been supplied together (Wittwer et al. 1967). In soybean plants, inclusion of urea in the foliar spray of FeIDHA (Fe-iminodisuccinic acid) resulted in better re-greening (Rodriguez-Lucena et al. 2010b). It is believed that urea facilitates the cuticular penetration of foliarly-sprayed ions (Yamada et al. 1965; Swietlik and Faust 1984). By increasing the plant N status (e.g., grain N concentration, Table 2.3), use of urea together with foliar Fe fertilizers may also promote phloem transport and seed deposition of Fe as discussed above. Additional studies are required to increase understanding of the urea effect on leaf absorption and grain accumulation of the foliarly-sprayed Fe.

Table 2.3. Changes in grain yield and grain N and Fe concentrations in plants treated by various foliar Fe fertilizers with and without 1% (w/v) urea in the spray solution. Foliar sprays of Fe fertilizers were done at the booting and early milk stages. All Fe fertilizer sprays contained the same amount of Fe that was present in the 0.25% (w/v) FeEDTA. Values are means of three independent replicates.

Foliar applications	Grain yield	Grain concentration	
		N	Fe
	($g\ plant^{-1}$)	($g\ kg^{-1}$)	($mg\ kg^{-1}$)
Control	2.71	37	36
Control + Urea	3.34	38	36
FeSO4	2.73	38	38
FeSO4 + Urea	2.69	41	43
FeEDTA	3.07	35	38
FeEDTA + Urea	3.38	36	42
FeEDDHA	3.11	36	35
FeEDDHA + Urea	2.61	39	39
Fe Citrate	2.54	37	36
Fe Citrate + Urea	2.91	39	37
CV (%)	18.5	4.7	8.1
F test*	n.s.	*	*
$LSD_{0.05}$	-	3	5

* F test: ** = Significant at (P =0.01) level
* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

The results presented here may have important timplications for human nutrition. Iron deficiency problem is a growing public health problem in human populations, associated with reduced dietary Fe intake (Bouis and Welch 2010; Cakmak et al.

2010a). There is an urgent need for improving Fe concentrations in food crops to minimize Fe-deficiency related health problems in human populations. Based on the results presented in this study, it can be suggested that N fertilizer management and spraying Fe together with urea may represent important agronomic practices to contribute to increasing grain Fe (and Zn) concentrations in food crops. The plant N status deserves a special attention in efforts to biofortify food crops with Fe and Zn.

CHAPTER 3

INCLUSION OF UREA IN THE FOLIAR ^{59}FeEDTA SOLUTION STIMULATED LEAF PENETRATION AND TRANSLOCATION OF ^{59}Fe IN WHEAT

3.1. Abstract

Increasing iron (Fe) concentration of food crops by using agricultural tools represents an important approach to improving dietary intake of Fe. As an agricultural practice, foliar application of Fe fertilizers remains, however, inadequate in increasing grain Fe. In this study, role of urea in translocation of ^{59}Fe from the ^{59}FeEDTA-treated leaves was studied in durum wheat (*Triticum durum* L.) which was grown for 2 weeks in nutrient solution or until grain maturation in soil culture. Five-cm long tips of the first leaf of the young wheat seedlings or the flag leaves at the early milk stage were immersed daily for 10 second in the ^{59}FeEDTA solutions containing increasing amounts of urea (0, 0.2, 0.4, 0.8% w/v) over 5 days. In the experiment with young wheat seedlings, urea inclusion in the ^{59}Fe-EDTA treatment significantly increased translocation of ^{59}Fe from the treated leaf into roots and the untreated part of shoots. When the ^{59}Fe-treated leaves were induced into senescence by keeping them in the dark, there was strong ^{59}Fe translocation from these leaves. Adding urea to the ^{59}Fe solution did not result in an additional increase in Fe translocation from the dark-induced senescent leaves. In the experiment conducted in soil culture until grain matuıration, translocation of ^{59}Fe from the flag leaves into grains was also strongly promoted by urea, whereas ^{59}Fe translocation from flag leaves into untreated shoot was very low, and not affected by urea. The urea treatments resulted in an increase in soluble free amino

acids in the treated leaf sections. In conclusion, urea contributes to transportation of the leaf-absorbed Fe into sink organs. Probably, the nitrogenous compounds formed after assimilation of foliarly-applied urea (such as amino acids) contributed to Fe chelation and translocation in wheat.

3.2. Introduction

Iron (Fe) deficiency is a well-documented problem in cultivated soils and responsible for impairments in yield capacity and nutritional quality of crop plants, especially in alkaline soils (Borg et al. 2009; Walker and Waters 2011). Plants growing in soils with limited availability of Fe are not able to accumulate adequate amounts of Fe in edible parts, leading to reductions in dietary intake of Fe in human populations that rely on staple food crops, such as cereals (Rengel et al. 1999; White and Broadley 2009). Iron deficiency problem in human populations represents a global micronutrient deficiency disorder and is associated with serious health complications such as anemia, birth defects and impairments in cognitive development and function (Carter et al. 2010; Gibson et al. 2010)

