# Effect of nitrogen fertilization on zinc and iron uptake and yield components of wheat

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# Effect of nitrogen fertilization on zinc and iron uptake and yield components of wheat

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# Abstract:

This study was performed to assess the role of nitrogen (N) rate and application time in combination with zinc (Zn) and iron (Fe) supplement in soil and foliage at booting stage of wheat (*Triticum aestivum* var. Krabat) for augmentation of protein, Zn and Fe in grain. Eighty four plastic pots of wheat containing 8 plants in each pot were grown in a climatically controlled growth room. The rates of N supply were 28.57, 42.86 and 57.14 mg N $kg^{-1}$ soil, equivalent to 80, 120 and 160 kg N $ha^{-1}$, respectively and for Zn and Fe, the rate was 10 mg $kg^{-1}$ soil and additional 30% through foliar spray at the designated N treatments, a total of 21 treatments. Plants missed tillering reducing overall grain and straw yields.

Grain and straw yield $pot^{-1}$, 1000 grains weight, number of grains $pot^{-1}$, whole grain protein content, concentration and total uptake of Zn and Fe in grains were determined. Analysis of variance demonstrated that soil applied N at sowing interacted often with Zn-Fe- treatments resulting differences in number of grains $pot^{-1}$, straw yield, grain Fe- concentration and uptake of Zn and Fe in grain. A tendency of higher yield was seen when increasing N rate at sowing was applied but the tendency was reduced at higher dose (160 kg N $ha^{-1}$ in this study). Relatively higher grain yield, protein content and the uptake of Zn and Fe in grain were obtained at split N equivalent to 160 kg N $ha^{-1}$ (applying 70% of allocated N at sowing and 30% at stem elongation). But lower N rates at sowing resulted in reduced grain yield with higher concentrations of Zn and Fe in grain. At soil plus foliar supply of zinc sulfate ($ZnSO_4$) and ferric ethylenediamine tetraacetic acid (Fe-EDTA) together enhanced grain protein content, concentration of Zn and Fe up to 46% and 64% and their total uptake in grain by 35% and 42%. For the localization of Zn and Fe in grain, LA-ICPMS and MA-XRF were used. Scanning of half grain along the crease pointed co-localization of Zn and Fe at germ, crease and aleurone. Split application of N with sufficient dose at sowing and stem elongation or beyond in combination with soil plus foliar application of Zn and Fe can be a good agricultural practice to enhance protein, Zn and Fe content in wheat.

Key words: Zinc and iron uptake, nitrogen fertilization, foliar spray of zinc and iron, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICPMS), wheat, wheat grain protein, grain components.

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I am glad to declare that this thesis is my own work and it has not been submitted for a degree at any other institutions.

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**List of symbols and abbreviations:**

| | |
|---|---|
| °C | Degree Celsius |
| % | Percent |
| n | Numbers of replicate |
| N | Nitrogen |
| Zn | Zinc |
| Fe | Iron |
| Zn+Fe | Zinc plus iron |
| DALYs | Global disability-adjusted life years |
| MA-XRF | Macro- X-ray florescence |
| LA-ICPMS | Laser ablation-inductively coupled plasma mass spectrometry |
| DW | Dry weight |
| TGW | Thousand grains weight |
| WGP | Whole grain protein |
| SRM | Standard Reference Material |
| RSD | Relative standard deviation |
| LOD’s | Lower detection limits |
| LOQ’s | Lower quantification limits |
| SD | Standard deviation |
| SE | Standard error |
| Avg. | Average |
| R-sq | Coefficient of determination |
| Fig. | Figure |
| µm | Micrometer |
| kV | Kilovolt |
| mA | Milliampere |
| W | Watt |
| Hz | Hertz |
| mg | Milligram |
| DMA | 2-deoxymugineic acid |
| NA | Nicotinamine |
| Fe-EDTA | Ferric ethylenediamine tetraacetic acid |
| NOx | Nitrogen oxide |
| Emb | Embryo |
| Endo | Endosperm |
| CDF | Cation diffusion facilitator |
| < | Less than |
| > | Greater than |
| kg | kilogram |
| $kg^{-1}$ | Per kilogram |
| g | Gram |
| mg | milligram |
| ml | Milliliter |
| $m^2$ | Meter square |
| Mm | millimeter |
| $Pot^{-1}$ | Per pot |
| $Spike^{-1}$ | Per spike |
| $ha^{-1}$ | Per hectar |
| Eq. | Equation |

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# 1 INTRODUCTION:

## 1.1 Background:

Micronutrient fortification of food started more than 80 years ago in developed countries to overcome the loss of some of the B-vitamins due to food processing (Allen et al 2006) like the milling of wheat to make common flour (FAO 1996). The fortification of wheat flour and its products is long practiced to solve contemporary micronutrient deficiency in human. In the United States, the enrichment of flour and bread with vitamins and minerals started in 1940 (Bishai and Nalubola 2002). Wheat is a source of flour and a staple food since its domestication about 10000 years back (Lev-Yadun et al. 2000). It is cultivated in range of climate and geography occupying the largest area than any other cereals and harvested after maize and rice amounting 701.5 million tons on year 2011/12 (FAO 2013). It is a most treaded staple ever (Wheat Initiative 2013). All in all, wheat stood as one of the important foods globally and estimated to be increased by 60% to meet the demand by 2050 (Alexandratos and Bruinsma 2012). Globally, 19 % of daily human calorie is fulfilled by wheat. Developing world produce > 60% of global wheat and it fulfills > 50% of daily diet where the micronutrient deficiency including zinc (Zn) and iron (Fe) is obvious human health challenge (CGIAR 2012; Cakmak 2008). It demands for growing mineral rich wheat grains to solve Zn and Fe deficiency and assure future global health.

Cereals are genetically low in Zn and Fe concentration and have reduced bioavailability (Graham et al. 2001; Cakmak 2002). About half of the world cereal is cultivated in soil low in plant available Zn (Cakmak 2002) which has worsen the quality of cereal staple in terms of Zn. Similar is the situation for Fe deficiency in cereals. In modern cultivated wheat, the seed concentration of Zn and Fe were found less than in the wild wheat (Cakmak et al. 2004). Old wheat cultivars were claimed micro-nutrient rich in comparison to the modern cultivars. May be old varieties were more efficient for micronutrient absorbance and their translocation to grain than today’s semi-dwarf, high yielding varieties that were introduced after mid 1960s (Fan et al. 2008). However a research carried at similar time showed wheat was rich in Zn and Fe than the maize (Ortiz-Monasterio et al 2007) establishing the importance of wheat as human food.

## 1.2 The role of Fe and Zn:

Zinc and Fe are important minerals required for various metabolic functions. It is obvious for both animals and plants. Zinc is responsible for protein synthesis, gene expression, proper growth and immune system. Physically Zn deficiency is manifested as stunting, common health problem in children like diarrhea, low birth weight, high rate of infection, skin lesions and impaired wound healing (Mutangadura 2004; Samman 2007b). Similarly, Fe is important for the production of Red blood corpuscles (RBC) in blood and carries oxygen to every tissue where $O_2$ is used for combustion of food to produce energy. This makes body metabolism keep going and healthy. Major symptoms of iron deficiency are anemia and decreased aerobic fitness. It causes behavioral disturbances and impairment of both cognitive function and psychomotor development to children (Samman 2007a). In plant, Zn and Fe deficiency reduces the growth, yield, and overall quality of edible part. Soil with low micronutrient concentrations produces grains with low concentration of for example Zn (Rengel 2002) and Fe concentration (Singh 2009).

## 1.3 Iron and Zn malnutrition:

There are more than 3 billion people suffering from malnutrition: mainly Zn and Fe (Welch and Graham 2004). About one third of the developing countries' population and approximately 10% Americans and Canadians are living with Zn deficiency or risk (Hotz & Brown, 2004) erasing the geographic and political boundaries. Every year Fe deficiency causes deaths of about 800000 children and 2.4% of global disability-adjusted life years worldwide (DALYs). Zinc is equally responsible for child death and 1.9% of DALYs in a global scale (Ezzati et al. 2002; Mutangadura 2004) and even more in the developing regions. In World Health Report 2002, Zn deficiency was ranked as 5th risk factor for 3.2% DALYs in developing countries with high child and high or very high adult mortality. Similarly, Fe deficiency was ranked at 6th position causing 3.1% of DALYs (Mutangadura, 2004). On percentage basis, Fe deficiency alone is responsible for affecting more than 47% preschool aged children and about 25% of the world population (de Benoist et.al. 2008). In addition, zinc deficiency is responsible for 4.4% deaths in children below 5 years of age (Black et.al. 2008). In a study of low-income African American and Hispanic children in Atlanta, the prevalence of Zn deficiency and anemia was high in low-income family (Cole et al. 2010).

The consumption of white flour made dominantly from endosperm of wheat grain discarding bran in milling process has even worsened the degree of Zn and Fe malnutrition. This is because of the Zn and Fe accumulate in some specific locations within wheat grain, particularly higher concentration in embryo and aleurone layer than in endosperm (Šramková, et al., 2009; Kutman et al., 2010; Cakmak et al., 2010). So, the contemporary researchers and authorized health organizations have urged to consume whole grain wheat rather than white wheat flour to increase the daily Zn and Fe intake. The agricultural fortification of staple crops including wheat can be one of the best and cheap options to increase the Zn and Fe supply in human food (Welch and Graham 2004; Bouis and Welch 2010). If mineral rich wheat and other cereals could be grown and marketed locally, that can solve the deficiency of Fe and Zn to large extent in the population of developing and emerging world.

Current study focuses on, how to increase the uptake and concentration of Zn and Fe in wheat grain using agricultural practices, taking the advantage of interaction between N, Zn and Fe. Nitrogen and Zn-Fe– fertilization were adopted as agricultural practices. Their interactive effect on yield, protein content and metal concentration in grain were analyzed through a pot experiment in environmentally controlled growth room. Further, scanning macro- X-ray fluorescence (MA-XRF) and laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS) were used to locate the hot spots for Fe and Zn in some wheat grains.

### 1.4 Research Objectives:

The study investigated the effect of nitrogen fertilization on uptake of Zn and Fe in wheat grain grown in environmentally controlled growth room. The specific objectives of the research were as follows:

1. To investigate the effect of nitrogen fertilization on yield components, total uptake of Zn and Fe and protein concentration in wheat grain.
2. To analyze the effect of soil and foliar applied Zn and Fe on total uptake of Zn and Fe in wheat grain.
3. To investigate the interactive effect of N- and Zn-Fe fertilization on wheat yield, protein and total uptake of Zn and Fe in wheat grain.
4. To identify the location of Zn and Fe accumulation in wheat grain using LA-ICPMS.

## 1.5 Research Hypothesis:

Above stated objectives were fulfilled by testing the following hypothesis.

1. N fertilization increases uptake of Zn and Fe in wheat grain.
2. Foliar spray of Zn and Fe increases their uptake and content in wheat grain.
3. N fertilization increases the overall wheat grain protein and yield components of wheat.

## 1.6 Limitations of result:

Higher and constant temperature (21°C) in growth chamber throughout the growth period of wheat made a unique growth environment in the current study. Consequently, wheat plants did not tillers leading to reduced grain yield. Still, the growth condition in the chamber was same for all treatments. Thus, comparison between treatments and computation of relation between different variables were possible.

# 2 REVIEW OF LITERATURE:

## 2.1 Basic on uptake and transport of Zn and Fe in wheat:

The uptake zinc and iron by roots of crop plants including wheat primarily occur in the ionic forms, for example $Zn^{2+}$ and $Fe^{2+}$ or $Fe^{3+}$ (mostly as divalent ions) or metal-legends via pores called divalent ion channels which are minute openings in root cell epidermis. Szatanik-kloc and Józefaciuk (2007) calculated the range of pore size in seedling roots of wheat in terms of radius varies from < 0.4 to < 1.6 nm. Earlier study by Whittaker and Muntus (1970) mentioned that the ionic radii of Zn and Fe less than 1 Å (= 0.1 nm), smaller than cell wall's pores. This hint easy passage of zinc and iron ions into apoplasm by diffusion or passive uptake which finally enters the nutrition transport vessels called xylem either by apoplastic route (along cell wall continuum) crossing through casparian stripe or symplastic (cell continuum) route (Fig. 2.1) and supplied to different parts of plant (Marschner, H. 1995; Campbell and Reece 2002). Often this is not the case and uses secondary active transport against higher root concentration (Tazi and Zeiger, 2010). The uptake Zn and Fe is facilitated by natural chelating compound called phytosiderophore (PS) (Takagi, et al., 1984; Römheld and Marschner, 1986) and synthesize more by wheat under Fe and/or Zn deficiency than at high available Zn and Fe (Tolay, et al., 2000).

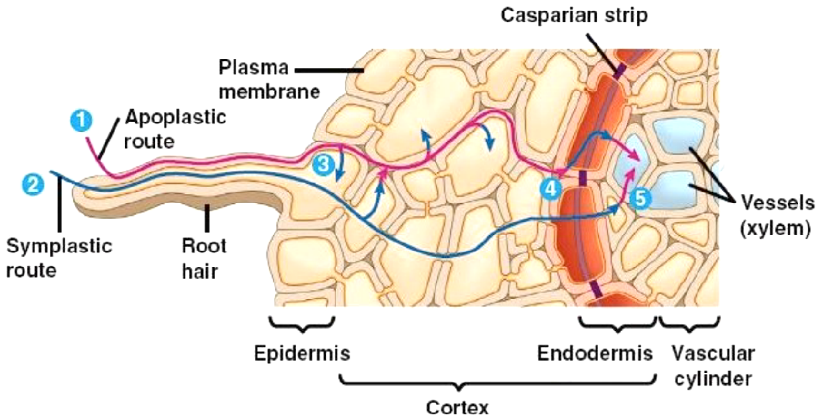

Fig. 2.1: The mineral and water uptake routes in plant roots and loading to vascular tissue. Apoplastic route (1), symplastic route (2); possible change of apolstic to symplastic route (3). Crossing the endodermis (4) minerals and water reach xylem (5) and get ready for long distacne transport when loaded to pholoem. The casparian strip acts as barrier. (Campbell and Reece 2002)

As water and minerals enters the vascular system, the xylem vessel transports them to stem, reproductive parts and leaves directing upward. Root pressure and evapo-transpiration regulate the continuous uptake and xylem transport. In contrast, phloem carries sugars, amino acids and minerals from leaves to other plant parts where the demand is high for example to the growing root tips, shoot tips, buds, and grains. Translocation of nutrients from the old to young leaves and leaves or stem to grains occur through phloem transport system and translocation ranges from utilization to storage sinks (Marschner, 1995; Campbell and Reece, 2002). In case of wheat plant, grain resembles the storage sink and rest as utilization sink. Thus the availability of Zn and Fe at later stage of plant development particularly at grain filling period could increase the uptake as well as concentration of these elements in wheat grain (sink). Many current and past researches pointed soil and/or foliage supplied Zn and Fe can increase the accumulation of Zn and Fe in wheat grain respectively (Yilmaz et al., 1997; Cakmak, 2008; Kutman et al., 2010; Habib, 2012, Kutman et al. 2012). In addition, uptake and transport of metals in plant is facilitated by metal transporter proteins located in different tissues of a plant (Hall and Williams, 2003).

Even though some mechanisms of uptake and transport of Zn and Fe in plants are known, wheat is poor in Zn and Fe content in grain (Cakmak, 2008). This suggested some complex or may be even unknown mechanism involved for limited transport of minerals like Zn and Fe into the wheat grain. The role of N, Zn and Fe fertilization on wheat and distribution of Zn and Fe in wheat grain are discussed below.

### 2.2 Nitrogen fertilization and growth of wheat:

Nitrogen plays important role in the vegetative as well as generative growth of wheat in many ways which ultimately affects straw and grain yields. Many researches were carried out in the past pointing the positive response of N availability in wheat plant in range of aspects, such as vegetative growth (Kanampiu et al., 1997; Oscarson 2000; Warraich et al., 2002), grain yield components (Oscarson 2000; Warraich et al., 2002; Abedi et al., 2010; Marino et al., 2011), assimilate formation and its translocation to grain (Rodrigues et al., 2000) and promotion of Zn and Fe accumulation in wheat grain (Kutman et al., 2010; Kutman et al., 2012). Initially applied N before wheat plantation or until tillering helps for the establishment and vigor of vegetative growth (Marschner 1995; Li et al., 2001; Brown et al., 2005). In case of wheat, initial N increases the number of spikes (Oscarson 2000) as the number of tillers per plant increases (Oscarson 2000; Li et al., 2001). Sufficient availability of N before the setting of spike primordial can increase the number of spikelets per spike and total number of grains in spike finally increasing the grain yield (Oscarson 2000) but over dose of N can lower the grain yield (Abedi et al. 2010). Similarly, Protic et al. (2007) observed increase in 1000 grains weight of wheat increased irrespective of varieties when increasing N rate from 0 to 60 kg ha$^{-1}$ but the 1000 grains weight decreased with further increment in N. Late application of N after stem elongation or beyond, particularly around anthesis enhance the size of grains only if the initial N was not sufficient to meet grain yield potential (Brown et al., 2005). Still, why does the over dose of N lowers the grain yield in wheat is questionable demanding further focused study for clarification.