In most cases, cereals develop adaptive root responses to improve solubility and root uptake of Fe, including root exudation of Fe-mobilizing phytosiderophores (Marschner et al. 1986; Murata et al. 2006) .Phytosiderophores are highly effective in chelation, transportation and subsequent uptake of poorly soluble Fe compounds in soils and correction of Fe deficiency chlorosis (Treeby et al. 1989; Takahashi et al. 2001). However, a role of root-adaptive responses in increasing grain Fe concentrations beyond the amounts needed for high grain production is not clear. In case of genetic engineering, excellent examples are available, showing that over expression of target genes controlling uptake, transport and seed deposition of Fe is associated with impressive increases in seed concentrations of Fe (Uauy et al. 2006b; Conte and Walker 2011; Waters and Sankaran 2010). However, the issue with changes in grain yield capacity of those transgenic lines deserves careful consideration (Cakmak, 2008; Zhao et al. 2009). It is also important to elaborate the need for the application of

micronutrient fertilizers to those transgenic lines in order to maintain their genetic capacity for accumulation of targeted micronutrients in their seeds when grown under field conditions.

Cereal grains are inherently low in concentrations of total and bioavailable Fe to meet human requirements. The most common range of Fe concentrations in wheat is estimated to be between 25 and 35 mg kg^{-1} (Rengel et al. 1999; Cakmak et al. 2010a). In order to achieve significant measurable health effects, grain Fe concentrations should be more than 50 mg kg^{-1} (Graham et al. 2007). Application of soil and/or Fe fertilizers might be a solution to improving Fe concentrations of cereal grains. However, when applied to soils, Fe is often rapidly converted to poorly soluble forms, and its acquisition by roots is limited (Marschner and Romheld 1994; Rengel et al. 1999). Foliar applications of Fe fertilizers also remain less effective in terms of increasing grain Fe concentrations, probably due the poor penetration through the leaf cell walls and limited phloem transportation of Fe (Grusak et al. 1999; Rengel et al. 1999). In case of foliar sprays of Zn fertilizers, grain Zn is increased up to 3-fold (Cakmak, 2008; Cakmak et al. 2010b), whereas foliar sprays of inorganic or chelated Fe fertilizers has little impact on grain Fe (Gupta, 1991; Zhang et al. 2010; Aciksoz et al. 2011b). However, adding urea to inorganic or chelated forms of foliar Fe fertilizers at 1 % (*w/v*) had a positive impact on increasing grain Fe concentrations in wheat (Aciksoz et al. 2011b).

The mechanism by which urea improves foliar absorption and grain accumulation of Fe is not well understood. One possible explanation could be due to improving N nutritional status of plants (e.g., higher leaf or grain tissue protein concentrations) by spraying Fe fertilizers together with urea (Aciksoz et al. 2011b). Published data show that enhancements in the N nutritional status of plants result in significant increases in root uptake, transport and grain allocation of Fe (Kutman et al. 2010; Shi et al. 2010; Aciksoz et al. 2011b). In addition, urea is known to stimulate penetration of leaf-sprayed nutrients and pesticides through the leaf cuticula (Swietlik and Faust, 1984; Weinbaum, 1988; Bowman and Paul 1992). Previously it was shown that urea stimulated cuticular penetration of Fe in different plants (Kannan and Wittwer 1965; Wittwer et al. 1967). In industrial pharmacy and dermatology, urea is often applied as

penetration enhancer and acts as a facilitator for skin or tissue absorption of several compounds (Godwin et al. 1998; Ferrante et al. 2010). Urea is reported to easily permeate through the cuticular membranes (10 to 20 times better than inorganic ions) (Wojcik, 2004). The rate of urea penetration depends on how rapidly it is metabolized and assimilated into amino acids in the leaf tissue. Data are available showing that urea is rapidly absorbed by leaf tissue and converted to amino acids that are transported from the treated leaves (Dong et al. 2002). Amino acids produced right after urea absorption might be important in absorption and translocation of Fe.

To our knowledge, there is no published data about the impact of leaf-applied urea on translocation (partitioning) of the leaf-absorbed Fe in the whole plant. In the study conducted by Aciksoz et al (2011b) changes in grain Fe concentrations were studied in wheat plants which were sprayed by various Fe fertilizers with and without inclusion of 1 % urea. In the present work, 5 cm-leaf tips were immersed in a ^{59}Fe-labeled FeEDTA solution containing increasing amount of urea to monitor leaf absorption and translocation of ^{59}Fe in both young wheat seedlings and mature wheat plants. In case of young and mature wheat plants, the first leaf and the flag leaves were treated with ^{59}Fe-labeled FeEDTA solution containing varied concentrations of urea, respectively.

3.3. Materials and methods

Two solution culture and one soil culture experiments were conducted using durum wheat (*Triticum durum* cv. Balcali 2000) to study the effect of increasing urea concentrations in foliarly-applied FeEDTA solutions labeled with Fe-^{59} on translocation of Fe from the treated leaves to other plant parts such as grains and roots.

3.3.1. Plant Growth

Seeds of durum wheat were germinated in perlite moistened with saturated $CaSO_4$ for 5 days. After the seedling emergence, 10 plants were transferred to 2.7-L nutrient

solution in black plastic pots; 2 days after the transfer, seedlings were thinned to 4 plants per pot. After this pre-culture for 7 days, each individual seedling was used for the treatment with ^{59}FeEDTA treatments as described below. The nutrient solutions were continuously aerated and had the following composition: 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.9 mM K_2SO_4, 0.2 mM KH_2PO_4, 0.5 mM $MgSO_4.7H_2O$, 0.1 mM KCl, 1 µM $ZnSO_4$, 0.1 µM H_3BO_3, 0.5 µM $MnSO_4.H_2O$, 0.2 µM $CuSO_4.5H_2O$, and 0.14 µM $(NH_4)6Mo_7O_{24}.4H_2O$. All plants were treated with a low supply of Fe (i.e. 2 µM FeEDTA). The plants were grown in a growth chamber under controlled climatic conditions (e.g., light/dark regimes of 16/8 h at 22/18 °C, 60/70% relative humidity). The light intensity during the light cycle was 400 µmol m^{-2} s^{-1}. Nutrient solutions were renewed every 3 to 4 days.

3.3.2. Experiment 1: Treatments of Leaf Tips with FeEDTA

The first translocation experiment was performed on young wheat plants grown in nutrient solution to test the effect of urea inclusion in the Fe-labeled FeEDTA solution on translocation of Fe from the oldest leaf of the seedlings to the reminder of the shoot and roots. Leaf application of the Fe was started on the intact first leaves of 7- days old seedlings. Five cm long leaf tips were immersed into 0.1 % Fe-EDTA solution labeled with 925 kBq Fe containing increasing urea concentrations of 0, 0.2, 0.4 and 0.8 % (*w/v*). Leaf tips were immersed in the treatment solution for 10 s, once early in the morning, once in the late afternoon. The foliar treatment has been repeated every day over 5 days of period. Treatment solutions contained Tween-20 as a wetting surfactant at a concentration of 100 mg L^{-1}.

3.3.3. Experiment 2: Fe Translocation from Senesced Leaves

In this experiment, the treatment of the 5-cm-long leaf tips with ^{59}FeEDTA as well as the growth chamber conditions were the same as described above. For the senescence treatment, the ^{59}FeEDTA-treated leaves were covered by using aluminum foil for 5 days

during the ^{59}FeEDTA treatment period to induce leaf senescence. The remaining plant parts were kept uncovered. At the end of the dark-induced senescence period, the aluminum-covered leaves were chlorotic and analyzed for chlorophyll concentrations (see below).

3.3.4. Experiment 3: Fe Translocation into Grain

For the greenhouse experiment, the seeds of the same durum wheat cultivar were sown in plastic pots containing 3.1 kg of soil that was collected from a Zn-deficient location in Central Anatolia. The soil used was calcareous (18% w/w $CaCO_3$) and alkaline (pH 8.0 in dH_2O) with clayey loam texture and low organic matter content (15 g/kg) (Cakmak et al. 1996). The diethylenetriamine pentaacetic acid (DTPA)-extractable Fe concentration was 2.1 mg kg^{-1} soil, measured by using the method described by Lindsay and Norvell (1978). Before potting, following basal nutrients (per kg dry soil) were homogenously incorporated in the soil: 250 mg nitrogen (N) in the form of $Ca(NO_3)_2.4H_2O$,100 mg phosphorus (P) as KH_2PO_4, 25 mg sulfur (S) as K_2SO_4 and 2 mg Zn as $ZnSO_4$ $7H_2O$. No Fe was applied. During the heading stage a second 250 mg N in the form of $Ca(NO_3)_2.4H_2O$ per kg soil was applied to all pots. Ten seeds were sown in each pot, and pots were thinned to 3 per pot. All tillers except main stem were removed at the booting stage to study the ^{59}Fe transport from flag leaves to the main spike in order to eliminate competing sink activity.The subsequently developing new tillers were also removed. The plants were harvested at full grain maturity.

Application of ^{59}FeEDTA labeled with 925 kBq ^{59}Fe was done on the intact flag leaf of the main stem as described above for the young wheat leaves. Five-cm long tips of the flag leaves were immersed in solutions containing ^{59}FeEDTA (0.1% *w/v*) and urea at concentrations of 0, 0.2, 0.4 or 0.8% (*w/v*) for 10 s twice daily over 5 days period during the early milk stage. At grain maturation, the ^{59}Fe-treated flag leaves were washed first in deionized water for 3 min and then with 10 mM $CaSO_4$ solution. The treated flag leaves, the rest of shoot (straw) parts and grains of the experimental plants were separately harvested and subjected to analysis of ^{59}Fe activity by using a Perkin

Elmer 2480 WIZARD2 Automatic Gamma Counter. Each single plant has been separately harvested and analyzed.

3.3.5. Measurement of Free Amino Acids

The urea-treated leaf sections were extracted in 0.2 M sodium citrate buffer (pH 2.2). Following centrifugation at 15,000 *g* for 30 min samples were filtered through syringe-tip filters with polyethersulfone (PES) membrane (0.22 μm pore size). All samples and standards were analyzed by an automated amino acid analyzer (Biochrom 32 Oxidised Hydrolysate System, Biochrom Ltd., Cambridge, U.K.) with post-column ninhydrin derivatization as described in Kabaha et al. (2011). The results were expressed as mg of total free amino acids per g of fresh leaf material.

3.3.6. Analysis of Fe and N

For measurement of Fe and N, the leaf and grain samples were dried at 70ºC and 45ºC, respectively, and were then subjected to acid digestion in a closed microwave system (MarsExpress; CEM Corp., Matthews, NC, USA) by using 1 mL of 30% v/v H_2O_2 and 5 mL of 65% v/v HNO_3. Iron concentration of the digested samples was determined by ICP-OES (inductively coupled plasma optical emission spectrometry) (Vista-Pro Axial; Varian Pty Ltd., Mulgrave, Australia.) Measurement of N concentration in samples was done by a LECO TruSpec C/N Analyzer (Leco Corp., St Joseph, MI, USA). Measurements were checked against the certified standard reference materials obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA).