In a field experiment of wheat, both vegetative and generative growth increased with increasing N supplement rate (Warraich et al., 2002). During this study, Warraich et al. (2002) investigated the number of tillers per plant, leaf area index and dry weight of straw for vegetative growth. Similarly, they also studied grain filling rate (DW), grain filling duration (days), number of grains per unit area, 1000 grains weight and finally grain yield for the generative growth of wheat. All these parameters were increased with increase in the supplement of N. In addition, they also found increase in net assimilation rate (g cm$^{-2}$ day$^{-1}$) with increasing N rate. It might be due to the increment of total chlorophyll (a + b) content in leaves as evidenced in the sunflower leaves when N availability was higher (Nasser 2002). Similar results were experienced in hydrophonic culture of wheat to full maturity except for unit weight of grains which was not affected to significant level by increasing N rate (Oscarson, 2000). Oscarson (2000) mentioned the increment in leaf area index and dry weight of all vegetative parts including mass of tiller, main shoot and root when N availability was increased. Other than increment in the weight of vegetative parts, number of ear-bearing tillers increased linearly from 0.1 to 2 tillers per plant and the number of spikelets per spike increased more than 25% for N rate increment from 20 to 56 mg N plant$^{-1}$. But the straw weight, main shoots' height and number of spikelets decreased when the N per plant was higher than 56 mg (Oscarson 2000). In a study for the interactive effect of N fertilization and inoculation with *Azospirillum*, Rodrigues et al. (2000) observed, grain and straw dry weight increased and post-anthesis translocation of assimilate (DW) from vegetative parts to grains also increased at higher N application rate and contributed > 52% of the final grain weight (DW) at 60 kg N ha$^{-1}$. The contribution of assimilate translocation to grain weight was calculated based on the loss of vegetative dry matter between anthesis and maturity.

### 2.3 Zinc and iron fertilization and growth of wheat:

Zinc and iron are essential functional and structural components in many types of proteins and enzymes in plants and are equally important micronutrients for the proper development of higher plants (Marschner 1995) including both vegetative as well as generative development. Some earlier studies claimed that Zn fertilization affects the vegetative development and grain yield of wheat. For instance, Langeragan and Webb (1993) defined the role of Zn deficiency in relation to N availability, stating that the response of Zn deficiency do not prevail both on vegetative and reproductive development until the N rate is high. But the deficiency of Zn lowers the vegetative growth as well as grain yield of wheat when N supply is high (Cakmak and Engels 1999; Salvagotti and Miralles 2007; Kutman et al. 2010; Kutman et al. 2011). Further Yilmaz et al. (1997) argued that wheat grain yield could be more sensitive than the straw yield when encountered soil Zn deficiency. Similarly, soil applied Zn induce the higher leaf area index and photosynthetic rate (Nadim et al. 2012; Jiang et al. 2013) as Zn availability result vigorous growth of plant (Kutman et al. 2010). Nadim et al. (2012) also recorded the significant increase in grain yield but not in 1000 grains weight with increasing soil application of Zn to the rate of 10 kg ha$^{-1}$ in comparison to soil applied Fe. But, Jiang et al. (2013) indicated increment of 1000 grains weight in response of soil supplement of Zn at the rate of 200 mg kg$^{-1}$ in the form of $ZnSO_4.H_2O$ along with increase in the number of grains per spike and grain yield. Similarly, Habib (2012) obtained significant increase in 1000 kernels weight when Zn and Zn+Fe supplied on foliage at grain filling period of wheat in comparison with Fe supplement without affecting grain numbers per spike. Zeidan et al. (2010) recorded significant increase in all grain yield parameters and straw yield when Zn and Fe were sprayed on foliage at tillering and booting stage. In contrast with findings of Zeidan et al. (2010), when Zn and Fe applied on foliage at anthesis straw yield did not increased as the mass of straw is determined before the development of reproductive phase (Kutman et al. 2011).

### 2.4 Role of nitrogen on uptake of Zn and Fe in wheat:

In addition to the role of N in growth of wheat plant, N can play equally important role in root uptake and translocation of micronutrients like Zn and Fe finally accumulating in wheat grain. Earlier studies have demonstrated that the uptake and translocation of Zn and Fe in wheat grain increases with higher N status in plant or seed and external supply of N at different phonological stages (Kutman et al., 2010; Shi et al., 2010; Cakmak 2010; Cakmak et al., 2010a; Cakmak et al., 2010b; Kutman et al., 2011). But the translocation of Zn and Fe in grains particularly in endosperm is hindered due to the presence of physiological barriers between stem and grain and crease vascular tissue to endosperm (Wang et al., 2011). When wheat was grown in Zn deficient and Zn adequate soil, with higher application rate of Zn (50 kg $ha^{-1}$ in soil and 0.5% $ZnSO_4.7H_2O$ as foliar supplement), maximum enrichment of wheat grain with Zn as well as Fe was found in treatments supplied with sufficient N (urea as a source of N) as via soil and/or foliar (Cakmak 2010). In the green house study to analyze the effects of externally supplied N and Zn in durum wheat on the total uptake and remobilization of Zn, Fe and N showed that high supply of N and Zn enhanced the uptake of Zn and Fe per plant up to 4- fold but the plant growth did not increased in similar proportion (Kutman et al., 2011). In the same study, at high N and Zn application Kutman et al. (2011) found that about 60% and 40% of total Zn and Fe accumulated in vegetative parts before anthesis were retranslocated to grains respectively. In climatically controlled hydroponic study of durum wheat, Kutman et al. (2012) examined the effect of Zn availability at post-anthesis stage and nitrogen nutrition to point out the responsible mechanism of Zn accumulation in grain. Results indicated that when Zn supply was stopped at pre-anthesis, the remobilization of Zn from vegetative parts to grain was found responsible for the accumulation of Zn in grain but when Zn supply via solution was continued even after anthesis, root uptake of Zn after anthesis was responsible for grain Zn. In both situations, higher rate of N increased the grain filling period extending the Zn supply time favoring the accumulation of more Zn in grain.

In addition to higher grain uptake of Zn induced by longer grain filling period of wheat, N is expected to involve chemically in the formation of metal chelating compounds (Kutman et al., 2010) like nicotianamine (NA) as observed in tobacco plant (Takahashi et al., 2003) and 2-deoxymugineic acid (DMA) mainly for the translocation of Fe and Zn from flag leaves in wheat to grain (Barunawati et al., 2013). In a comparative study for the effect of ammonium and nitrate fertilizers on wheat, Barunawati et al. (2013) found increase in the total content of Fe, Zn and copper in wheat grain was not related to the extent of metal translocation from flag leaves even for the increase in NA due to increased N. But it could be a major role of DMA in translocation of metals from flag leaf to the grain in modern high-yielding wheat as evidenced by higher proportion of DMA than NA in flag leaf and in flag leaf exudates.

Further the uptake and transport of metals in plant is also regulated by some special transporter proteins situated in different tissues of root, stem, leaf and reproductive parts. Many of them are specific in transporting Zn and Fe. For example Nramps transport Fe and CDF family transport Zn, while ZIP family carries both Zn and Fe (Hall and Williams, 2003). Heavy metal ATPases are supposed to be present everywhere in a plant (Hall and Williams, 2003). Thus in wheat, the uptake of Zn and Fe from soil and their transport from stem or leaf to grain is facilitated by transporter proteins and N supplement to plant probably fetch positive effect as N is an important constituent of proteins as well as the plant N nutritional status most likely affect the transporter proteins Cakmak et al. (2010a). Peterson et al. (1986), Peleg et al. (2008) and some recent studies reported positive correlation between grain concentration of Zn or Fe with protein in wheat.

### 2.5 Grain yield components:

Grain yield in wheat is the combined effect of number of grains per unit area and the unit weight of grains (Mishra et al., 2001). But many previous studies (Waddington et al., 1987; Protic et al., 2007; Peltonen-Sainio et al., 2007; Zhang et al., 2010) indicated the dominating role of number of grains per unit area than that of the unit weight of grains. This could be because the unit weight of grains is the genetic trait of wheat and do not differ in general but get influenced by other environmental factors (Kausar et al., 1993) like the availability of water and temperature during the grain filling period. Fertilization can also affect the unit grain weight to some extent as (Protic et al., 2007) mentioned nitrogen fertilization increased the grain yield of wheat by positive effect on the number of grains per unit area as well as 1000 grains weight, but 1000 grains weight decreased as N was supplied beyond 60 kg ha$^{-1}$. Similarly, in separate studies, Nadim et al. (2012), Habib (2012) and Jiang et al. (2013) noticed significant increase in 1000 grains weight in response of Zn or Fe fertilization.

### 2.6 Protein content in wheat grain:

In contrast to the positive influence of initial N application on straw and grain yields, late application of N after stem elongation or beyond increases the availability of N particularly at late stage of wheat development, as a consequence the protein content in wheat grain increases (Brown et al., 2005). This is possible only if the initial N is sufficient to meet the grain yield potential (Ottman et al., 2000; Brown et al., 2005; Weber et al., 2008). In the irrigated field condition, Brown et al. (2005) observed both increasing as well as decreasing wheat grain protein with increasing grain yield. Although they could not noticed a significant relation between protein and irrigated wheat grain yield, but interesting results came out after the analysis performed dividing the data into sites that contain protein higher and lower than 12.5%. Finally, they concluded that increase in grain protein with higher grain yield was due to the N limitation to meet the yield potential and decrease in grain protein with higher grain yield was possible due to the availability of adequate N for maximizing yield. In sum, Nitrogen deficiency at the early phase of wheat growth not only reduce the grain and straw yield but also lowers the protein content in grain due to the competition for available N at vegetative as well as at reproductive phase of plant (Li et al., 2001; Fowler 2003; Brown et al., 2005), eventually reducing the overall value of grain.

As stated in earlier Zn and Fe are essential functional and structural components in many types of proteins and enzymes in higher plants (Marschner 1995), the importance of Zn and Fe supplement for the production of protein rich wheat grains is obvious particularly in soil with lower availability of Zn and Fe. Earlier studies (Peleg et al., 2008; Kutman et al., 2010; Cakmak et al., 2010a; Velu et al., 2011) obtained positive correlation between Zn- or Fe- concentrations in wheat grain with grain protein content. Similarly, Kutman et al. (2010) and Cakmak et al. (2010 a) demonstrated the co-existence of Zn, Fe and protein in durum wheat grain suggested that the wheat grain protein might be a sink for Zn and Fe and expected the presence of some kind of synergetic relation between Zn, Fe and N for co-segregation in grain when higher rate of N provided.

Other than the availability of N, Zn and Fe for wheat growth, environmental factors also prevail equally to determine the concentrations of protein and metals in grains. When the grain yield potential get decreased in response of environmental limitation like drought and higher temperature at grain filling period the size of grain reduced showing higher concentration of protein (Fowler 2003). In contrast, the grain protein content dilutes in relation to larger grain size as supply of non-protein compounds like assimilate (e.g. starch) increases during grain filling period (Pleijel et al., 1999) when growth temperature will be higher and water availability will be enough. Similar situation prevail for the Zn and Fe concentration in grain.

### 2.7 Role of Fe and Zn fertilization on uptake of Zn and Fe in wheat:

It is obvious that the external supplement of Fe and Zn compounds either in soil or leaves increase the availability of these elements for wheat plants finally increasing the uptake and concentration in grain. Yilmaz et al. (1997) reported that Zn and Fe fertilizers can increase Fe and Zn concentration in wheat grain up to 3- or 4- folds. Cakmak et al (2010) suggested the positive role of soil and foliar applied Zn and Fe to increase respective metal concentrations in durum wheat grain. In addition Cakmak et al. (2010) claimed increased activity of Zn and Fe in source (flag leaf and stem) during grin filling could be increased by additional Zn and Fe application in soil or on foliage, as a consequence increase the uptake as well as concentrations of Zn and Fe in grain. Habib (2012) showed that combined application of Zn and Fe can increase the concentrations of Zn and Fe in grain than at separate application of Zn and Fe. The dilution of grain concentration of Zn and Fe depend on the size of wheat grains (Velu et al., 2011) and numbers of grain per spike (Nowack et al., 2008) as these parameters determine the size of sink in wheat. Foliar sprayed Zn and Fe absorb in plant leaves by diffusion and translocate to other parts of wheat as observed for foliar applied Zn Haslet et al. (2001). Haslet et al. (2001) demonstrated the role of phloem transport of Zn in wheat plants by performing stem girdling, that $^{65}$Zn supplied on upper leaf transported to lower leaves and root tip.

### 2.8 Distribution and translocation of Zn and Fe in wheat grain:

A wheat grain is oval shaped, slightly elongated having bulged dorsal portion and fissured at ventral side. Fissured portion is known as crease. In general, a wheat grain consists of 2-3% embryo (dormant seedling), 80-85% starchy endosperm, aleurone layer and the testa (seed coat) fused with the pericarp (fruit coat) forming about 13-17% in dry weight basis (Belderok et al., 2000). The structure and different parts of a wheat grain is presented below (Fig. 2.2).

The concentration of minerals differs within a grain from one to other portion. For example, wheat endosperm consists of about 15 mg kg$^{-1}$ Zn, while germ and aleurone holds about 150 mgkg$^{-1}$ Zn (Šramková, et al., 2009) which is 10 times higher than in endosperm. More recent study of wheat grain under LA-ICPMS, Wang et al. (2011) depicted higher concentration of Zn in aleurone layer and crease vascular tissue with decreasing gradient of Zn from crease vascular tissue to the endosperm, suggesting translocation of Zn occurred through crease vascular tissue into the endosperm. Similar distribution and translocation was noticed for Zn as well as Fe by (Cakmak et al., 2010a) when analyzed wheat grains under LA-ICPMS. The protein rich grains accumulate higher amount of Zn as well as Fe in wheat (Ozturk, et al., 2009), who found higher Zn and Fe concentrations in grain for wheat variety having high protein grains (i.e. Fe = 71 mg kg$^{-1}$ and Zn = 57 mg kg$^{-1}$ in average), while lower concentrations (i.e. Fe = 36 mg kg$^{-1}$ and Zn = 30 mg kg$^{-1}$ in average) in wheat variety having low protein grain. This showed that higher protein or nitrogen content favors the accumulation of Zn and Fe in considerable level in wheat grain (Ozturk, et al., 2009; Kutman et al., 2010).

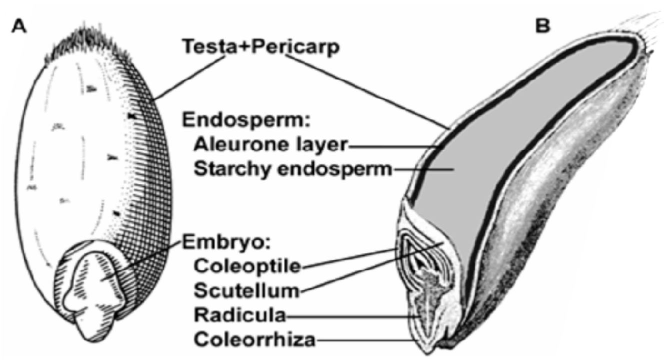

Fig. 2.2: Structure of a wheat grain. (A) Dorsal view (B) dissected view along crease (Leubner, 2007).

# 3 MATERIALS AND METHODS:

Most of the work for this study, from wheat growth experiment to the analysis of grain samples was carried out in the Department of Plant and Environmental Sciences (IPM) of the Norwegian University of Life Sciences (UMB) ÅS, Norway during 2012 and 2013. MA-XRF and LA-ICPMS of wheat grains for the hot spots (higher concentration area) of Fe and Zn within a grain were performed in the University of Antwerp, Belgium.

## 3.1 Control chamber experiment and experiment design:

### 3.1.1 Growth chamber setup:

Wheat plants grew in control growth chamber at IPM-UMB from May to August 2012. The temperature of the growth chamber was regulated at about 21°C. The duration of day length was 8 hours and of dark period was 16 hours. The source of light was Halogen metal halide lamps by POWERSTAR HQI-BT 400W/D. Pots with wheat plants were put on seven wheeled tables each containing 12 pots. To avoid shading effect, the position of tables was changed at every two days interval.

### 3.1.2 Design of the experiment and fertilizer rates:

The experiment was setup based on a complete randomized factorial design so as to reduce confounding effect as well as to minimize the unknown errors. The experiment contained two major treatment factors: N-treatments and Zn-Fe treatments. It was further divided into two groups: experiments with soil application of all treatment factors (5 N- treatments × 3 Zn-Fe- treatments = 15 soil treatments) and experiments with soil plus foliar spray of Zn and Fe (2 N- treatments × 3 Zn-Fe- treatments = 6 soil plus foliar treatments). Both treatment factors were incorporated into same experiment to see the combined effect of them. . There were five N- treatments. Three with single soil application, nitrogen solution mixed in soil just before sowing at rates equivalent to 80 kg N $ha^{-1}$, 120 kg N $ha^{-1}$ and 160 kg N $ha^{-1}$ and two split soil applications equivalent to 120 kg N $ha^{-1}$ and 160 kg N $ha^{-1}$. The allocated amount of total nitrogen in split application treatment was divided into 70% (i.e. 84 and 112 kg N $ha^{-1}$ respectively for 120 and 160 kg N $ha^{-1}$) at sowing and 30% (i.e. 36 and 48 kg N $ha^{-1}$ respectively for 120 and 160 kg N $ha^{-1}$) after complete emergence of 4th leaf which coincides with stem elongation phase in this study. Similarly three Zn-Fe treatments included Zn and Fe applied separately and together (Zn+Fe). The rate of Zn and Fe application in soil at sowing was at 10 mg $kg^{-1}$ soil (20.15 mg $pot^{-1}$) in all cases as per treatment described just before. While, 30% higher Zn and Fe than that applied in soil equal to 6 mg $pot^{-1}$ was sprayed on foliage during booting stage of wheat at N treatments: N1N120 and N2N120 that were considered during the experiment with soil application of nutrients.

To understand the effect of foliar spray of Zn and Fe (together and separate) on wheat grain Zn- and Fe- concentration and yields, separate experiment was conducted taking two N-treatments having single and split application of 120 kg N $ha^{-1}$. For this purpose, 30% higher Zn and Fe in addition to that applied in soil was sprayed as solution at boot stage of wheat growth. Then the effect was compared with an equivalent experiment for 120 kg N $ha^{-1}$ having only soil application of N as well as Zn and Fe treatments.

All together 21 treatments were performed at 84 growth pots with four replicates of each treatment. A complete picture of design and treatment combinations with fertilization rates is presented in the Table 3.1 followed by abbreviations of treatment and their illustrations. Timing of treatment application is described in Table 3.2.