Data analysis was conducted by JPM software, and comparison of means was performed by using the Student's *t* test, whenever ANOVA (using general linear model) indicated a significant treatment effect.

3.4. Results

3.4.1. Experiment 1

In the first experiment, young wheat plants were used. The distribution of total ^{59}Fe (as a percentage per organ) as dependent on urea concentration in the treatment solution is presented in Table 3.1. The ^{59}Fe retranslocation from the first (oldest) leaf to the remainder of shoot was 6.5% without urea treatment. Supplying 0.2% w/v urea in the treatment solution significantly enhanced translocation to 13.3% from 6.5 %. Additional increases in urea concentration further improved the translocation of ^{59}Fe to the shoot, but not significantly. Irrespective of the urea treatment, greater amounts of retranslocated ^{59}Fe were found in the treated part of the leaf and the remainder of shoot compared to roots.

The experimental plants were supplied well with N in the growth medium and had sufficient leaf N concentrations (e.g., 55 g kg^{-1} N). Accordingly, the shoot and root dry weights were not influenced by the short-term urea treatments (Table 3.1). Similarly, short-term feeding of leaf tips with urea did not cause any change in total N concentration of shoots and roots (data not shown).

3.4.2. Experiment 2

In the second experiment, dark-induced leaf senescence was achieved by covering the treated leaves with aluminum foil for 5 days during the treatment with ^{59}Fe solution (Table 2). As found in the previous experiment (Table 3.1), inclusion of urea at the rate of 0.8% (w/v) in the ^{59}Fe treatment solution significantly increased absorption and translocation of ^{59}Fe (Table 3.2).

Table 3.1. Effect of increasing concentration of urea on relative distribution of ^{59}Fe from the treated leaf to the remainder of shoot and the roots of 12-day-old durum wheat (*Triticum durum* cv. Balcali 2000) plants grown in nutrient solution (experiment 1). Immersion of the 5-cm-long tips of the first leaf into $^{59}FeEDTA$ solution (containing 0.1% FeEDTA, w/v) was performed daily for 10 seconds. The leaf treatment with the $^{59}FeEDTA$ solution started when the plants were 7 days old and was repeated daily for 5 days. Data represent means of twelve replicates with one seedling.

	Relative distribution of the absorbed ^{59}Fe				
Urea concentration in ^{59}Fe solution	Treated leaf	Remainder of shoot	Root	Dry weight	
				Shoot	Root
(w/v%)		(%)		(mg plant^{-1})	
0	89.8 a	6.5 b	3.7 b	133	69
0.2	79.8 b	13.3 a	6.5 a	115	65
0.4	81.7 b	13.7 a	4.9 b	125	71
0.8	79.3 b	15.7 a	4.9 b	125	74
CV (%)	6.4	36.3	24	21.9	23
F test*	**	**	**	n.s.	n.s.
$LSD_{(0.05)}$	6.3	5.4	1.4	-	-

* F test: ** = Significant at (*P*=0.01) level

* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

When the treated leaves were senescent by keeping under dark conditions and without urea treatment, there was a significant enhancement in ^{59}Fe translocation from the senescent-leaves, when compared to the non-senescent leaves. The stimulated Fe transport through senescence tended to be higher with the urea inclusion; but, this urea effect was not significant (Table 3.2).

Table 3.2. Relative distribution of ^{59}Fe in the treated leaf, remainder of shoot, and root of 12-day-old wheat seedlings (*Triticum durum,* cv. Balcali 2000) as dependent on senescence of the $^{59}FeEDTA$- treated leaf and inclusion of urea in the treatment solution (experiment 2). Immersion of the 5-cm-long tips of the first leaf into $^{59}FeEDTA$ solution (containing 0.1% FeEDTA, w/v) was performed daily for 10 seconds. The leaf treatment with the $^{59}FeEDTA$ solution started when the plants were 7 days old and was repeated daily for 5 days. Senescence of the ^{59}Fe-treated leaf was induced by covering it with aluminum foil for the duration of the ^{59}Fe treatment. Data represent means of twelve replicates with one seedling.

		Relative distribution of the absorbed ^{59}Fe		
Dark induced senescence	Urea concentration	Treated	Remainder of shoot	Root
	(% w/v)		(%)	
	0	85	8.7	6
No	0.8	69	23.3	10
	0	68	23	8.8
Yes	0.8	64	28.7	8.8
CV (%)		12.1	34.4	30.0
F test		**	**	**
$LSD_{0.05}$		7.1	5.9	2.1

* F test: ** = Significant at (*P*=0.01) level

* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

The differential urea and senescence treatments did not have a significant effect on the shoot and root dry matter production (Table 3.3). As expected there was a distinct decrease in chlorophyll concentration in the covered leaves (Table 3.2).

Leaves treated with and without 0.8% w/v urea were analyzed for total amount of free amino acids. The concentration of free amino acids increased non-significantly (about 13%) in the urea-treated leaf parts with respect to comparable non-treated leaf sections (Table 3.4).