### 3.1.3 Growth media preparation:

Artificially prepared growth media was used for growing plants represented a modified soil composition according to the OECD guideline 207 (OECD, 1984), where it was used 80% sand (< 2 mm), 10% peat (< 4 mm), and 10% kaolin on dry weight basis. In the absence of sphagnum peat, unfertilized and unlimed natural peat produced by Econova Garden AB, Sweden was used. Air dried peat was sieved through 4 mm wire mesh and average moisture content was determined by drying nine representative samples in oven at 105°C for 24 hours for the correction of moisture content in peat while preparing growth media. Average moisture content varied from 41 to 48% depending on peat delivery bags (Annex 1: Table 1). To maintain the soil pH at 6.5 ± 0.2, $CaCO_3$ was mixed at the rate of 0.5 g per 100 g soil mixture. The soil pH was determined using digital pH meter (ORION1, model SA720). The proportion of carbonate was determined through the development of liming curve for soil mixture using different proportion of powdered $CaCO_3$ from zero to 5 g per 100 g soil mixture (Fig. 3.1, Annex 1: Table 2). All constituents were placed in big and open stainless steel bowel and mixed thoroughly by hands. Finally, the homogeneous soil mixture was filled in three liters' plastic pots amounting to 2015 g soil (dry weight) in each pot. The same mass of soil in each pot was filled to minimize the fertilizer dilution due to variation in soil amount. The amount of nutrition and water was fixed when mixing nutrients, treatments and watering plants.

Table 3.1: Design of experiment and rate of fertilization as per treatment.

| **Experiments with soil application of nutrients (N, Zn and Fe)** | | | | | |
|---|---|---|---|---|---|
| **Zn-Fe-treatments** | **N- treatments** | | | | |
| | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** |
| **Zn** | N1N80Zn | N1N120Zn | N1N160Zn | N2N120Zn | N2N160Zn |
| **Zn+Fe** | N1N80Zn+Fe | N1N120Zn+Fe | N1N160Zn+Fe | N2N120Zn+Fe | N2N160Zn+Fe |
| **Fe** | N1N80Fe | N1N120Fe | N1N160Fe | N2N120Fe | N2N160Fe |
| **Experiments with soil plus foliar application of Zn and Fe** | | | | | |
| **Zns+f** | | N1N120Zns+f | | N2N120Zns+f | |
| **(Zn+Fe)s+f** | | N1N120(Zn+Fe)s+f | | N2N120(Zn +Fe)s+f | |
| **Fes+f** | | N1N120Fes+f | | N2N120Fes+f | |

Where,

**N-treatments:**

Single soil application of N at sowing:

N1N80 = single application of N at sowing equivalent to 80 kg N ha$^{-1}$ mixed with soil.

N1N80 = single application of N at sowing equivalent to 80 kg N ha$^{-1}$ mixed with soil.

N1N120 = single application of N at sowing equivalent to 120 kg N ha$^{-1}$ mixed with soil.

N1N160 = single application of N equivalent to 160 kg N ha$^{-1}$ mixed with soil.

Split application of N:

N2N120 = Split application of N equivalent to 120 kg N ha$^{-1}$. 70% (equivalent to 84 kg N ha$^{-1}$) of allocated N was applied at sowing time and 30% (equivalent to 36 kg N ha$^{-1}$) at the beginning stem elongation

N2N160 = Split application of N equivalent to 160 kg N $ha^{-1}$. 70% (equivalent to 112 kg N $ha^{-1}$) of allocated N was applied at sowing time and 30% (equivalent to 48 kg N $ha^{-1}$) at the beginning stem elongation.

**Zn-Fe treatments:**

Soil application of Fe and Zn at sowing:

Zn = Zn mixed with soil at sowing. .

Zn+Fe = Zn and Fe mixed with soil at sowing.

Fe = Fe mixed with soil at sowing.

Soil plus foliar application of Fe and Zn at booting stage:

Zns+f = soil application of Zn at sowing plus 30% of soil applied Zn as foliar spray.

(Zn+Fe)s+f = soil application of Zn and Fe at sowing plus 30% of soil applied zinc and iron as foliar spray.

Fes+f = soil application of Fe at sowing plus 30% of soil applied iron as foliar spray.

### 3.1.4 Addition of basic nutrients and soil treatment factors:

All basic nutrients and treatment factors (N, Zn and Fe) were applied in deionized water solution except calcium carbonate ensuring the application rate (Fig. 3.1). Powdered calcium carbonate was mixed in soil mixture to achieve a soil pH of 6.5 ± 0.2. The volume of nutrient as well as treatment factor solution was fixed to 25 ml in soil while mixing nutrients and later it was compensated during watering soil for the first time after sowing seeds.

All added nutrients were mixed manually to get homogeneous distribution. The second dose of nitrogen in split nitrogen treatments (N2N120 and N2N160) amounting to 30% of the total nitrogen was added directly in growth pot after 4th leaf stage at the beginning of stem elongation and watered immediately so that N could spread properly.

### 3.1.5. Calibration of spray and application of Fe and Zn on foliage:

Seven plant pots form experiment was sprayed with de-ionized (18 ohm) water after surrounding pot from bottom and around with paper having known weight. For each pot, 10 ml water was kept in locally available graduated spray and sprayed on plants from all side in full capacity. When water drops were seen falling off the leaf, spraying was stopped. When plants were dry, the spraying started again and repeated until all water was applied. The same procedure was adopted when spraying Zn and Fe solutions. Wet paper after each stoppage of spray was weighted to record how much water did not fall on plants and finally recorded the cumulative amount and calculated the spayed amount that fall on plant. During the calibration, water fall on soil and pot was not deducted so that it came under total water or solution fall on plants after spraying. From the calibration, a major question was answered as to how many times should the plant be sprayed for 10 ml of solution. When spray was used in full capacity, the spraying times to spray 10 ml water varied between 9 to 11 times with mode value 10 (Annex 1: Table 5). But only about 50% of the water retained on plants after spray (Annex 1: Table 6). So while applying Zn and Fe solutions, 20 sprayings per pot was adopted to ensure 6.0 mg of Zn and Fe each which was equivalent to 26.6 mg zinc sulfate and 33.2 mg Fe-EDTA amounting 30% of total Zn and Fe applied in the soil. The sprayed solution contained 10 ml water and surfactant called DP-Klebemiddel (at 0.5 ml per one liter solution, contained 90% alcohol ethoxylate produced by Norgesför AS. Separate solutions were made for Zn, Fe and Zn+Fe. Then the solutions were sprayed to designated pots as the design of the experiment, on leaves after complete emergence of flag leaf at booting stage in majority of plants.

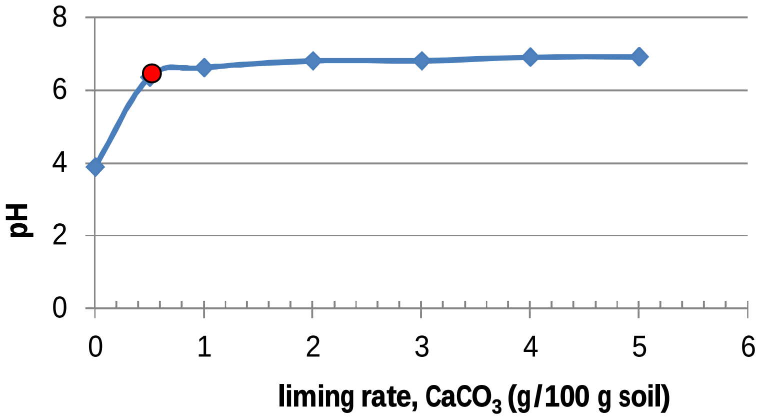

Fig. 3.1: pH curve at different liming rates. Red spot on curve represents selected liming rate and expected soil pH.

### 3.1.6. Test plant and germination rate of wheat seeds:

The test plant was hard red winter wheat variety which was breed by Graminor AS, Norway. It was accepted in Norwegian variety list in 2010 and is being grown in Norway thereafter. The breeder claims this variety is medium early in growth period, high yielding with good agronomical characteristics, medium protein, relatively good disease resistance and backing quality (personal communication with Jon Arne Dieseth, in Graminor AS, Norway.

The germination rate of wheat seed determined before sowing was 92 %. During the test, 60 seeds were randomly selected and placed on wetted filter paper base counting 20 seeds in each Petridis, three in total. The petridish was covered with clinging thin plastic sheet and placed inside wooden cardboard for 24 hours in dark. After 24 hours brought to light, plastic cover was removed and the germinated/sprouted seeds were counted after each 24 hours until four days (Annex 1: Table 4).

### 3.1.7 Growth of plants and watering:

Twenty seeds of winter wheat cultivar were sowed in each pot more or less in equal distance to surrounding seeds placing about a centimeter beneath the soil surface followed by watering soil to its 60% field capacity (equal to 569 ml water). After one week, each pot was thinned to eight plants. While thinning, healthy plants were kept at equal distance between neighboring plants. The field capacity of the soil was determined by wetting and drying of volumetric soil sample at the soil physics laboratory of IPM-UMB, ÅS, Norway. While watering for the first time, the amount of water contributed by peat and other liquid nutrition were taken into consideration resulting into a total weight of single pot to 2691 g (2015 g soil + 107 g pot + 569 g water). When plants reached 4th leaf stage, 6 thin bamboo sticks were used to manage plants which increased the total weight of a pot to 2711 g. To avoid the contamination and errors in the experiment de-ionized water was used while watering every two days in the initial weeks and the last weeks but every day in the middle of the growth stage while plants demanded more water. To water plants, growth pot was weighed and the loss in weight was compensated by adding water. The position of each wheeled tables holding growth pots were randomly changed position every 2nd day to avoid the shading effect within growth chamber. On the same day the general condition of plants used to be monitored and made notes which could finally produce the growth and event calendar for wheat (Table 3.2).

### 3.1.8 Harvest:

Grain yields were harvested on 17.08.2012. Every spike was cut at its peduncle with scissors and kept in separate sampling paper bags for each pot. After harvesting spikes straw was left standing and harvested on 27.08.2012. Straw was cut just at the base of first node above soil surface avoiding soil stick part due to splash of soil while watering plants. Harvested straw was further cut into small pieces and stored in separate sampling paper bags for each pot.

Table 3.2: Cultivation, growth and treatment calendar for wheat.

| Events | Date | Remarks |
|---|---|---|
| Mixing basic nutrients and treatment factors to soil | 10.05.2012 | |
| Seed sowing | 11.05.2012 | |
| Seedlings emerged | 13.05.2012 | |
| 3rd leaf stage | 28.05.2012 | No tillering yet but it was expected by this time. |
| 4th leaf stage | 04.06.2012 | Fist node came above the soil surface. |
| Yellowing of lowermost leaves | 07.06.2012 | Similar to all treatments. |
| Addition of split amount of nitrogen to soil | 10.06.2012 | |
| 5th leaf stage | 11.06.2012 | |
| 6th leaf stage | 15.06.2012 | By now, noticed more green leaf in N- treatments having 30% of N at stem elongation. |
| 7th or flag leaf stage | 20.06.2012 | |
| Foliar spray of Zn and Fe | 21.06.2012 | |
| Emergence of spike out of collar | 29.06.2012 | Minor leaf burn on 6th and flag leaves after foliar spray dominantly for Fe spray |
| Yellow stage flower | 04.07.2012 | |
| White stage flower | 09.07.2012 | |
| 6th leaf dry | 19.07.2012 | |
| Flag leaf dry (still stems green) | 25.07.2012 | |
| Plants dry completely | 10.08.2012 | |
| Spike harvest | 17.08.2012 | |
| Straw harvest | 27.08.2012 | |

Note: Dates of leaf stage and spike emergence was defined based on emergence of complete leaf or spike in more than 50% of plants.

## 3.2 Laboratory works and chemical analysis:

### 3.2.1 Grain and straw yield:

Grain and straw yields were determined on dry weight basis. Total grains and straw harvested from each growth pot was pre-weighed and dried in preheated oven at 75° C. After 48 hours, grains were let to cool at room temperature and weighed in digital balance with two digit accuracy. Then, thousand grains weight (TGW) was calculated on dry weight basis according to formula stated below.

$$\text{TGW (g)} = \{(\text{grain yield, g pot}^{-1}) / \text{number of grains pot}^{-1}\} \times 1000$$

### 3.2.2 Grinding of wheat grains:

About 2 g of wheat grain was powdered in mixture mill (Retsch MM301). The milling duration was 1 minute 30 seconds and the frequency 20 Hz. The milling ball and wall of containers were made of Zirconium to avoid contamination of samples. The powdered wheat was used as primary sample for chemical tests of whole grain protein, Zn, and Fe which are described in the following chapters.

### 3.2.3 Whole grain protein:

Analysis of total grain nitrogen was determined by dry combustion as described in Dumas method, reported by Brammer and Mulvaney (1982). About 200 mg oven dried and finely powdered whole grain wheat sample was burn at high temperature about 900°C in the presence of oxygen. Through this the produced nitrogen oxide (NOx) was reduced to $N_2$ gas by catalyst of copper and the concentration was measured by calibrated thermal conductivity detector called TC cell at analysis instrument LECO-CHN 1000. Finally the results were corrected for dry matter and the nitrogen percentage was converted into whole grain protein (WGP) by multiplying with a factor of 5.70 (ISO, 2009).

### 3.2.4 Grain Fe and Zn concentration:

#### 3.2.4.1 Sample preparation:

About 0.2 g of Mixture Mill (Retsch MM301) grounded wheat flour samples was digested in 5 ml conc. $HNO_3$ for about two hours in ultra clave microwave reactor (MLS-MILESTONE, ultraCLAVE III) at maximum 250°C and 160 bar pressure. The digested samples were transferred to vessel and diluted with 50 ml by adding double de-ionized water (B-pure, Barnstead). In total 84 wheat samples, 3 Standard Reference Material (SRM) (SRM1567a wheat flour) and 5 method blanks (5 ml $HNO_3$ solution) were digested and diluted for total analysis of zinc and iron.

### 3.2.4.2 Instrumentation and Measurement:

Concentrations of Fe and Zn in wheat grain were analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 5300 DV) for wheat samples, SRM, method blanks, standard and instrumental blank. One standard and next instrumental blank (10% HNO3) were analyzed just before and after the analysis of each 10 samples. The standard was 50 ml 10% HNO3 solution with concentration of 1mg/liter for both Fe and Zn. Repeated measurements were to monitor the instrumental drift during analysis and the concentration of standard offered the limit of measurement for Fe and Zn from concentration zero to 1 mg/L.

Finally, the metal concentration in ml/liter was converted to mg/kg on dry weight (DW) basis. For the assurance of data and validation of method, the concentration of Fe and Zn in SRM 1567a was tested against certified values by NIST (1987) for wheat flour. Total uptake of Fe and Zn in wheat grain was calculated according to formula stated below. The total uptake represents for the total amount of accumulated Fe and Zn in the grain.

$$\text{Total metal uptake in grain (mg pot}^{-1}) = \{(\text{metal concentration, mg kg}^{-1} / 1000), \text{mg g}^{-1}\} \times \text{dry weight of grains, (g pot}^{-1})$$

### 3.2.5 Quality assurance and Method Validation:

The ICP-OES was used to analyze the total concentration of Fe and Zn in ultraclave digested whole wheat grain samples. In addition, the ICP-OES analysis included three replicates of standard reference materials (SRM), standards and blanks.

The calibration of instrument was done by measuring the concentration of Fe and Zn in standard solutions which had a fixed concentration of 1 mg/L. If the measured concentration is 1 mg/L or very near to it, the instrumental drift is said to be minimal, which was fulfilled in this case and relative standard deviation (RSD) was < 5% (Table 3.3).

Lower detection limits (LOD's) and lower quantification limits (LOQ's) were determined for the concentration of Fe and Zn in the method (Table 3.4) and instrumental blanks (Table: 3.5). Measured concentrations of Fe and Zn in all samples were higher than LOD's (Average of blanks plus 3 times the SD) and LOQs (Average of blanks plus 10 times the SD).

The accuracy of analytical method was determined by the analysis of three replicates of standard reference materials (SRMs 1567a wheat flour). The measured concentrations of Fe and Zn in SRMs were in accordance to the certified concentration limits, 14.1 ± 0.5 mg kg$^{-1}$ for Fe and 11.6 ± 0.6 mg kg$^{-1}$ for Zn (NIST 1987) and the RSD was < 5% (Table 3.3). In overall the ICP-OES analysis conducted during this study was valid and provided accurate determination of Zn and Fe concentration.

Table 3.3: Average concentration and RSD for SRMs and standard solution.

| **Particulars** | **No. of rep.** | **Avg. con., mg/kg** | | **RSD %** | | **Certified con. mg/kg** | |
|---|---|---|---|---|---|---|---|
| | | **Fe** | **Zn** | **Fe** | **Zn** | **Fe** | **Zn** |
| **SRMs 1567a** | 3 | 14.10385 | 11.42502 | 2.32 | 1.01 | 14.1 ± 0.5 | 11.6 ± 0.6 |
| **Standard solution** | 10 | 1.0137 | 1.0125 | 1.3 | 0.9 | | |

Table 3.4: LODs and LOQs for method blanks.

| **Method blank** | **Concentration** | |
|---|---|---|
| | **Fe, mg/kg** | **Zn, mg/kg** |
| Blank | -0.0001 | 0.0004 |
| Blank | 0.0001 | -0.0005 |
| Blank | -0.0024 | 0.0001 |
| Blank | -0.0023 | -0.0007 |
| Blank | -0.0007 | -0.0004 |
| **Average** | **-0.00108** | **-0.00022** |
| **Stdev** | **0.0011967** | **0.000455** |
| **LOD** | **0.002510** | **0.001145** |
| **LOQ** | **0.013163** | **0.005005** |

The

Table 3.5: LODs and LOQs for instrumental blanks.

| **Instrumental blank** | **Concentration** | |
|---|---|---|
| | **Fe, mg/kg** | **Zn, mg/kg** |
| Blank | -0.0008 | -0.0008 |
| Blank | -0.0011 | 0.0001 |
| Blank | -0.0008 | -0.0008 |
| Blank | -0.0019 | -0.0009 |
| Blank | -0.0006 | -0.0007 |
| Blank | -0.0007 | -0.001 |
| Blank | -0.0009 | -0.0003 |
| Blank | -0.0013 | -0.0005 |
| Blank | -0.0011 | -0.0006 |
| **Average** | **-0.001022** | **-0.0006111** |
| **Stdev** | **0.0003962** | **0.0003408** |
| **LOD** | **0.000166** | **0.000411** |
| **LOQ** | **0.002939** | **0.002796** |

### 3.3 Localization of Fe and Zn in wheat grain:

Many earlier and contemporary studies have proved the importance of Zn and Fe for human health and plant development and also some parts of grain like bran of wheat rich in minerals is discarded in a milling process to make flour for human. Bearing this in mind, few wheat grains produced during this study were analyzed using modern techniques with aim to investigate the location of Zn and Fe within a grain. At first, the Environmental scanning electron microscope with dispersive X-ray spectrometry (ESEM-EDSX) technique was used available in the Norwegian University of Life Sciences (UMB) at ÅS, Norway but the sensitivity of SEM-EDS was not enough to detect and quantify target elements. It was realized that there is a need of 10 – 100 times higher sensitivity for detection and further study. Therefore, the analyses were performed using Scanning macro- X-ray fluorescence (MA-XRF) and laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS).

Selected six wheat grains representing different treatments and relatively higher in Zn (41.69 to 98.77 mg/kg) and Fe concentration (43.78 to 114.57 mg/kg) were mounted on plasticine and subjected first to MA-XRF, then to LA-ICPMS. MA-XRF created elemental distribution maps for Zn, Fe and some other elements. This allowed understanding the general pattern of elemental distribution in the grain, and in particular of Zn and Fe in this study. This work was carried out at the University of Antwerp, Belgium using a non-commercial self-assembled Scanning macro- X-ray fluorescence (XRF) and more precisely the setup named instrument C (Alfeld et al. 2011) was used. The elemental maps were recorded with a step size of 25 µm and dwell time of 400 ms per point; with tube settings of 50 kV and 1.0 mA (35 W) the beam size at the focal point was approximately 50 µm. The element distribution maps generated by MA-XRF were not quantitative because of many factors like absorption of X-rays by the sample material and by the air between sample and detector, surface roughness of sample and focus of the X-ray beam so decided to use only for the study of localization analysis of Zn and Fe in a grain. For a more detailed analysis of elements in grain, an additional technique was used as described below.

A more accurate localization of Zn and Fe and their relative abundance in different parts of a wheat grain was carried out by means of a laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS) in the University of Antwerp only in three grains out of six used in MA-XRF by means of a New Wave NWR193 ArF excimer laser and a Varian 7700 ICPMS using a quadrupole ICPMS. The ablation of sample was performed in helium gas (He) and transported to the plasma in argon gas (Ar). The flow rate was set to 0.4 l/min for carrier gas and

0.7 l/min for make-up gas. The forward power was set to 1350 watt. The line scan was executed at the speed of 10 um/s with dimension more than 3 mm length and 100 um beam diameter. The repetition rate of scan was 20 Hz at 90% energy capacity. The fluence was maintained at approximately 8 J/cm$^2$. The laser warm up at the beginning of scan lasted till 20 s and washout begin after about 290 s lasting until 350 s.

Net count was determined by subtracting average background counts for each element counts. Calculation of net counts can minimize the risk of over estimation of element counts and assure for more real estimation. Background count was the counts recorded along the scan line beyond the wheat grain which includes wash out generally after 290 s of scan time which falls at 2.9 mm of the scan line. While calculating background counts for each element counts at every scan line, raw counts obtained first hand were observed carefully, instrumental set up as described earlier and the length of scan line marked even in analyzed grains were considered together. So the location of sample lies in the middle of the scan line excluding warm up distance at the beginning and washout afterword. Net count was than corrected by replacing statistically not meaningful counts which were smaller than (average counts $_{\mathbf{background}}$ + (3×SD$_{\mathbf{background}}$)) with half of the 'average counts $_{\mathbf{background}}$' for all three elements K, Zn and Fe. Finally the normalized counts of Zn and Fe were determined by dividing their respective net counts with net counts for K. Potassium ($^{39}$K) was used as the normalizing element based on following considerations.

1. In the MA-XRF produced K distribution map of wheat grains in comparison to other analyzed elements, it was found more evenly distributed throughout the grain, and in particular in the crease (Fig. 3.16 D).
2. More than that the LA-ICPMS data for $^{39}$K showed the maximum counts within the sample (Fig. 3.17) among analyzed isotopes (counts for other elements not shown in the report) and a relatively persistent profile across the grain, once again with higher concentration in the crease.
3. The use of K-normalized counts for Fe and Zn, in contrast with net counts, will give us an indication of the positive increase of either element in spots/areas of the grain, independent from total ion count, laser focus, and surface roughness.

### 3.4 Data analysis and presentation:

The analysis of variance (ANOVA) was performed by 2-way ANOVA and the relation between variables were analyzed by regression model using Minitab 16. During the regression analysis, data for independent variables were centered on their average value when necessary. For centering of data, each observed value was subtracted from the average of respective variable. The comparison between all treatments considering the interaction of treatment factors and main effects of N- and Zn-Fe- treatments were carried out by Tukey comparison. In all cases, data were analyzed considering 5% level of significance ($p = 0.05$). Throughout the text, tables and graphs, values after the sign '±' are the standard error (SE) of the mean. Data are presented as bar diagrams and tables in the text. Values for grain and straw yield, number of grains, and total uptake of Zn and Fe in grains are presented on pot$^{-1}$ basis and each pot had eight wheat plants. Similarly, the concentrations of Zn and Fe in grain are presented as mg kg$^{-1}$ whole wheat grain and the protein concentration in percentage basis.

# 4 RESULTS:

Results presented in the following sections are based on the analysis of variance (ANOVA) considering N- and Zn-Fe- treatments as these factors were applied in the same pot (i.e. soil) to grow wheat. The purpose was to see the possible interactive effects of two treatment factors on wheat. F- value, p- value, correlation coefficient ($R^2$) and degree of freedom are presented in Table 4.1 and also mentioned in the text wherever necessary when main effects or interactions were significant. Whereas detail numerical tables for mean (± 1 SE, n = 4) of each treatment, comparison between all treatments considering interaction of treatment factors and main effects of treatments with their significance are presented in Annex 2 (Table 1 to 18).

## 4.1 Grain Dry yield:

The main effect of N- and Zn-Fe- treatment for grain dry yield was significant for experiment with soil application of nutrients ($p < 0.001$ respectively) but their interaction was not (Table 4.1). The grain yield pot$^{-1}$ increased while increasing N rate from 80 to 120 kg N ha$^{-1}$ then decreased when N was increased to 160 kg N ha$^{-1}$. Similar trend was observed even at split application of N from 120 to 160 kg N ha$^{-1}$. The single application of 120 kg N ha$^{-1}$ at sowing and split application of 160 kg N ha$^{-1}$ at sowing and stem elongation resulted similar and the highest yield (Fig. 4.1). Likewise, the soil application of Zn produced higher yield than soil applied Fe and Zn+Fe, particularly at application of 160 kg N ha$^{-1}$ at sowing (Fig. 4.1). In other N- treatments, soil applied Zn at sowing only showed a tendency of higher yield with respect to soil applied Fe and Zn+Fe (Annex 2: Table 1).

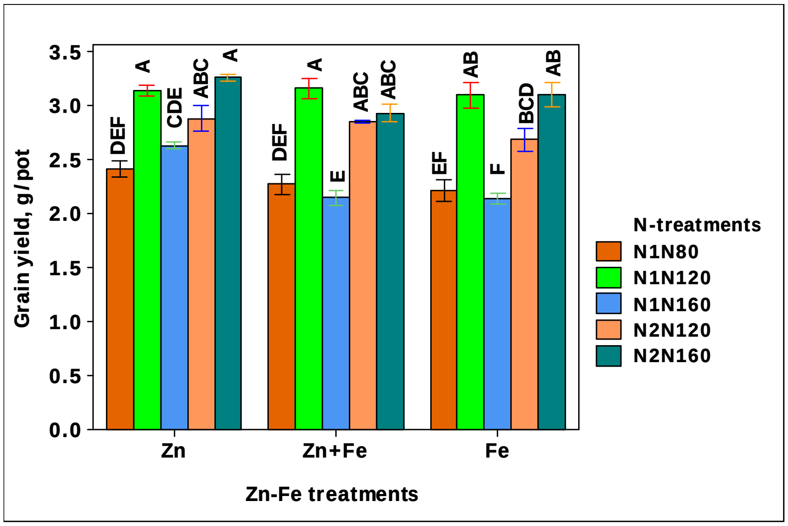

Fig. 4.1: Mean ± 1SE (n=4) bar plot of grain yield responses at experiment with soil application of nutrients. Zn, Zn+Fe and Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

Table 4.1: Variance for response of N- and Zn-Fe- treatments and their interaction.

Conditions for the ANOVA analysis were: N treatments, Zn and Fe treatments, N- × Zn-Fe-treatments. Analysis results are presented in same table side by side for treatments with soil application of nutrients and soil plus foliar application of Zn and Fe.

| **Treatments with soil application** | | | | | **Treatments with soil plus foliar application** | | | |
|---|---|---|---|---|---|---|---|---|
| **Response variables** | **Deg. of freedom** | **F-value** | **P-value** | **$R^2$, %** | **Deg. Of freedom** | **F-value** | **P-value** | **$R^2$, %** |
| **Grain yield:** | | | | | | | | |
| N-treatment | 4 | 72.52 | 0.000* | 87.93 | 1 | 10.92 | 0.002* | 54.06 |
| Zn-Fe treatment | 2 | 10.29 | 0.000* | | 5 | 1.97 | 0.107 | |
| Interaction | 8 | 2.14 | 0.052 | | 5 | 4.32 | 0.004* | |
| **No. of grains:** | | | | | | | | |
| N-treatment | 4 | 90.61 | 0.000* | 89.76 | 1 | 10.43 | 0.003* | 52.54 |
| Zn-Fe treatment | 2 | 6.96 | 0.002* | | 5 | 1.82 | 0.133 | |
| Interaction | 8 | 2.26 | 0.040* | | 5 | 4.06 | 0.005* | |
| **TGW:** | | | | | | | | |
| N-treatment | 4 | 9.82 | 0.000* | 50.73 | 1 | 0.07 | 0.798 | 46.99 |
| Zn-Fe treatment | 2 | 1.22 | 0.304 | | 5 | 5.32 | 0.001* | |
| Interaction | 8 | 0.58 | 0.791 | | 5 | 1.05 | 0.403 | |
| **Whole grain protein:** | | | | | | | | |
| N-treatment | 4 | 17.48 | 0.000* | 69.03 | 1 | 5.85 | 0.021* | 76.94 |
| Zn-Fe treatment | 2 | 13.00 | 0.000* | | 5 | 21.78 | 0.000* | |
| Interaction | 8 | 0.55 | 0.811 | | 5 | 1.08 | 0.388 | |
| **Grain iron con.:** | | | | | | | | |
| N-treatment | 4 | 28.38 | 0.000* | 81.32 | 1 | 2.19 | 0.152 | 61.82 |
| Zn-Fe treatment | 1 | 0.55 | 0.463 | | 5 | 10.33 | 0.000* | |
| Interaction | 4 | 4.13 | 0.009* | | 5 | 1.89 | 0.158 | |
| **Grain iron uptake:** | | | | | | | | |
| N-treatment | 4 | 7.57 | 0.000* | 61.61 | 1 | 5.48 | 0.028* | 55.01 |
| Zn-Fe treatment | 1 | 0.54 | 0.469 | | 5 | 6.42 | 0.002* | |
| Interaction | 4 | 4.33 | 0.007* | | 5 | 1.53 | 0.233 | |
| **Grain zinc con.:** | | | | | | | | |
| N-treatment | 4 | 8.75 | 0.000* | 59.01 | 1 | 0.61 | 0.443 | 48.50 |
| Zn-Fe treatment | 1 | 0.03 | 0.865 | | 5 | 5.77 | 0.004* | |
| Interaction | 4 | 2.04 | 0.113 | | 5 | 1.56 | 0.225 | |
| **Grain zinc uptake:** | | | | | | | | |
| N-treatment | 4 | 42.25 | 0.000* | 89.50 | 1 | 1.13 | 0.299 | 46.98 |
| Zn-Fe treatment | 1 | 3.08 | 0.089 | | 5 | 5.15 | 0.007* | |
| Interaction | 4 | 20.89 | 0.000* | | 5 | 1.57 | 0.224 | |
| **Straw yield:** | | | | | | | | |
| N-treatment | 4 | 104.70 | 0.000* | 90.92 | 1 | 128.91 | 0.000* | 81.14 |
| Zn-Fe treatment | 2 | 2.79 | 0.035* | | 5 | 0.68 | 0.642 | |
| Interaction | 8 | 3.27 | 0.008* | | 5 | 4.51 | 0.003* | |

p values with sign * are statistically significant at 5% level of significance.

In case of soil plus foliar spray of Zn and Fe, the interaction effect ($p = 0.004$) and main effect of N- treatment ($p = 0.002$) were significant. The soil plus foliar application of 30 % higher Zn and Fe single or together did increase the grain yield in comparison to their soil application at sowing. The single application of 120 kg N $ha^{-1}$ resulted in significantly higher yield (3.02±0.04 g $pot^{-1}$) than the split application of 120 kg N $ha^{-1}$ (2.87±0.04 g $pot^{-1}$) (Table 4.2) in all combinations of Zn and Fe except for soil plus foliar spray of Zn. The Zn-Fe treatment did not show significant difference in mean grain yield for experiments with soil plus foliar application of Zn and Fe ($p = 0.107$).

Table 4.2: Grain yield (g $pot^{-1}$) at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average**** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | **(n = 24)** |
| **N1N120** | 3.14±0.052 A | 3.16±0.092 A | 3.1±0.117A | 2.9±0.066 AB | 2.79±0.058 AB | 3.045±0.058 AB | **3.02±0.04a** |
| **N2N120** | 2.88±0.119 AB | 2.85±0.007 AB | 2.69±0.107B | 3.1±0.072 A | 2.84±0.042 AB | 2.85±0.091AB | **2.87±0.04b** |
| **Avg. # (n=8)** | **3.01±0.077a** | **3.01±0.204a** | **2.89±0.107a** | **3.0±0.0591a** | **2.81±0.035a** | **2.95±0.177a** | |

****** Significant at $p = 0.01$; # Not significant at $p = 0.05$; Avg. = Average; Treatment means ± 1SE ($n = 4$) followed by same upper case alphabet are not significantly different for N- × Zn-Fe-treatments. Average means followed by same lower case alphabet are not significantly different for respective N- or Zn-Fe- treatments. Tukey comparison was performed at 5% level of significance. N1 and N2 stand for single and split application of N. N120 stands for soil application of 120 kg N $ha^{-1}$. Zn, Zn+Fe and Fe without suffix for soil application of Zn-Fe-treatments and with suffix 's+f' for soil plus foliar application.

## 4.2 Number of grains:

The number of grains $pot^{-1}$ responded significantly to main and interaction ($p < 0.05$) effects of both N- and Zn-Fe- treatments (Table 4.1). The number of grains $pot^{-1}$ increased while increasing N rate from 80 to 120 kg N $ha^{-1}$ then decreased at 160 kg N $ha^{-1}$ as observed for grain yield $pot^{-1}$. Single soil application of N equivalent to 120 kg N $ha^{-1}$ and split application equivalent to 160 kg N $ha^{-1}$ produced higher and similar number of grains for single as well as combined Zn and Fe supplied in soil (Table 4.3). Similarly, at N- treatments N1N80 and N1N160, yield significantly lower number of grains $pot^{-1}$. In respective N- treatment, soil applied Zn and Zn+Fe resulted higher number of grain $pot^{-1}$ than at Fe application, except at split application of 160 kg N ha$^{-1}$ (Table 4.3). In total, soil applied Zn at sowing significantly increased the number of grains pot$^{-1}$with respect to Fe and Zn+Fe.

Table 4.3: Number of grains pot$^{-1}$ at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average **** |
|---|---|---|---|---|---|---|
| **Zn-Fe- treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | **(n=20)** |
| **Zn** | 90.50±4.17DEF | 122.0±2.20 A | 95.75±0.479 CDE | 108.75±4.25ABC | 124.25±0.946 A | **108.25 ± 3.31a** |
| **Zn+Fe** | 88.25±4.48 DEF | 124.0±2.48A | 77.75±1.49 F | 109.50±2.10 ABC | 114.25±4.05AB | **102.75 ± 4.12b** |
| **Fe** | 86.25±4.31 EF | 118.5±3.84AB | 76.75±2.39F | 103.50±3.84 BCD | 120.25±2.84 A | **101.05 ± 4.20b** |
| **Avg.*** (n = 12)** | **88.33 ± 2.32 c** | **121.50 ±1.68 a** | **83.42 ± 2.77c** | **107.25 ± 2.01b** | **119.58 ± 1.96a** | |

*** Significant at $p = 0.001$; ****** significant at $p = 0.01$; Avg. = Average; Treatment means ± 1SE (n = 4) followed by same upper case alphabet are not significantly different for N- × Zn-Fe- treatments. Average means followed by same lower case alphabet are not significantly different for respective N- or Zn-Fe- treatments. Tukey comparison was performed at 5% level of significance. N1 and N2 stand for single and split application of N. 80, 120 and 160 stands for rate of N in kg ha$^{-1}$. Zn, Zn+Fe and Fe represent the soil application of Zn-Fe- treatments.