Table 3.3. Shoot and root dry weights and leaf chlorophyll (SPAD values) of 12-day-old wheat seedlings grown in nutrient solution (experiment 2). Data represent means of 6 replicates.

Dark induced senescence	Urea concentration	Dry weight		Chlorophyll
		Shoot	Root	
	(% w/v)	(mg plant^{-1})		(SPAD)
	0	134	55	27.2
No	0.8	144	62	29.6
	0	136	52	7
Yes	0.8	126	49	7.1
CV (%)		28.1	31	16.3
F test		n.s.	n.s.	**
LSD $_{0.05}$		-	-	1.6

* F test: ** = Significant at (*P*=0.01) level
* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

Table 3.4. Changes in total free amino acids in urea treated leaf parts of 12-days old durum wheat plants. Five cm-long tips of the first leaves of young durum wheat plants were daily immersed daily twice in the 0.8% urea solution 10 seconds over 5 days of period. Data represent means of three independent replicates.

Leaf Treatment	Free amino acids
	(mg g^{-1} fresh wt.)
Without urea	7.88
With urea (0.8 %)	8.93
CV (%)	6.8
LSD $_{(0.05)}$	-
F test	n.s.

* F test: ** = Significant at (*P*=0.01) level
* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

3.4.3. Experiment 3

In the soil experiment, the impact of urea on ^{59}Fe retranslocation was studied during the generative period. Intact flag leaves of the plants at early milk stage were immersed in the ^{59}Fe solutions containing increasing amount of urea from 0 to 0.8 % (Table 3.5). Although it was not statistically significant, inclusion of urea at a rate of 0.2% w/v in the ^{59}Fe solution increased the ^{59}Fe activity in grains by about 2-fold. When the amount of urea in the treatment solution increased from 0 to 0.4 and 0.8%, ^{59}Fe activity of the grains very significantly enhanced (Table 3.5). Similar to grains, shoot ^{59}Fe activities were also enhanced by the treatment, but the amount of ^{59}Fe transported into shoot parts (excluding a flag leaf) was much lower than the amounts found in grains (Table 3.5). As expected, the level of ^{59}Fe activity was the highest in the ^{59}Fe-treated leaves, and increases in urea concentration in the treatment solution further increased ^{59}Fe activity in the treated flag leaves.

Table 3.5. Effect of increasing urea concentration in the ^{59}FeEDTA treatment solution on the activity of ^{59}Fe in the treated flag leaves, shoot (straw) and grain, and on the relative distribution of absorbed ^{59}Fe to the grain and straw of the mature durum wheat plants (experiment 3). Immersion of the 5-cm-long tips of the flag leaf into ^{59}FeEDTA solution (containing 0.1% FeEDTA, w/v) was performed daily for 10 seconds over 5 days of period, and started at the beginning of the early milk stage.Data represent means of twelve replicates with one seedling.

Urea concentration in ^{59}FeEDTA solution	^{59}Fe activity			Relative proportion of absorbed ^{59}Fe	
	Treated flag leaf	Remainder of shoot	Grain	Grain	Shoot
(% w/v)		(CPM)		(%)	
0	3224	71	265	8.8	2.4
0.2	2929	62	584	22.2	2.1
0.4	4494	93	1485	34.6	2.2
0.8	7507	116	1907	28.1	1.6
CV (%)	27	29.8	37.3	41.6	39.9
F test *	**	**	**	**	n.s.
$LSD_{0.05}$	608	27	456	11.6	-

* F test: ** = Significant at (*P*=0.01) level

* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

The ^{59}Fe-mobilization ratio calculated by dividing ^{59}Fe activity in grains or shoots with the values in the flag leaves was higher in grains than shoots (Table 3.5). Inclusion of urea in the immersing solution resulted in significant increases in ^{59}Fe mobilization from flag leaves into grains, especially at the 0.4% urea treatment. In case of shoots, urea treatment did not have any significant effect. Under given experimental conditions, short-term urea treatments did not alter shoot dry matter production and grain yield (Table 3.6).

Table 3.6. Effect of leaf applied radiolabeled Fe on grain yield and shoot dry weight of durum wheat (*Triticum durum* cv. Balcali 2000) at maturity (experiment 3).

Urea concentraton in ^{59}Fe solution	Dry weight Shoot	Grain
(% w/v)	(g plant^{-1})	
0	2.79	1.31
0.2	2.53	1.08
0.4	2.59	1.13
0.8	2.51	1.14
CV (%)	16.5	26.1
F test*	n.s.	n.s.
$LSD_{0.05}$	-	-

* F test: ** = Significant at (P=0.01) level
* = Significant at (P=0.05) level, n.s. = not significant at (P=0.05) level

3.5. Discussion

This study demonstrated that urea inclusion in the foliar Fe-EDTA treatment solution significantly improved leaf penetration and translocation of Fe from the treated leaf at both the early (Table 1) and the reproductive growth stage (Table 5). When applied during the reproductive growth stage, the proportion of ^{59}Fe found in grains increased from 8.8 to 34.6% by including urea in the treatment solution, but such an enhancing urea effect was not observed for ^{59}Fe translocation into shoots (Table 5). This result implies that transport of leaf-applied Fe into grains is, at least partly, a sink-driven process because developing grains are better competitors than shoots at the reproductive growth stage (Marschner, 2012). Indeed, wheat shoots (straw) are rather a source of micronutrients during seed formation (Garnett and Graham 2005; Grusak et al. 1999; Kutman et al. 2011; Pearson et al. 1995).