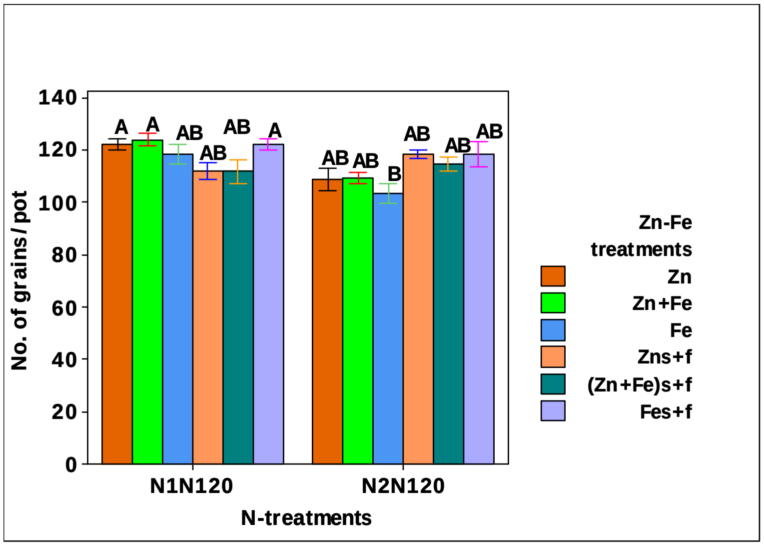

Fig. 4.2: Mean ± 1SE (n=4) bar plot of number of grains pot$^{-1}$ at experiment with soil plus foliar application of Zn and Fe. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N ha$^{-1}$. Zn, Zn+Fe and Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

In the experiment with soil plus foliar application of 30% higher Zn and Fe, single application of 120 kg N ha$^{-1}$ at sowing produced significantly higher number of grains (118.38±1.53, n = 24) than the split application of 120 kg N ha$^{-1}$ (112.21±1.68, n = 24) ($p < 0.01$). Particularly the case was noticed for single application of 120 kg N ha$^{-1}$ at sowing when Zn and Zn+Fe applied in soil and soil plus foliar Fe as compared with soil application of Fe at N2N120 (Annex 2: Table 4). Within split application of 120 kg N ha$^{-1}$, soil plus foliar applied Zn and Zn+Fe produced higher number of grains pot$^{-1}$ than at soil applied Fe. Otherwise main effect of Zn-Fe treatment was not significant ($p > 0.05$) but able to interact ($p < 0.01$) with N-treatments ensuring the distinct difference in mean number of grains pot$^{-1}$ (Fig.4.2).

### 4.3 Thousand grains weight (TGW):

Thousand grains weight (TGW) is the quality parameter for yield. Higher TGW, larger is the size and/or weight of individual grain higher will be yield as it is considered as one of the major factor in determining final grain yield. There was a significant difference in mean TGW only between N- treatments ($p < 0.001$) for the experiments with soil application of nutrients. Single application of N equivalent to 160 kg N ha$^{-1}$ (N1N160) was the best (27.66 ± 0.28 g/1000 grains, n = 12) among all N-treatments at separate as well as combined soil application of Zn and Fe. It was significantly higher compared to N1N120, N2N160 at soil Zn+Fe and N1N80 at soil Fe (Fig. 4.3). Effects of other treatments were more or less similar.

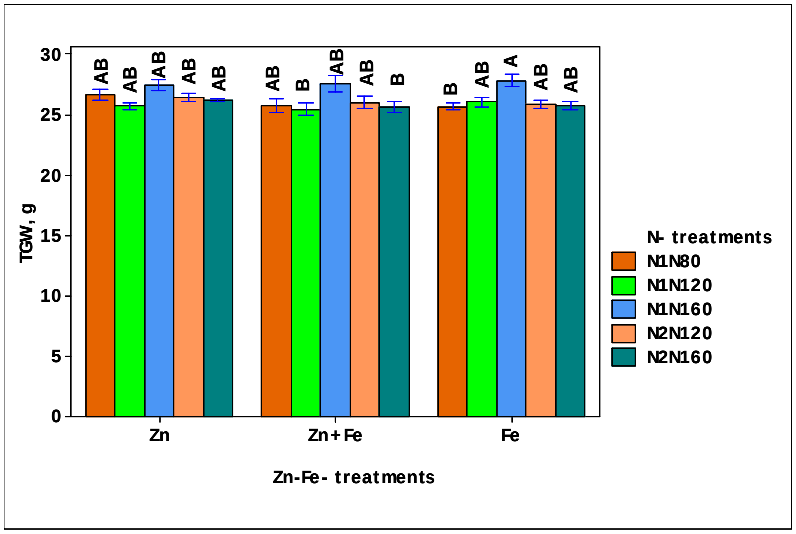

Fig. 4.3: Mean± 1SE (n=4) bar plot of TGW (g) at experiment with soil application of nutrients. Zn, Zn+Fe and Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

In contrast to the experiment with soil application of all nutrients, the foliar spray of 30% higher Zn and Fe in addition to soil applied amount, resulted in significant difference in mean TGW ($p = 0.001$) for the experiments with soil plus foliar application of Zn and Fe. Soil plus foliar sprayed Fe significantly lowered TGW in comparison with soil as well as soil plus foliar application of Zn at split application of N equivalent to 120 kg N ha$^{-1}$ (Fig. 4.4), whereas the responses were in par among other treatments. The interactive effect of N- and Zn-Fe- treatments remained non-significant at 5% level of significance.

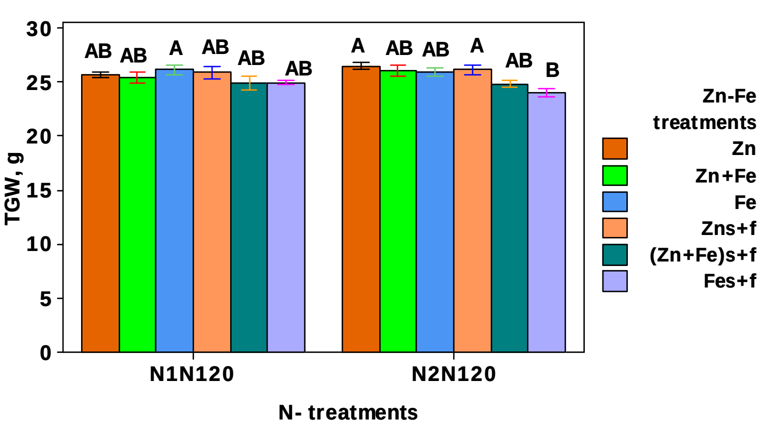

Fig. 4.4: Mean ± 1SE (n=4) bar plot of TGW (g) at experiment with soil plus foliar application of nutrients. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N ha$^{-1}$. Zn, Zn+Fe and Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

### 4.4 Straw yield:

Straw yield represents oven dried (75°C for 48 hours) aerial biomass about 1 cm above soil surface and devoid of spike.

In experiment with soil application of nutrients, straw yield responded to the main effect of N-treatment and N-treatment × Zn-Fe treatment (Table 4.1). When Zn and Fe were applied in separate or together in soil, single application of 120 kg N ha$^{-1}$ at sowing produced the largest amount of straw followed by split application of 160 and 120 kg N ha$^{-1}$. As observed for grain yield and number of grins pot$^{-1}$, straw yield also increased with increasing N rate at sowing from 80 to 120 kg N ha$^{-1}$, then decreased when further increase to 160 kg N ha$^{-1}$ (Fig. 4.5). For instance, the lowest and similar straw yield was in connection to single application of the highest (160 kg N ha$^{-1}$) and lowest (80 kg N ha$^{-1}$) rates of N at sowing (Fig. 4.5). Within soil applied Zn-Fe- treatments, the average straw increased significantly when Zn was mixed in soil at sowing than supplying Zn+Fe but the difference in yield was not significant in comparison with Fe. The lowest yield was recorded for application of Zn+Fe at single application of 160 kg N ha$^{-1}$.

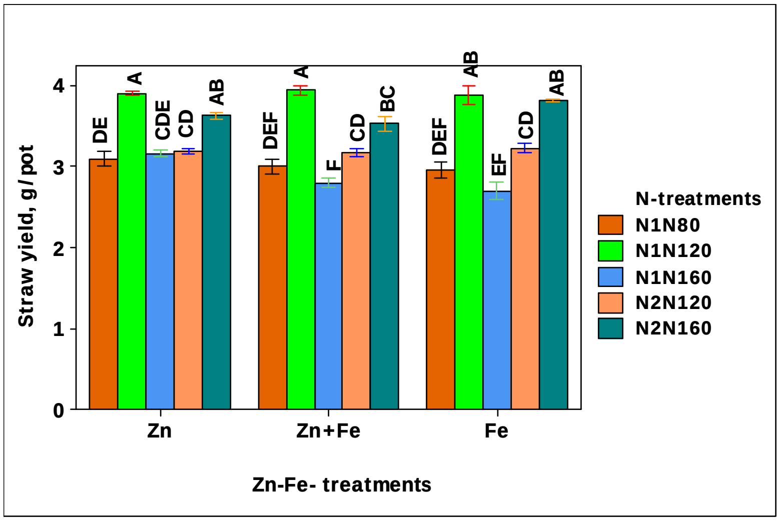

Fig. 4.5: Mean± 1SE (n=4) bar plot of straw yield (g pot$^{-1}$) at experiment with soil application of nutrients. Zn, Zn+Fe and Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

When Zn and Fe were applied both in soil and foliage, at single and split application of 120 kg N ha$^{-1}$, the interaction of N and Zn-Fe treatment ($p < 0.05$) and N ($p < 0.001$) main effects produced significant difference in mean straw yield. The N effect was larger and positive for all Zn and Fe treatments under single application of 120 kg N ha$^{-1}$ than at split application of N (Fig. 4.6). The main effect of Zn-Fe- treatment was not significant ($p > 0.05$) rather tend to lower at single application of 120 kg N ha$^{-1}$ and increase slightly when Zn and Zn+Fe was applied both in soil and of foliage at split 120 kg N ha$^{-1}$ (Fig 4.6).

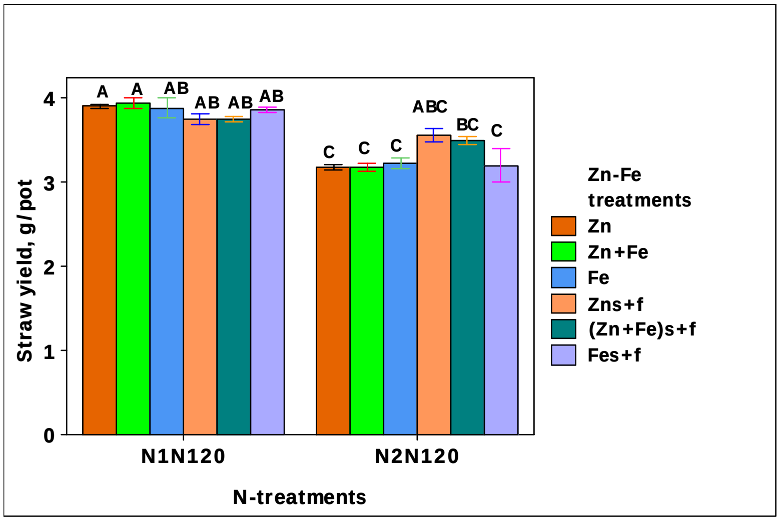

Fig. 4.6: Mean ± 1SE (n=4) bar plot of straw yield (g) at experiment with soil plus foliar application of Zn and Fe. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N ha$^{-1}$. Zn, Zn+Fe and Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

### 4.5 Relation between grain yield parameters, straw yield and treatment factors.

Encompassing all treatments and replicates, a multiple linear regression (Eq. 1) showed significantly positive role of TGW and number of grains pot$^{-1}$, suggesting increase in grain yield was combined effect of number of grains pot$^{-1}$ and unit weight of grain. In Eq. 1, data for number of grains pot$^{-1}$ and TGW were centered on their sample averages 107.5 grains pot$^{-1}$ and 25.98 g respectively. The coefficient of determination (R-sq) defined more than 99.71% of the variability in grain yield (g pot$^{-1}$). Although, TGW tend to increase the grain yield, in a regression analysis for TGW and number of grains pot$^{-1}$, TGW was negatively correlated with the number of grains pot$^{-1}$ (Eq. 2), suggesting unit weight of grain tended to decrease when the number of grains per spike increased, finally minimizing the importance of TGW to increase the grain yield. In addition, irrespective of proportional variation of grain yield and number of grain pot$^{-1}$, TGW responded more or less equally to all treatments except for significant increase at soil application of 160 kg N ha$^{-1}$ at sowing but unable to increasing the grain yield in par with other N-treatments (Annex 2: Table 5). Considering above mentioned relations and data, it signified grain yield of wheat var. Krabat was dependent on the number of grains per pot$^{-1}$ and little on unit weight of grains as indicated by TGW. Similarly, grain yield correlated positive and significantly with the straw yield (Fig. 4.7). The positive correlation demonstrated that when the straw yield increased, the grain yield also tended to increase.

$$\text{Grain yield (g pot}^{-1}\text{)} = 2.78 + 0.026 \text{ number of grains pot}^{-1} + 0.105 \text{ TGW (g)} \ldots\text{. (Eq. 1)}$$
$$p<0.001, \text{R-sq (adj)} = 99.71\%$$

$$\text{TGW (g)} = 25.99 - 0.04 \text{ number of grains pot}^{-1} \ldots\ldots\ldots\ldots\ldots\text{. (Eq. 2)}$$
$$P<0.001, \text{R-sq (adj)} = 29.76\ \%, r = -0.55$$

### 4.6 Whole grain protein (WGP):

In this study, whole grain protein (WGP) stands for the concentration of protein in whole grain flour estimated based on the percentage of nitrogen present in the flour. The range of protein concentration in grain varied from 7.3% to 9.3%. The interaction effect of N- and Zn-Fe-treatments was not significant but their main effects were significant at 5% level of significance in each experiment with soil application of all nutrients as well as soil plus foliar spray of Zn and Fe (Table 4.1).

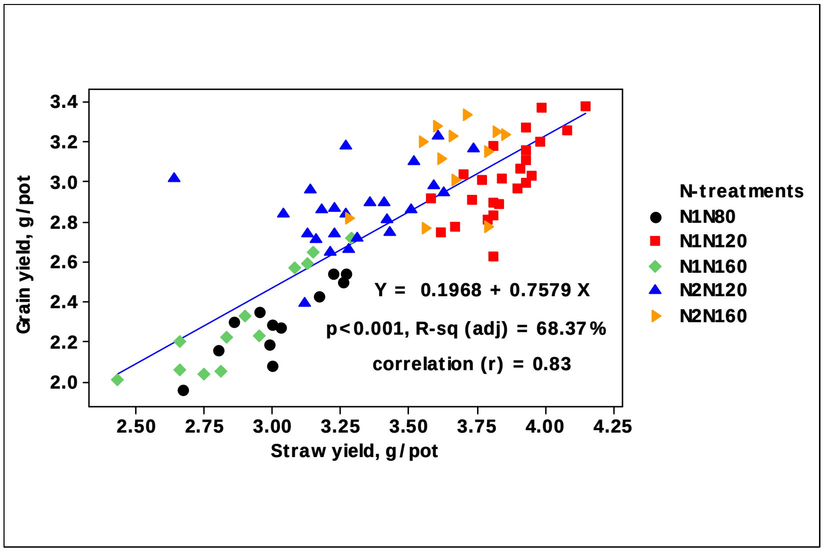

Fig. 4.7: Regression plot of mean grain yields against mean straw yield. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$.

In the experiment with soil application of nutrients, N- treatments, N2N160 and N1N80 resulted in comparatively higher whole grain protein (WGP) than rest of the N treatments (Table 4.4). Within the single application of N at sowing, the highest WGP was recorded for the lowest rate of N (80 kg N/ha) followed by intermediate equal to 120 kg N ha$^{-1}$ and the least for the highest amount of N applied at the rate of 160 kg N ha$^{-1}$ (Table 4.4). While comparing single and split application of N, split application brought the positive and higher protein content in wheat grain. It was obvious for both levels of N, N equivalent to 120 kg N ha$^{-1}$ showed a tendency for higher protein and 160 kg N ha$^{-1}$ was significantly higher when Zn and Fe applied in soil together or separate. As a main effect, within Zn-Fe- treatments, the combined application of Zn and Fe followed by soil applied Fe at sowing stood with significantly higher WGP than the single soil applied Zn. Among all treatments the relatively higher mean protein was recorded at split application of 160 kg N ha$^{-1}$ with soil applied Fe followed by Zn+Fe (Table 4.4).

The foliar application of 30% higher Zn and Fe in addition to that applied in the soil at sowing resulted in significantly higher WGP against soil application ($p = 0.021$) (Table 4.1) at single as well as split N equivalent to 120 kg N ha$^{-1}$ (Table 3.5). Irrespective of N treatments, average increment of WGP for soil plus foliar application of Zn+Fe, Zn and Fe was 8.38%, 6.5% and 7% with respect to protein at soil applied Zn+Fe, Zn and Fe. Among all treatments, the soil plus foliar application of Zn+Fe at split 120 kg N ha$^{-1}$ produced the most protein rich grains (8.96 ± 0.133 %), once again supporting split N as better method of achieving higher protein in wheat grain. While the Zn applied alone in soil at single application of 120 kg N ha$^{-1}$ ended up with the lowest rank in protein content (7.95±0.06 %) (Table 3.5). Irrespective of the Zn ad Fe treatments, the split application of N at the rate of 120 kg N ha$^{-1}$ produced about 2% higher WGP with respect to same rate of N supplied once at sowing as a main effect of N ($p<0.001$) (Table 4.5). But within Zn-Fe treatments, the effect of N was found not to be significant except for soil plus foliar applied Zn.

Table 4.4: Mean ± 1SE for whole grain protein (%) in wheat grains at experiment with soil application of nutrients.

|  | **N-treatments** |  |  |  |  | **Average***** |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | **(n = 20)** |
| **Zn** | 8.09±0.083BCDE | 7.9±0.108DE | 7.64±0.133E | 8.01±0.061CDE | 8.28±0.045ABCD | **7.984±0.0611b** |
| **Zn+Fe** | 8.56±0.075 ABC | 8.13±0.101BCDE | 8.02±0.148CDE | 8.32±0.108ABCD | 8.62±0.09AB | **8.331±0.0681a** |
| **Fe** | 8.35±0.172 ABCD | 8.0±0.503CDE | 7.91±0.086DE | 8.21±0.154ABCD | 8.75±0.164A | **8.245±0.0860a** |
| **Avg. *** (n=12)** | **8.333±0.0842ab** | **8.012±0.0542cd** | **7.857±0.0817d** | **8.180±0.0717 bc** | **8.550±0.0828 a** |  |

*** Significant at p = 0.001; Avg. = Average. The significance of letters after mean and treatments are explained in Table 4.3.