Urea facilitated penetration of Fe into and translocation from the treated leaf to the other plant parts. The result with the urea- promoted penetration of Fe into the leaf is in good agreement with the published reports (Swietlik and Faust 1984; Rodriguez-Lucena et al. 2010). In tomato plants, leaf absorption of ^{59}Fe from ^{59}Fe-labeled $FeSO_4$ was strongly stimulated when $FeSO_4$ and urea were supplied together (Wittwer et al. 1967). Similarly, re-greening of Fe-deficient chlorotic leaves was better when urea was added to the foliar Fe fertilizers (Rodriguez-Lucena et al. 2010). In durum wheat, spraying various Fe fertilizers to plants at booting and milk stage resulted in significant increases in grain Fe concentrations when foliar Fe fertilizers were applied together with urea (Aciksoz et al. 2011b). The study by Aciksoz et al (2011a) together with the results presented in this paper indicates that urea inclusion is highly effective in translocation of leaf-absorbed Fe into roots and seeds.

One possible explanation of the positive effect of urea on Fe translocation would be related to N (and protein) status of plants. Increasing evidence is available showing a positive impact of improved N nutritional status on shoot and grain concentrations of Fe and also Zn. Enhancements in N supply were found to promote root uptake and particularly translocation of Zn and Fe in plants. In short-term experiments with ^{65}Zn in

durum wheat seedlings, Erenoglu et al. (2011) showed that increases in N supply in growth medium enhanced root-to-shoot translocation of ^{65}Zn more than the root uptake of ^{65}Zn. Similarly, in an experiment using ^{59}Fe-complexed phytosiderophore (^{59}Fe-PS) increasing N supply to wheat plants had a greater impact on the root-to-shoot translocation than the root uptake of ^{59}Fe-PS (Aciksoz et al. 2011a). Growing plants under increasing N supply also promoted retranslocation of Fe from vegetative tissues into grain (Kutman et al. 2011).

Given that transport of Fe in plants occurs in a chelated form (Marschner, 2012; Von Wiren et al. 1999), it can be suggested that leaf urea treatments may have an important role in chelation and phloem transport of the leaf-absorbed Fe. Diverse nitrogenous compounds (such as amino acids, phytosiderophores, nicotianamine and peptides) have been proposed to contribute to transport and phloem loading of Fe (Grusak et al. 1999; Haydon and Cobbett 2007; Kruger et al. 2002). The pool of those nitrogenous compounds might be positively affected by the urea treatments, at least in the urea-treated leaf sections. In addition, possible increases in grain N (and protein) concentration through urea treatments may also increase seed strength for Fe transport. Higher level of seed protein is believed to attract Fe transportation from vegetative tissues into seeds by creating storage and binding capacity for Fe (Cakmak et al. 2010a; Kutman et al. 2011; Waters and Sankaran, 2011).

A quick absorption and assimilation of foliar-applied urea is well-documented in the literature (Klein and Weinbaum, 1984; Nicoulaud and Bloom 1996; Dong et al., 2002). In the present study, the short-term urea treatments of the flag leaves were very short and not able to affect grain N (protein) concentrations. Similarly, also short-term feeding of leaf tips in young wheat plants did not cause measurable change in total concentration of N both in roots and reminder part of shoots. There was, however, an increase in concentration of soluble amino acids in the treated section of the flag leaves (Table 4). Treatment of leaf tips with 0.8% urea resulted in about 13% increase in soluble amount of amino acids in the treated sections of the leaves. Similarly, quick increases in concentrations of free amino acids in response to foliar urea applications were reported in young apple trees (Dong et al. 2002), tomato plants (Bal and Bloom 1996), and peach trees (Rosecrance et al. 1998). According to Dong et al. (2002)

conversion of urea into amino acids takes place in sprayed leaves, and then transport of N occurs in the form of amino acids.

In the present study, the nitrogenous compounds formed right after assimilation of foliarly-applied urea such as amino acids and amines might have contributed to chelation and translocation of Fe in wheat plants. Published data show that nicotianamine is a key compound in phloem translocation of Fe in plants (von Wiren et al. 1999; Takahashi et al. 2003; Trampczynska et al. 2010). Over-expression of nicotianamine synthase is associated with high levels of nicotianamine and accumulation of Fe in seeds, indicating a important role of nicotianamine in Fe transport in plants (Lee et al. 2012; Usuda et al. 2009). Hence, in the present study, nicotianamine and/or other related Fe-chelating nitrogenous compounds (such as amino acids and amines) produced during the urea assimilation in leaves might have contributed to Fe transport to grain (Table 4) and roots (Table 1). In the future, concentration of individual amino acids and amines should be measured in the urea-treated wheat plants, and their relation to Fe transport should be evaluated.

Leaf senescence is known to be associated with transport of most mineral nutrients, especially micronutrients, into seeds (Marschner, 2012). Dark-induced leaf senescence is highly effective in stimulating Fe and Zn translocation from the senescing leaf tissue to other shoot parts (Zhang et al. 1995; Erenoglu et al. 2011). It is likely that amino acids are involved in transportation of Fe and Zn through phloem. Leaf senescence increases concentrations of free amino acids in affected tissues as well as the phloem sap (Caputo et al. 2001; Soudry et al. 2005; Couturier et al. 2010). Remobilization of Fe and Zn from leaf tissues into wheat grain is closely associated with leaf senescence and increased concentration of free amino acids in senescing leaves (Kade et al. 2005). The NAC transcription factor regulates leaf senescence in wheat and contributes greatly to grain Zn and Fe concentrations as well as protein (Uauy et al. 2006a; Cantu et al., 2011). Down-regulation of the NAC transcription factor delayed senescence and simultaneously reduced grain Zn and Fe concentrations (Uauy et al. 2006a; Waters et al. 2009). A positive impact of leaf senescence on Fe translocation was also shown in the present study (Table 2). When urea was not included in the ^{59}Fe treatment solution, dark-induced senescence increased ^{59}Fe transport

from 8.7 to 23% to the remainder of shoots and from 6 to 8.8% to roots. The urea inclusion in the ^{59}Fe treatment solutions did not influence ^{59}Fe transport from the senesced leaves, indicating that urea inclusion and senescence improve Fe transport in a similar way, probably by affecting pools of Fe-chelating nitrogenous compounds.

In conclusion, spraying Fe together with urea contributed not only to improved absorption, but also translocation of absorbed Fe into sink organs such as seeds. If confirmed in the field trials, urea inclusion into foliar Fe treatment solutions would represent a useful agronomic practice for biofortification of cereal grains with Fe, and may have significant implications for nutritional quality of wheat-based food products and thus human nutrition.

C. CONCLUSION AND SUMMARY

The results in obtained under this PhD Thesis indicate clearly that N nutritional status of durum wheat plants has a significant impact on shoot and grain accumulation Fe. Improving N status of plants very positively affected root uptake and tissue accumulation of Fe. This N effect may have significant implications for nutritional quality of wheat-based food products and thus for human nutrition.

As illustrated below in Fig 1, at least one of following mechanisms contributes to N-induced grain accumulation of Fe:

i) release of Fe-mobilizing compounds from roots (such as phytosiderophores) and improving Fe acquisition by roots,

ii) improving root absorption capacity for Fe by affecting pool and activity of transporter proteins which mediates Fe uptake and transport in root cells,

iii) facilitating Fe transport within plants through xylem and phloem (re-translocation) as result of better chelation and loading into xylem and phloem thanks to Fe- chelating nitrogenous compounds (e.g., amino acids and nicotianamine) and transporter proteins responsible for Fe transport into xylem and phloem

iv) increasing seed/grain sink capacity by enhancing Fe-binding proteins for Fe.

It has been reported that Zn, Fe and proteins are co-localized in the same seed parts (Cakmak et al. 2010a), suggesting existence of a close relation between these nutrients regarding their transportation and deposition. Published molecular evidence suggests that amino acids, Zn and Fe are transported into seeds through similar molecular and physiological mechanisms (Uauy et al. 2006a; Distelfeld et al. 2007) and

a common genetic systems (NAC transcription factors) regulates this tansportation (Uauy et al. 2006a) The well-known positive relationship between the grain concentrations of Zn, Fe and N became stronger when N and Zn supply are sufficiently high (Kutman et al. 2010). This result lead to suggest that N and micronutrients act synergistically in improving the grain micronutrient concentration when both nutrients exist at sufficient amounts either in the growth medium or in the vegetative tissues (Kutman et al. 2011; Cakmak et al. 2010a).

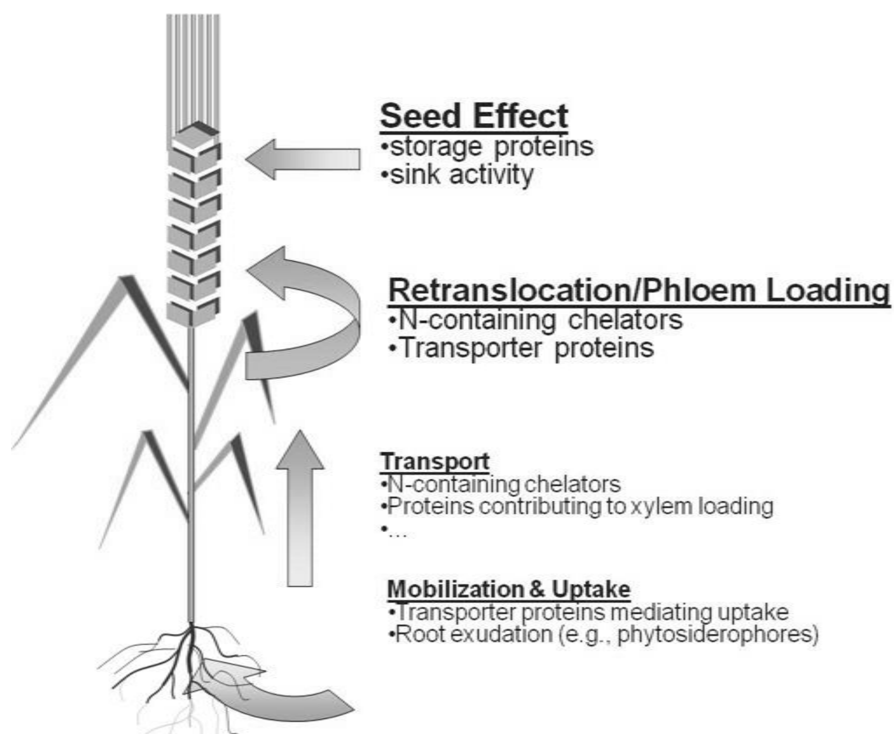

Fig. C.1. Critical steps affecting Fe uptake and transport in plants which are possibly under influence of N nutrition concentration in wheat grain (developed from Cakmak et al. 2010a; Kutman et al. 2011)