Table 4.5: Mean ± 1SE (n = 4) whole grain protein (%) in wheat grains at experiment with soil plus foliar application of Zn and Fe.

|  | **Zn-Fe treatments** |  |  |  |  |  | **Average *** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | **(n=24)** |
| **N1N120** | 7.9±0.108 C | 8.13±0.101BC | 8.0±0.042BC | 8.51.±0.103AB | 8.88±0.0.122A | 8.46±0.096AB | **8.315±0.0788 b** |
| **N2N120** | 8.01±0.061BC | 8.326±0.108BC | 8.21±0.154BC | 8.44±0.114ABC | 8.956±0.133A | 8.87±0.131A | **8.468±0.0836 a** |
| **Avg.*** (n=8)** | **7.95±0.061d** | **8.23±0.078cd** | **8.11±0.084d** | **8.48±0.073bc** | **8.92±0.085a** | **8.67±0.108ab** |  |

*** Significant at p = 0.001; ***** significant at p = 0.05; Avg. = Average. The significance of letters after mean and treatments are explained in Table 4.2.

## 4.7 Iron concentration in wheat grain:

During the analysis of data for the concentration and uptake of Fe in grain, among Zn and Fe treatments, the application of Zn alone in soil or along with 30% higher zinc as foliar spray were avoided since these treatments did not provide external iron to plants.

In the experiment with soil application of all nutrients, Fe concentration of whole grain was significantly affected by soil supplied N ($p < 0.001$) and the interaction ($p < 0.01$) with soil applied Zn and Fe but not by soil Zn-Fe- treatments itself ($p > 0.05$) (Table 4.1). The main effect of increasing N applied rate at sowing from 80 or 120 to 160 kg N $ha^{-1}$ resulted in significantly higher Fe concentration in grain. But the concentration was reduced while increasing N from 80 to 120 kg N $ha^{-1}$ (Fig. 4.8) when grain yield increased (Fig. 4.1). The Fe concentration in grain did not responded positively to the split application of N. For instance, the Fe concentration reduced by about 20.5% at split 160 kg N $ha^{-1}$ but did not differ at split 120 kg N $ha^{-1}$ in comparison with their respective single application of N at sowing (Annex 2: Table 11).

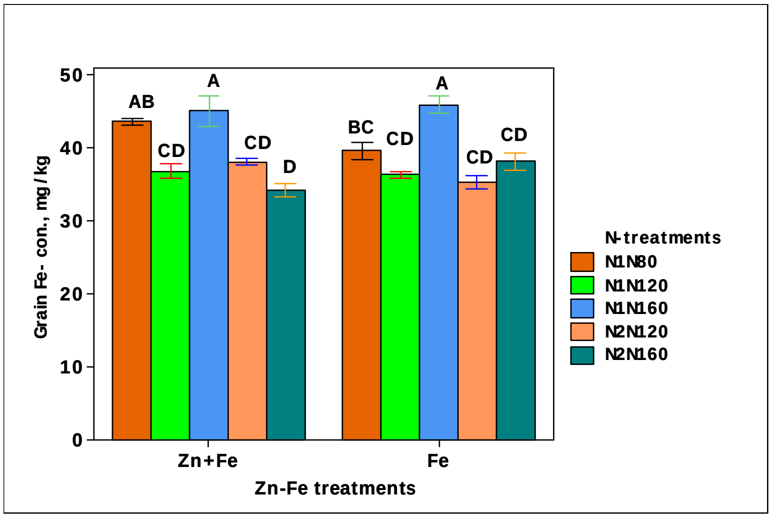

Fig. 4.8: Mean ± 1SE (n=4) bar plot of grain Fe-concentration (mg $kg^{-1}$) at experiment with soil application of nutrients. Zn+Fe and Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg $ha^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

The application of 30% higher Fe and Zn+Fe as foliar spray in addition to soil application caused positive and statistically significant ($p < 0.001$) increase in Fe- concentration in wheat grain (Table 3.1). The increment was by 34% and 64% respectively in comparison with soil applied Fe and Zn+Fe (Annex 2: Table 12). Either in soil or soil plus foliar Zn-Fe- treatments, the application of Zn+Fe revealed a trend of higher Fe- concentration in wheat grain against its respective soil application. But only soil plus foliar spray of Zn+Fe at single application of 120 kg N $ha^{-1}$ at sowing stood with significantly higher level of Fe- concentration with respect to all other treatments (Fig. 4.9). The interaction of Zn- Fe- and N- treatments ($p > 0.05$) and main effects of N ($p > 0.05$) were not significant in spite of some distinct tendency of higher Fe concentration for single against split application of N equivalent to 120 kg N $ha^{-1}$ (Table 4.1; Annex 2: Table 12).

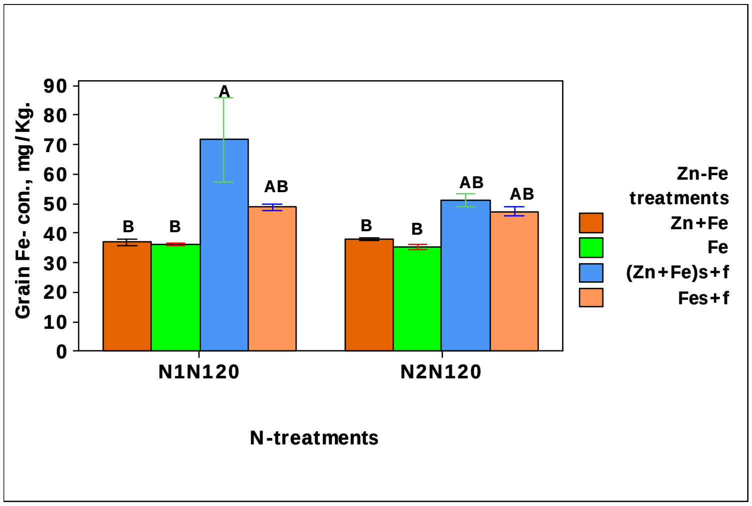

Fig. 4.9: Mean ± 1SE (n=4) bar plot of grain Fe-concentration (mg $kg^{-1}$) at experiment with soil plus foliar application of Zn and Fe. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N $ha^{-1}$. Zn+Fe and Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

### 4.8 Total Fe uptake in wheat grain:

Iron uptake in the grain stands for the total Fe accumulated in the grain on dry weight basis. In the experiment with soil application of all nutrients, total Fe- uptake in grain reacted significantly to the main effects of soil supplied N ($p < 0.001$) and the interaction ($p < 0.01$) with soil applied Zn and Fe (Table 4.1). But, the main effect of Zn and Fe treatment was not significant ($p > 0.05$) to increase total Fe- uptake in grain.

In spite of some variation, the effect of N application was not significant at soil applied Zn+Fe but it was significant at soil application of Fe. For example, split N at the rate of 160 kg N $ha^{-1}$ revealed significantly higher Fe uptake than at N- treatments, N1N80 and N2N120 when Fe was supplied in soil at sowing (Fig. 4.10). Increasing N rate from 80 to 120 kg N $ha^{-1}$ at sowing, the total Fe- uptake in grain increased when Zn+Fe or Fe was applied in soil. But the uptake decreased at 160 kg N $ha^{-1}$ as observed for the number of grains $pot^{-1}$ and grain yield. In general, split application of N equivalent to 120 kg N $ha^{-1}$ at sowing and stem elongation tended to lower Fe- uptake in grain with respect to single soil application of 120 kg N $ha^{-1}$. But, the uptake increased at split application of 160 kg N $ha^{-1}$ with respect to single soil application of 160 kg N $ha^{-1}$. It indicated, when the rate of N at sowing was higher, split N raised the uptake of Fe in wheat grain.

Under the single and split application of 120 kg N ha$^{-1}$, the foliar spray of Fe and Zn+Fe increased Fe uptake over soil applied Fe and Zn+Fe. The mean Fe uptake was 40% and 42% higher respectively due to the foliar spray of 30% higher Fe and Zn+Fe than their respective soil application. The significant increase was only noticed for combined application of Zn and Fe in soil and on foliage (Fig. 4.11). Interaction between N- and Zn-Fe- treatments ($p > 0.05$) was not able to bring any significant difference in Fe- uptake for the experiments with soil plus foliar application of nutrients (Table 4.1).

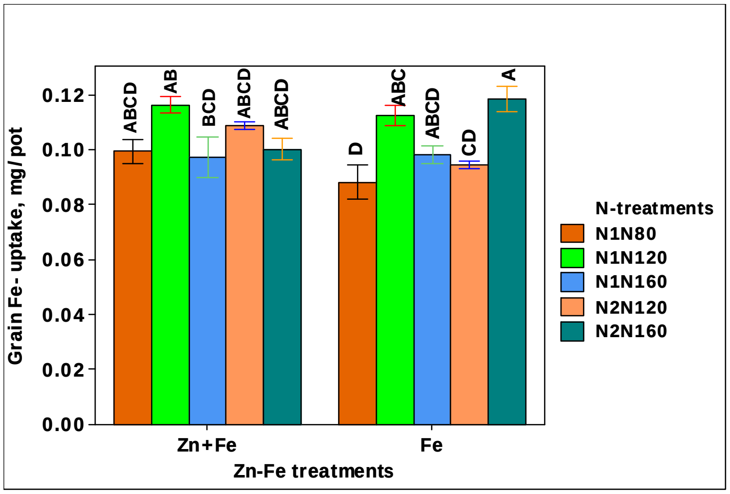

Fig. 4.10: Mean± 1SE (n=4) bar plot of total Fe-uptake in grain (mg pot$^{-1}$) at experiment with soil application of nutrients. Zn+Fe and Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

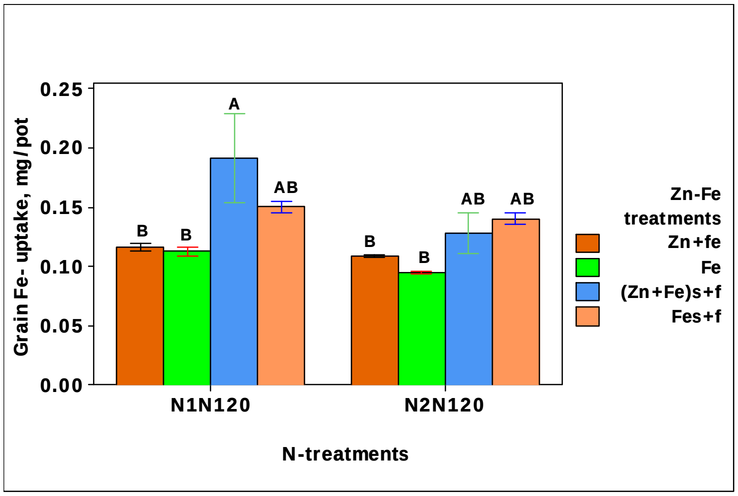

Fig. 4.11: Mean ± 1SE (n=4) bar plot of total Fe- uptake in grain (mg pot$^{-1}$) at experiment with soil plus foliar application of Zn and Fe. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N ha$^{-1}$. Zn+Fe and Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

## 4.9 Zinc concentration in wheat grain:

For the analysis of the concentration and uptake of Zn in grain, among the Zn and Fe treatments, the application of iron alone in soil or along with additional iron as foliar spray were avoided as they did not have external zinc supply to plants.

Grain Zn- concentration responded significantly for the main effect of N-treatment ($p < 0.001$) in experiments having soil application of nutrients. When N rate at sowing increased from 80 to 120 kg N ha$^{-1}$, the concentration of Zn in grain tend to decrease (Fig. 4.12). For instance, single application of 120 kg N ha$^{-1}$ at sowing and split application of 160 kg N ha$^{-1}$ produced about 9% and 10% less Zn concentration, respectively in comparison to single application of 80 and 160 kg N ha$^{-1}$ at sowing. Under the application of Zn+Fe, at single application of 80 and 160 kg N ha$^{-1}$ at sowing produced higher Zn- concentration in wheat grain than at single application of 120 kg N ha$^{-1}$ at sowing and split application of 160 kg N ha$^{-1}$ (Fig. 4.12) which had produced higher number of grains (Table 4.2) and grain yield pot$^{-1}$ (Fig. 4.1). All other combination of treatments produced similar concentration. Grain Zn- concentration did not responded at significant level for the main effect of Zn-Fe- treatments and its interaction with N- treatments (Table 4.1).

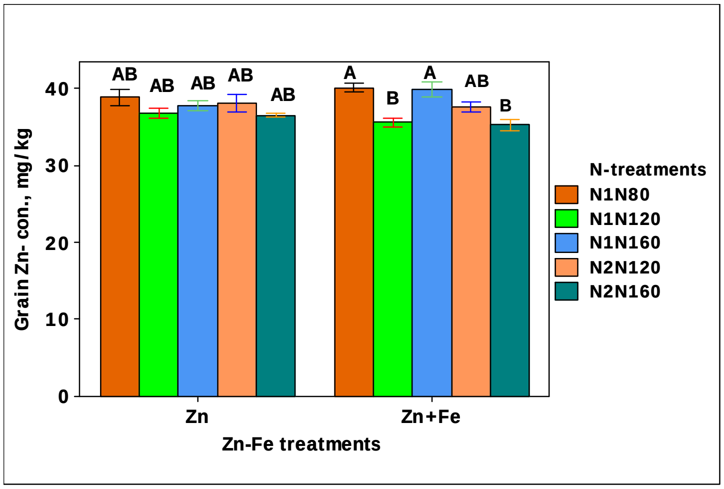

Fig. 4.12: Mean± 1SE (n=4) bar plot of grain Zn- concentration (mg kg$^{-1}$) at experiment with soil application of nutrients. Zn and Zn+Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

In the experiments with soil plus foliar spray of Zn and Fe, Zn- concentration in wheat grain responded significantly positive to the main effect of Zn-Fe- treatments ($p < 0.01$). But, Zn- concentration did not respond to the main effect of N- treatments and the interaction between N- and Zn-Fe- treatments (Table 4.1). Increase in the grain Zn- concentration on foliar sprayed treatments was higher than at treatments without foliar spray in both, single and split application of 120 kg N ha$^{-1}$. But, significantly higher Zn- concentration was noticed only for the soil plus foliar spray of Zn+Fe at single 120 kg N ha$^{-1}$ (Fig. 4.13). Irrespective of N- treatments, soil plus foliar supply of Zn and Zn+Fe resulted in 17 % and 46 % higher Zn- concentration in grain compared with the soil application of Zn and Zn+Fe respectively (Annex 2: Table 16) .

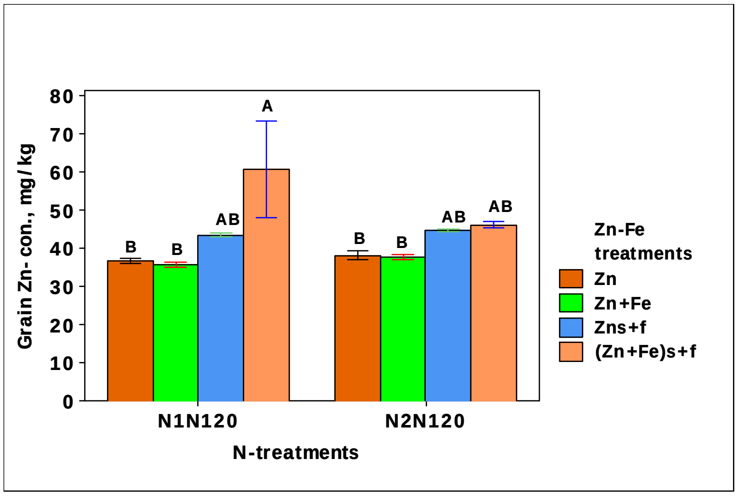

Fig. 4.13: Mean ± 1SE (n=4) bar plot of grain Zn- concentration (mg kg$^{-1}$) at experiment with soil plus foliar application of Zn and Fe. N1 and N2 respectively stand for single and split application of N. N120 stands for 120 kg N ha$^{-1}$. Zn and Zn+Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

### 4.10 Total Zn uptake in wheat grain:

In the experiments with soil application of all nutrients, the main effect of N- treatments was significant ($p < 0.001$) but not of the Zn-Fe treatments (Table 4.1). In general, total Zn- uptake in grain was higher when N- rate at sowing increased from 80 to 120 kg N ha$^{-1}$ but decreased at 160 kg N ha$^{-1}$ at sowing as observed for the total Fe- uptake in grain, grain yield and number of grains pot$^{-1}$. In comparison with single application of 120 kg N ha$^{-1}$, split application of 120 kg N ha$^{-1}$ tended to lower Zn- uptake at soil applied Zn and Zn+Fe. But, Zn- uptake was increased at split application of 160 kg N ha$^{-1}$ when compared with single application of 160 kg N ha$^{-1}$ at sowing. Particularly, at the soil application of Zn, the split application of 160 kg N ha$^{-1}$ significantly increased total Zn- uptake with respect to single application of 80 and 160 kg N ha$^{-1}$ (Fig. 3.14). Although the main effect of Zn and Fe treatments was not significant, total Zn- uptake in wheat grain was influenced to some extent by the interaction with N treatment ($p < 0.001$) (Table 4.1). For instance, the total Zn- uptake for 160 kg N ha$^{-1}$ at sowing was increased at soil applied Zn+Fe, but the uptake get reduced for all other N- treatments at soil applied Zn+Fe as compared with respective N- treatments at soil supplied Zn (Fig. 4.14).