Application of Fe fertilizers to plants has been considered as an important agronomic tool to improve grain Fe concentrations as demonstrated several times for Zn (Cakmak, 2008; Cakmak et al. 2010b; Zhang et al. 2010). However, the experiments realized under this thesis showed that application of various soil or foliar Fe fertilizers (even in chelate forms like FeEDTA and FeEDDHA) had either a little positive effect or remained ineffective on shoot or grain Fe. When the plant N status is improved by soil N application, grain concentration of Fe is increased significantly. We noticed that the relative increase in shoot Fe content by N was around 125%, whereas an increase in

shoot dry matter production by N was only 42%. Considering much greater increases in shoot Fe accumulation (about 3-fold) than the shoot dry matter accumulation by N, it has been suggested that increases in shoot growth caused by increased N application cannot be a major reason for increased shoot content of Fe. Probably, as highlighted above, high N nutrition increased activity and abundance of Fe transporter proteins in root cell membranes which positively contributed to root uptake and shoot transport of Fe.

It was also interesting to notice that the response of grain Fe concentrations to foliar Fe fertilization has been very positively affected when Fe fertilizers were sprayed together with 1% (w/v) urea. To our knowledge, this is first report showing a positive impact of urea on grain Fe concentration when sprayed onto foliage together with Fe fertilizers. The mechanism behind this positive impact of urea on allocation of Fe into grain is not well understood. As studied and discussed in the Chapter 3, probably urea facilitates the cuticular penetration of the foliarly-sprayed Fe, improve chelation of the leaf-absorbed Fe and promote its transportation from the treated leaves into sink organs such as seeds. In the experiments using the radiolabelled Fe fertilizer (e.g., ^{59}FeEDTA), adding urea in the foliar ^{59}Fe-solution increased the proportion of ^{59}Fe in grains very significantly, but did not affect the amount of ^{59}Fe in shoot (straw) parts, indicating preferential transport of leaf-absorbed ^{59}Fe into developing grains rather than shoot (straw). This result pointed out that urea-stimulated transport of leaf-applied Fe into grains is, at least partially, a sink-driven process, Sudden increases in amino acid pools right after foliar spray of urea is known in literature (Dong et al. 2002), and has been also shown in this paper (Chapter III). Probably, the amino acids formed right after urea application contributed to better translocation of Fe into seeds of the in urea-treated plants.

As shown in the Chapter I, improving the plant N status had also a significant impact on release of phytosiderophores (PS) from roots, eventually by increasing the amount of nitrogenous substrates and activity of enzymes contributing to PS biosynthesis. This thesis showed that not only the root release of PS, but also mobilization of Fe from insoluble Fe-hydroxide and root uptake and shoot translocation of Fe(III)-PS in wheat were markedly enhanced by improving N nutritional status of

plants. To our knowledge, there was no published data in literature on the effects of N nutrition on PS release, root uptake and shoot translocation of Fe(III)-PS.

Reports available in literature showing that high grain Fe or Zn concentrations are very much affected from environment which seriously affect selection and breeding activities and ranking genotypes of a given germplasm for their Fe or Zn concentrations (Uauy et al. 2006a; Morgounov et al. 2007; Gomez-Beccerra et al 2010). One of the reason for high dependency of the genotypes to environment regarding their variable Fe concentrations might be N fertilization regime of the soils and/or N nutritional status of the genotypes. It is, therefore, believed that the results presented in this Thesis are very important and helpful for the on-going breeding programs aiming at increasing grain Fe concentrations. Based on the results in this Thesis an important attention should be given to the following breeding-related issues.

i) inherent N concentration (and N use efficiency) of the selected parental lines used in breeding programs should be known before the start of the breeding programs

ii) N fertilization regime of the soils where breeding programs are on-going should be clarified and known.

iii) it would be interesting to study the response of the advanced breeding lines to soil and foliar and N fertilizations regarding grain Fe concentrations, and

vi) breeding materials should not be analyzed only for micronutrients, but also for N.

Finally, the results presented in thesis have important implications for the nutritional quality of cereal-based foods and thus human nutrition. Iron deficiency problem is a well-documented global public health problem in human populations, and, mainly, resulted from reduced dietary intake of Fe (Bouis and Welch 2010; Gibson et al. 2010). There is, therefore, an urgent need for improving Fe concentrations of food crops to minimize Fe-deficiency related health problems in human populations such as anemia, birth defects, impairments in cognitive development and function and increases in maternal mortality (McLeon et al. 2009; Carter et al. 2010; Gibson et al. 2010). Today, enhancement in concentration of Fe in staple food crops is an important

humanitarian challenge. Based on the results presented in this study, it can be suggested that N fertilizer management and spraying Fe together with urea represent an important agronomic practices to contribute to increasing grain Fe concentrations in food crops. It can be concluded that he plant N status deserves a special attention in efforts to biofortify food crops with Fe and Zn.

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