The soil plus 30% higher foliar application of Zn and Fe generated significant difference in total Zn- uptake in grain ($p < 0.01$). Under the single and split application of 120 kg N ha$^{-1}$, the foliar spray of Zn and Fe increased total Zn- uptake over soil applied Zn and Fe. The average Zn- uptake was 17% and 35% higher for additional 30% foliar spray of Zn and Zn+ Fe, respectively than their respective soil applications. But, within single application of 120 kg N ha$^{-1}$ at sowing, the uptake of Zn was higher by 48% for 30% higher spray of Zn+Fe than in soil application of Zn+Fe (Fig. 4.15). Interaction between N- and Zn-Fe- treatments ($p > 0.05$) and main effect of N-treatment ($p>0.05$) were not able to bring any significant difference in Zn- uptake for the experiments with soil plus foliar application of nutrients (Table 4.1). Only a tendency of higher Zn- uptake was noticed for N1N120 at foliar applied Zn+Fe (Annex 2: Table 18).

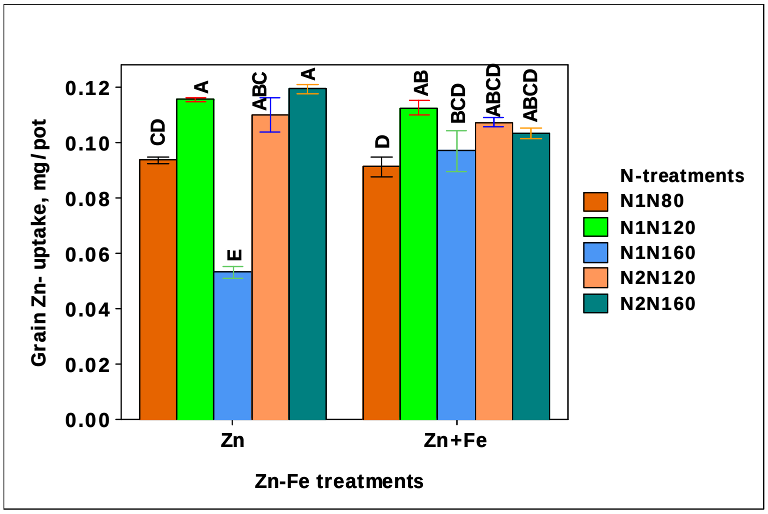

Fig. 4.14: Mean± 1SE (n=4) bar plot for the responses of total Zn- uptake in grain (mg pot$^{-1}$) at experiment with soil application of nutrients. Zn and Zn+Fe applied in soil at sowing. N1 and N2 for single and split N. 80, 120 and 160 stand for rate of N in kg ha$^{-1}$. Bars with same alphabet at head are not significantly different at 5% level of significance.

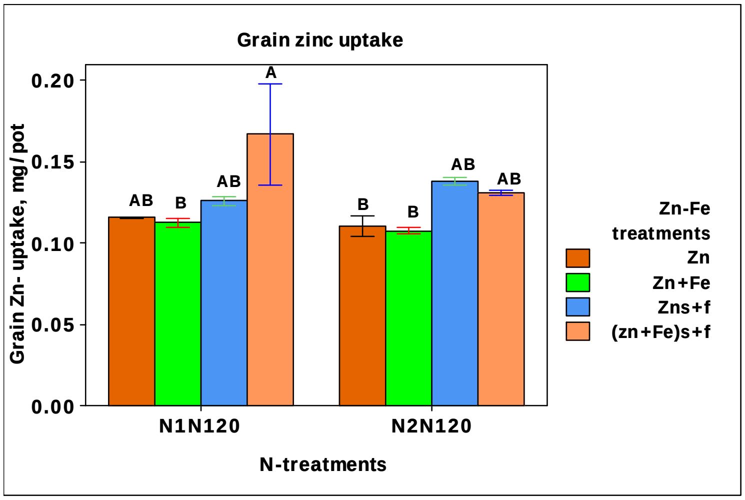

Fig. 4.15: Mean ± 1SE (n=4) bar plot of total Zn- uptake in grain (mg pot$^{-1}$) at experiment with soil plus foliar application of Zn and Fe. N1 and N2 respectively stand for single and split application of N; N120 for soil application of 120 kg N ha$^{-1}$. Zn and Zn+Fe without suffix for soil application and with suffix s+f for soil plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

### 4.11 Relation for WGP, grain yield parameters, Zn and Fe concentrations in wheat grain:

The WGP was not significantly correlated ($p > 0.05$) with grain yield pot$^{-1}$ although a trend for positive correlation was seen (insignificant regression equation not presented in the text). But, significant correlations exist with 1000 grains weight ($p < 0.001$) (Eq. 3) and number of grains pot$^{-1}$ ($p < 0.05$) (Eq. 4). A negative correlation with TGW indicated that larger size of wheat grains tend to lower the protein content in the grain. Similarly, a positive correlation with Number of grains pot$^{-1}$ indicated that when increase in number of grains pot$^{-1}$ or spike$^{-1}$ tend to increase the protein content in wheat grain probably higher number of grains produce smaller grains. While searching relations with Zn- and Fe- concentrations in wheat grain, Fe-concentration showed statistically valid positive correlation with WGP (Eq. 5) indicating that wheat grain protein tend to increase when grain Fe- concentration increased. Gain Zn-concentration did not evidence significant relation with WGP except a positive tendency.

WGP, % = 13.3044 - 0.1914 TGW, (g) ................................ (Eq. 3)
$p < 0.001$ (0.024) R-Sq (adj.) = 29.8%

WGP, % = 7.659 + 0.0062 No. of grains pot$^{-1}$ ....................... (Eq. 4)
$p < 0.05$ (0.024) R-Sq (adj.) = 4.9%

WGP, % = 7.742 + 0.0156 Grain Fe- concentration, (mg kg$^{-1}$) ...... (Eq. 5)
$p < 0.001$ R-Sq (adj.) = 25.6%

## 4.12 Relation for Fe- and Zn- concentration, their total uptake in grain and grain yield parameters:

A multiple linear regression including all measurements for 84 growth pots provided a valid relationship ($p < 0.001$) between Fe- concentration in grain and TGW, number of grains pot$^{-1}$ and grain Fe- uptake (Eq. 6) rather than with single variable. The coefficient of TGW and number of grains pot$^{-1}$ were negative, but positive for grain Fe- uptake. It indicated that grain Fe- concentration had a tendency to increase when total Fe- uptake in the grain increased but tend to lower with increase in grain yield parameters: TGW and number of grains pot$^{-1}$.

Grain Fe- concentration (mg kg$^{-1}$) = 69.888 - 0.959 TGW (g) - 0.321 No. of grains pot$^{-1}$ + 261.459 Grain Fe-uptake grain (mg pot$^{-1}$) …….. (Eq. 6)

$p < 0.001$ (for regression model, No. of grains pot$^{-1}$, Grain Fe- uptake); $p > 0.05$ for TGW; R-sq (adj) = 48.41%

Similarly, a regression analysis of Zn concentration in grain with TGW, number of grains pot$^{-1}$ and grain Fe- uptake in together showed a significant relation ($p < 0.01$) (Eq. 7). The regression model defined about 72 % of the variability in the grain Zn- concentration indicating the role of other variables in its determination. Positive coefficients of TGW and total Zn- uptake in grain indicated that grain Zn- concentration tend to increase with these factors and negative coefficient for number of grains pot$^{-1}$ hint for decrease in grain Zn- concentration when number of grains pot$^{-1}$ tended to increase.

Grain Zn- concentration (mg kg$^{-1}$) = 45.625 – 0.646 TGW (g) - 0.211 No. of grains pot$^{-1}$ + 120.98 Grain Zn-uptake (content) in grain (mg pot$^{-1}$) …….. (Eq. 7)

$p < 0.001$ (for regression model, Grain Zn- uptake; No. of grains pot$^{-1}$); $p > 0.05$ (for TGW); R-sq (adj) = 72.28 %

A significantly positive correlation ($p < 0.05$) existed between Zn and Fe concentrations in grain (Eq. 8). A positive correlation suggested that when Fe concentration increased in the grain, Zn concentration also tended to increase and vice-versa.

Grain Zn- concentration (mg kg$^{-1}$) = 24.899 + 0.288 Grain Fe- concentration (mg kg$^{-1}$)

(Eq. 8)

$p < 0.001$; R-sq (adj.) = 12.96%

### 4.13 Relation of Fe and Zn uptake with grain yield components:

A multiple linear regression including TGW and number of grains pot$^{-1}$ implied that uptake of Fe in grain was negatively correlated with TGW (Eq. 9) and grain Zn- uptake was positively correlated with number of grain pot$^{-1}$ (Eq. 10). It means, the uptake of Fe tended to decrease, when TGW decreased and grain Zn- uptake tended to increase when the number of grain pot$^{-1}$ increased. In all cases, coefficient of determination (R-sq) was small defining weaker relationships.

Grain Fe- uptake (mg kg$^{-1}$) = 0.465844 + 0.0002 number of grains pot$^{-1}$ - 0.0147 TGW, (g)
… ... (Eq. 9)
$p < 0.001$ (for regression model; TGW); $p > 0.05$ (for No. of grains pot$^{-1}$); R-sq (adj) = 25.54 %

Grain Zn- uptake (mg kg$^{-1}$) = -0.0506 + 0.0006 number of grains pot$^{-1}$ + 0.0031 TGW, (g)
…… (Eq. 10)
$p < 0.05$ (for regression model; No. of grains pot$^{-1}$); $p > 0.05$ (for TGW); R-sq (adj) = 5.82 %

### 4.14 Localization of Zn and Fe in wheat grain:

Element distribution map of half wheat grains along creases (Fig. 3.16) generated by the MA-XRF depict the pictures for distribution of Fe and Zn. Within a grain, relatively bright spots represent higher X- ray signal from respective elements that is influenced by many factors, of which element concentration is the main one, but the relation is not straightforward. Analyzed surface of grains were slightly irregular which could give somewhat hazy depiction of the element distribution but still some important information were gleaned.

It is obvious that Fe and Zn concentration varies inside the wheat grain which was also visible in this study (Fig. 4.16; 4.17; & 4.18). MA-XRF showed Fe and Zn seem to have a similar localization within grains. Iron was found concentrated mainly in embryo and to some extent along aleurone layer in the crease area (Fig. 4.16. B). Element distribution map of Zn depicted presence of Zn in embryo and along crease just outside the endosperm (Fig. 4.16. C).

To overcome the hazy depiction of elemental distribution and qualitative analysis offered by MA- XRF technique, LA-ICPMS was performed on three out of six samples that were analyzed by MA-XRF for its higher sensitivity and quantitative measurements based on elemental or isopitic counts per second at ablated location in the scan line. More precisely, LA-ICPMS provided relatively semi-quantitative comparison on the normalized counts of Zn and Fe elements along scan lines. Representative plots along two scan lines for normalized counts of Fe and Zn (Fig. 4.18) clearly showed:

1. Generally, the peaks of normalized counts for Zn and Fe vary together;
2. Higher metal concentration was located at an embryo and aleurone layer at the two ends of the scan line with minimum at endosperm in the middle;
3. Higher normalized counts can be seen for Fe than Zn at the end of scan line (represent bran area in general) point out relatively higher concentration of Fe, in particular at the ends of the grain but higher counts of Zn in the middle.
4. Output from both MA-XRF and LA-ICPMS, represented the co-localization of Fe and Zn in a wheat grain mainly at embryo, crease and/or aleurone.

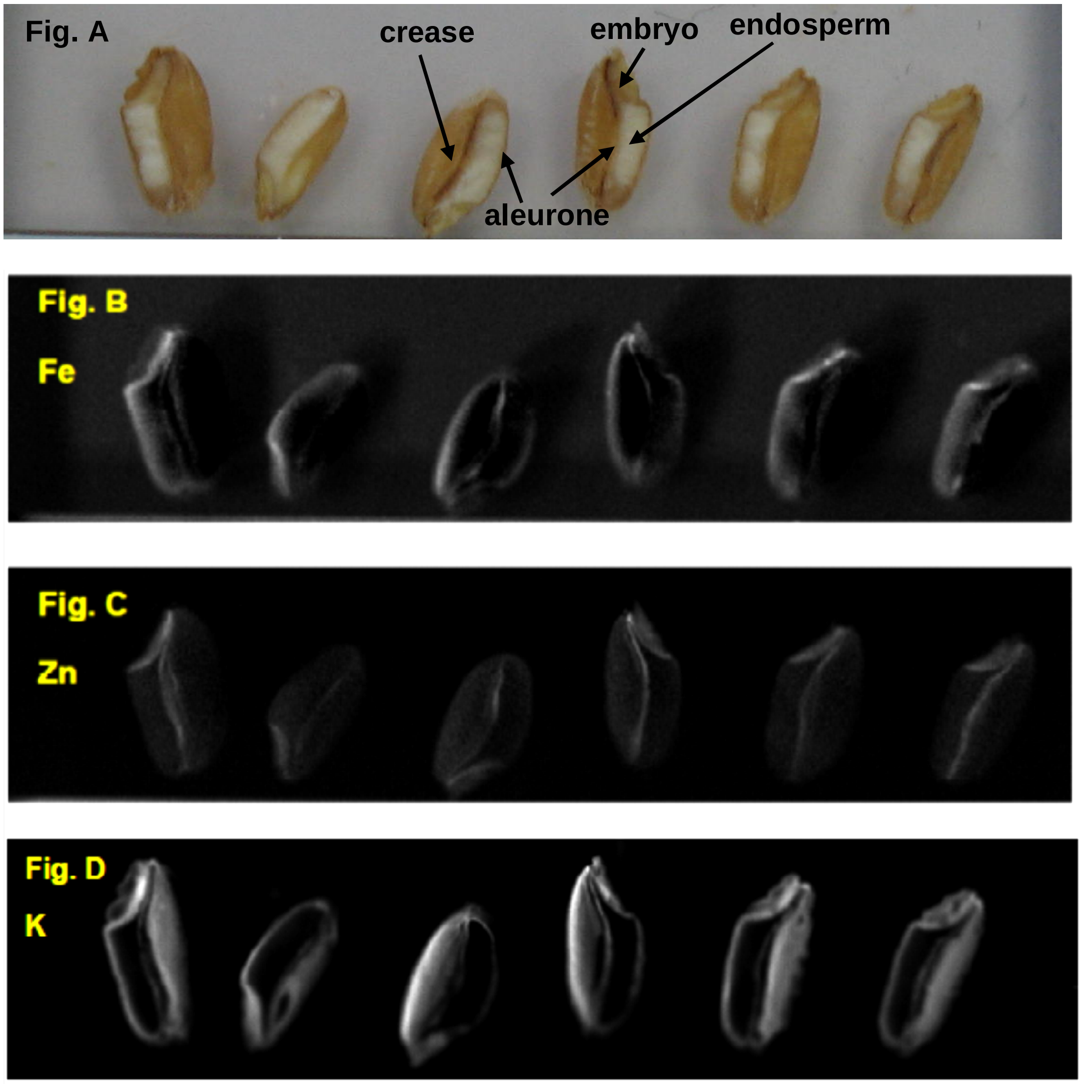

Fig. 4.16: Half wheat grains analyzed under MA-XRF Instrument C. The relief effects were not normalized and figures are not in scale. Grains from left to right represent sample 77, 7, 31, 64, 67 and 66.Sample numbers were picked from the pot number during the experiment. A: Position of wheat grains subjected to MA-XRF. B C & D: Element distribution maps (1400 × 400 pixels) of wheat grains. B. Iron, C. Zinc and D. Potassium.

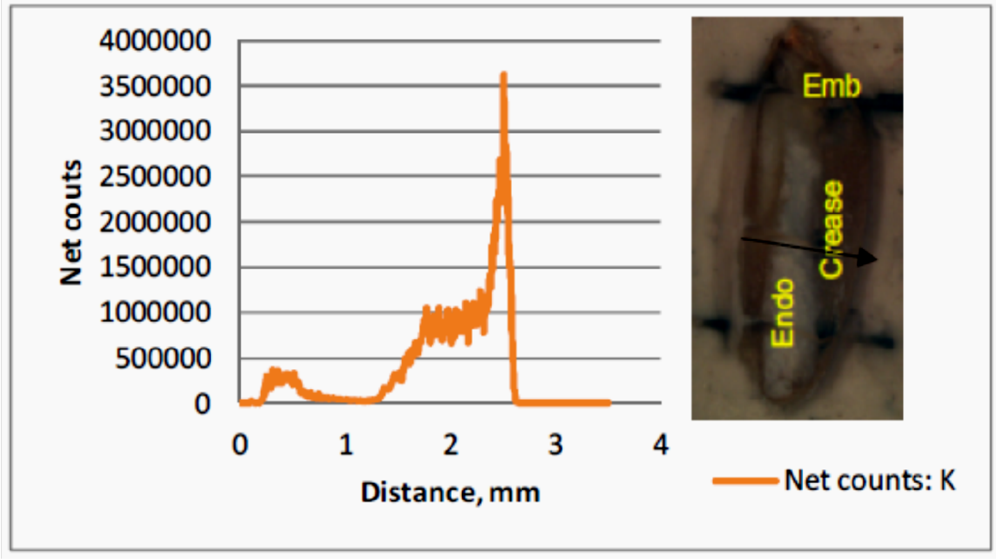

Fig. 4.17: Plot of net counts for 39K vs. distance. Scan line is represented by arrow in the wheat picture at right of plot and arrow head shows the direction of scan. Emb: embryo; Endo: endosperm.

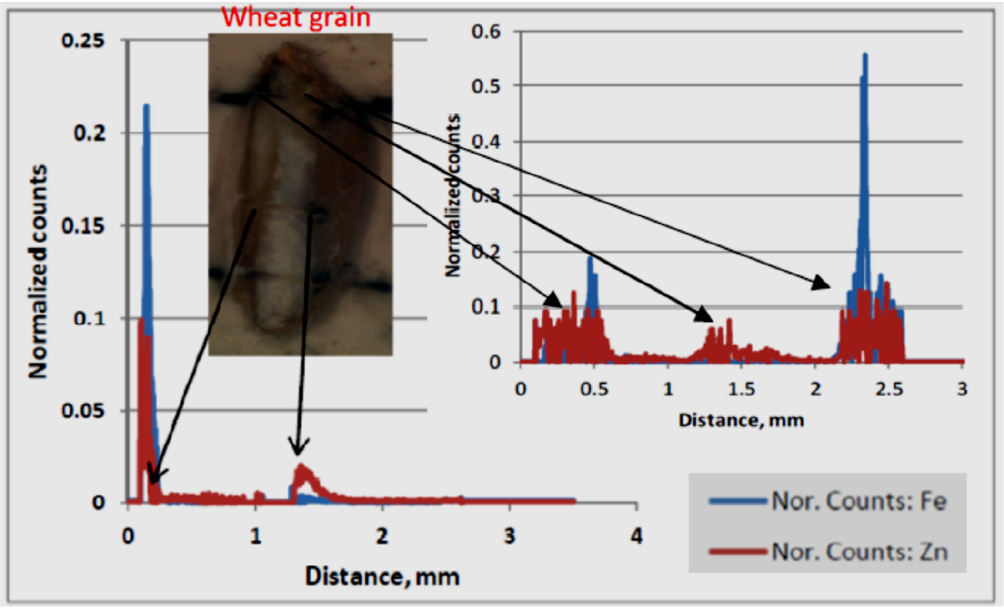

Fig. 4.18: Normalized counts for Fe or Zn vs. distance. Scan line for plot at top right corner passes through embryo at the middle of scan line and at bottom left corner through bran at two sides and endosperm at the middle. Normalized counts represent the relative count intensity of Zn and Fe with respect to K. Arrows indicate approximate location for elevated Zn and Fe concentration in the grain fall on scan line. White part in the picture of wheat grain is endosperm and bran on either sides of endosperm. Germ is at the top the grain. Bran at right hand side of endosperm is crease of wheat grain. Wheat grain was subjected to laser ablation-inductively coupled plasma mass spectrometry (LA-ICPMS) analysis.

# 5 DISCUSSIONS:

## 5.1 Why did lower yield and protein experienced in overall?

Other than the consequence of no tillers, the overall production of lower grain yield and protein in grain during this study could be due to the combination of poor availability of N particularly at the flowering and grain filling periods (Green 1984) as wheat was grown in nutritionally poor growth media (80% medium grain sand, 10% kaolin and 10% peat). The availability of N may further be depleted due to competitive consumption of initially applied N for the vigor of vegetative growth (Marschner 1995; Li et al., 2001; Brown et al., 2005) leaving less N for late growth stage when N was supplied only once at sowing or insufficient split dose (as observed at split of 120 kg N ha$^{-1}$). The range of split N- rate in this study was not enough to determine the optimum N- rate required for split application. Higher temperature (21° C) and enough moisture due to continuous water supply (in every two days interval) and the deficiency of N might became more worse through $NH_3$ loss (not measured) by the process called volatilization (FAO 2001; Søgaard et al., 2002) reducing available N at grain filling in comparison to assimilate supplied to grain (Brown et al., 2005) resulting lower protein in grain (7.3% to 9.3%). Nitrogen loss through leaching was not possible in this experiment as wheat was grown in plastic pots. In addition to adequate N at sowing, necessity of more N at late stage of wheat growth was felt for better yield and protein at least in N deficient condition. As an illustration, a tendency of increasing yield as well as protein content was seen in the highest rate of split N (160 kg N ha$^{-1}$), allocating 112 kg N ha$^{-1}$ at sowing and 48 kg N ha$^{-1}$ at stem elongation against comparatively similar yield but significantly lower grain protein when 120 kg N ha$^{-1}$ was provided once at sowing with in N. In addition, at split 120 kg N ha$^{-1}$ (84 kg N ha$^{-1}$ at sowing and 36 kg N ha$^{-1}$ at stem elongation) less number of grains pot$^{-1}$ and lower yield with lower protein was experienced.

## 5.2 Grain yield and yield components:

Irrespective of proportional variation of grain yield and number of grain pot$^{-1}$, TGW responded more or less equally to all treatments (Waddington et al. 1987; Peltonen-Sainio et al., 2007; Zhang et al., 2010) except for significant increase at single soil application of 160 kg N ha$^{-1}$ but without increasing grain yield or decrease at soil plus foliar application of Fe. It signified grain yield of wheat var. Krabat was largely dependent on the number of grains per pot$^{-1}$ and little on unit weight of grains (Kausar et al., 1993) as indicated by TGW at least in this study. Kausar et al. (1993) reported that grain weight is determined by genetic trait of a wheat variety and influenced by growth environmental parameters particularly during grain filling period. A multiple linear regression (Eq. 1) showed significantly positive role of number of grains pot$^{-1}$ and TGW, suggesting increase in grain yield was combined effect of number of grains pot$^{-1}$ and unit weight of grain (Mishra et al., 2001) who claimed the contribution of 1000 grains weight and number of grains per unit area in determining the wheat grain yield. More than 99% of variability in wheat grain yield (g pot$^{-1}$) was defined by the number of grains pot$^{-1}$ and TGW (Eq. 1). Although, TGW tend to increase the grain yield, in a regression analysis for TGW and number of grains pot$^{-1}$, TGW was negatively correlated with the number of grains pot$^{-1}$ (Eq. 2), suggesting unit weight of grain tended to decrease when the number of grains per spike increased, finally minimizing the importance of TGW to increase the grain yield. Similar finding was achieved in many earlier investigations claiming dominant role of number of grain per unit area than the unit weight of grains (Waddington et al., 1987; Protic et al., 2007; Peltonen-Sainio et al., 2007; Zhang et al., 2010). In a long term study from 1960 to 1984, Waddington et al. (1987) reported grain yield improvement was based on linear increase in number of grains m$^{-2}$ but TGW remained steady irrespective of cultivars. Whereas Zhang et al. (2010) pointed out grain yield increased with increased number of grain m$^{-2}$ but grain weight remained relatively stable even when half of the spikelet was removed from the main stem irrespective of higher availability of potential assimilate than average grain yield. It signified sink size represented by the number of grains per spike was a limiting factor to yield potential. This was one possible answer, how the relation of availability of assimilates (source) and number of grains (sink) determine the yield in modern wheat cultivars including wheat var. Krabat. Peltonen-Sainio et al. (2007) mentioned that the final grain yield depend more on the number of grains rather than on unit weight of grain when wheat grains are produced smaller and lighter. This could be a practical and simple justification for defining the dominating role of the number of grains pot$^{-1}$ over TGW in this study.

Beyond the overall relationship analysis between grain yield and its components, the interactive response between N- and Zn-Fe- treatments was experienced by grain and straw yield and number of grains pot$^{-1}$ except TGW. This indicated that more complex response of fertilization rate, type of nutrition, phonological stage of wheat for fertilization and method of nutrition supply rather than the straight forward responses of the main effects of nutrients.

Among treatments, grain yield and number of grains pot$^{-1}$ were relatively higher when N rate at sowing was about 120 kg N ha$^{-1}$ (N- treatments: N1N120 and N2N160) but lowered when applied 80 or 160 kg N ha$^{-1}$. This suggested positive response for increasing N application rate on yield parameters (Oscarson 2000; Rodrigues et al.,2000; Warraich et al., 2002) up to a definite rate only (Abedi et al., 2010; Marino et al., 2011). Further, higher or lower rate of N application to the critical N rate resulted negative yield effect as observed by Abedi et al. (2010). Increase in the yield parameters with increase in the supplement of N rate might be related with increase in total chlorophyll (a + b) content of leaves with increase in leaf area index as observed in sunflower leaves (Nasser 2002) and as a consequence increased the assimilation rate and its supplement to wheat grain Warraich et al. (2002). Protic et al. (2007) noticed that increasing N rate to 60 kg ha$^{-1}$, 1000 grains weight of wheat increased irrespective of varieties but further increment lowered TGW. Split application of 160 kg N ha$^{-1}$ (112 kg N ha$^{-1}$ applied at sowing and 48 kg N ha$^{-1}$ at stem elongation) produced higher number of grains pot$^{-1}$, and grain yield than at split application of 120 kg N ha$^{-1}$ (84 kg N ha$^{-1}$ applied at sowing and 36 kg N ha$^{-1}$ at stem elongation) but TGW did not change to a significant level. Similarly, in a study of emmer crops conducted at Mediterranean conditions in Italy, Marino et al. (2011) irrespective of varied rate and time of N application suggested that the number of grains and grain yield increased with increasing N rate but individual grain weight remain unaffected showing a dominating role of number of grains m$^{-2}$ in determining final grain yield.

Although the main effect of soil applied Zn and Fe at sowing did not affect the TGW (Table 4.1; Annex 2: Table 5), the grain yield and number of grain pot$^{-1}$ were significantly higher at soil application of Zn against soil applied Fe and Zn+Fe (Annex 2: Table 1 & 3). The results are in line with past studies (Silspour 2007; Nadim et al., 2012; Jiang et al., 2013). Nadim et al. (2012) recorded significant increase in leaf area index, photosynthetic rate and consequently grain yield also increased but TGW did not increased with the soil applied Zn (in the form of $ZnSO_4$) at the rate of 10 kg ha$^{-1}$ at sowing in comparison to soil applied Fe. It was further supported by the results of Jiang et al. (2013) that increasing soil supplement of Zn with $ZnSO_4.H_2O$) to 200 mg kg$^{-1}$ soil promoted leaf area index and photosynthetic rate as a result the number of grains per spike, TGW and grain yield were increased. As illustrated before about the effect of N on yield parameters, the increase in grain yield at soil applied Zn was also associated with the increased number of grain pot$^{-1}$. Ekiz et al. (1998) noticed significant increase in wheat and other cereals grain yield when Zn (7 kg ha$^{-1}$) was applied in Zn- deficient soil but the positive effect of Zn get reduced when higher rate was supplied.

Regardless of N application, number of grains pot$^{-1}$ and grain yield did not respond to the main effect of soil plus foliar supplied Zn and Fe (Annex 2: Table 2 & 4) but soil plus foliar applied Fe significantly lowered TGW at split application of 120 kg N ha$^{-1}$ (Annex 2: Table 6) as the flag leaf and other leaves showed mild burn after the spray of nutrient solution at booting stage. Leaf burn most probably impeded the photosynthesis as well as the supply of assimilate from flag leaf lowering the karnel weight as described by Jackson and Williams (2006). It is widely accepted that the number of grains per spike is determined before the stem elongation starts (Li et al., 2001; Acevedo, et al 2002). In our experiment, foliar spray was executed at booting stage of wheat growth so it was obvious to see least or no effect of late sprayed Zn and Fe on grain yield parameters. In contrast, Zeidan et al. (2010) recorded significant increase in all grain parameters and straw yield when Zn and Fe were sprayed on foliage at tillering and booting stage. Comparatively soil plus foliar applied Zn+Fe and Zn demonstrated a relative increase in TGW with respect to Fe but still not statistically significant. But, Habib (2012) obtained significant increase in 1000 kernel weight when Zn and Fe+Zn supplied on foliage at grain filling period of wheat in comparison to Fe without affecting number of grains per spike.

Although some researchers as stated before talked about the negative or null effect of initial higher N dose on grain yield in various growth conditions and further a positive role of initial N for the vigor of plant growth, mechanism involved in the reduction of number of grains per spike, grain and straw yield even at sufficiently high N rate (160 kg N ha$^{-1}$ in this study) applied at sowing time remain still unclear. For better understanding more focused study is needed.

### 5.3 Straw yield:

Straw yield increased when the rate of N at sowing was high. The results are in agreement with the findings of Kanampiu et al. (1997); Oscarson (2000); Warraich et al., (2002); Kutman et al. (2010) and Kutman et al. (2011). A team of Kutman and colleagues find positive effect of soil applied N rates on dry weight of wheat straw. A positive correlation ($p < 0.001$, $r = 0.83$) in between straw yield and grain yield (Fig. 3.7) was found as obtained by Mishra et al. (2001). It may be due to increased area of photosynthesis and supply of assimilates when straw including leaves were more abundent (Yilmaz et al. 1997; Rodrigues et al., 2000).

Straw yield as well as grain yield reduced even at the highest rate of soil applied N (160 kgN/ha) at sowing. This could be explained by the interaction of N with Zn and Fe application. It contrasted with Kutman et al. (2010) who noticed, when Zn was deficient in soil, increasing N supply did not increase the grain yield rather improved the straw yield. When the findings of Laneragan and Webb (1993) and others studies put together, an explanation for above results come in consensus. Laneragan and Webb (1993) stated that the effect of Zn deficiency do not prevail at lower N rate in soil both on vegetative and reproductive development but it could decrease grain and/or straw yield in wheat when the soil N supply is higher (Cakmak and Engels 1999; Salvagiotti and Miralles, 2007; Kutman et al. 2010; Kutman et al. 2011). Further Salvagiotti and Miralles (2007) described the positive effects of increasing N, initiate more straw production and tiller formation in wheat. Yilmaz et al. (1997) argued wheat grain yield could be more sensitive than the straw yield when encountered soil Zn deficiency.

A tendency of higher straw yield was recorded for soil or soil plus foliar applied Zn when N was applied at adequate rate (120 kg N ha$^{-1}$) in soil at sowing time, indicating a positive role of soil applied Zn for better production of straw yield. This was supported by the findings of Nadim et al. (2012) and Jiang et al. (2013), higher leaf area index and photosynthetic rate is increased by soil applied Zn resulting vigorous growth of plant (Kutman et al. 2010). Zeidan et al. (2010) noticed the positive role of foliar applied Zn in increasing the straw yield along with number of grains per spike, TGW and finally the grain yield than Fe spay. Similarly, in separate studies Kanampiu et al. (1997); Oscarson (2000) and Warraich et al. (2002) mentioned increase in all components of vegetative growth for supply of N, from number of tillers to height of shoots and leaf area index. But, excessive N (160 kg N ha$^{-1}$) at sowing did not increase the straw yield, rather it decreased the production of straw as observed for number of grains pot$^{-1}$ and grain yield. When Zn and Fe were applied in soil plus foliage, straw yield did not increase (Kutman et al. 2011). Most probably the mass of straw is determined before the boot stage.

From the above results and discussion, better straw yield can be achieved by applying optimum amount of N (about 120 kgN/ha at least in this study) together with Zn at sowing. Although, the application of Fe either in soil or soil plus foliage some time resulted in slightly higher straw yield (Annex 2: Table 7 & 8), but the results were not consistent.

### 5.4 Whole grain protein:

In overall, the protein (7.3% to 9.3% DW) content of wheat grain was lower than claimed for hard red spring wheat, 10% to 13.5% DW (Beuerlein 2001) and limited in feed category (i.e.

below 10% DW) (Thune et al. 2013) based on wheat quality standard practiced by Felleskjøpet Agri., Norway for season 2013/14. This has been discussed at the beginning of this chapter at topic ‘Why did lower yield and protein experienced in overall?’ In this section only the treatment effects and relation of WGP with other grain parameters and consequences are discussed.

Whole grain protein responded to the main effects of N- and Zn-Fe-treatments rather than interaction between treatments. This indicated the importance of independent role of both N- and Zn-Fe- treatments to determine the protein content in wheat grain. It was also true when Zn and Fe were applied both in soil and foliage.

Although WGP did not show any significant direct relation with grain yield, TGW was negatively (Eq. 3) and number of grains pot$^{-1}$ was positively (Eq. 4) correlated with WGP. A negative relation with TGW suggested that the dilution effect was created by larger size grain (Pleijel et al. 1999) and positive relation with number of grains pot$^{-1}$ suggested the WGP tended to increase when number of grains increased. Bearing in mind, a negative correlation between the number of gains per pot and TGW (Eq. 2), the dilution or concentration effect of yield on WGP may be defined by the competition between grains for available N (Fowler 2003) and assimilates (Fowler 2003; Brown et al., 2005). When the number of grain pot$^{-1}$ was more, comparatively smaller grains were formed and larger grains when the number of grains pot$^{-1}$ was less. In addition, Pleijel et al. (1999) described dilution of grain protein could be due to the non-nitrogen compounds in the grain which increase the growth of grain and accelerate the starch accumulation as observed in N- treatment: N1N160, that had highest TGW. But if the available N during grain filling period was deficient, the protein concentration could lower irrespective of grain yield (Fowler 2003; Brown et al., 2005). Peltonen-Sainio et al. (2007) and Valkama et al. (2013) in separate studies performed at high latitude locations, stated smaller protein response in cereals including wheat was due to the yield dilution of proteins as protein synthesis did not increase proportionally as did the yield biomass.

In the experiment with soil application of nutrients, N- treatments, N2N160 and N1N80 resulted in comparatively higher WGP than rest of the N treatments (Table 4.4). Each of these N-treatments represented different grain yield groups in this study. At N2N160, the grain yield and number of grains per pot was among the highest and N1N80 represent for lower yield with less number of grains pot$^{-1}$. At N1N80, TGW was slightly more than at N2N160 but not statistically significant. At N2N160, the increase in protein with increasing grain was supported by increased available N when 48 kg N ha$^{-1}$ was supplied at stem elongation since change in protein with change in yield mainly depends the available N (Brown et al. 2005). In addition, Brown et al. (2005) illustrated that both increasing and decreasing grain protein with higher grain yield may be due to N limitation at first and N surplus at second case. Similarly, at N1N80, higher protein was associated with lower yield and lower number of grains, suggesting the concentration effect (Marschner 1995). The higher concentration might not be the result of sufficient N for maximizing it but could be due to the reduction in grain yield potential by limited available N and environmental limitation (Fowler 2003) or diluted by relatively larger grain size in relation to increased supply of non-protein compound like assimilate during grain filling period (Pleijel et al., 1999) when growth temperature was high (21° C) and water availability was enough.

Late N application at stem elongation in split N- treatments enhanced grain protein in comparison with single N application at sowing (Elhanis et al., 2000; Ottman et al., 2000; Weber et al., 2008; Abedi et al., 2011). When initial N rate at sowing was enough, the increment in both yield as well as protein was assured by the split N applied at stem elongation period. For example, split application of 160 kg N ha$^{-1}$ (70% at sowing and 30% at stem elongation) increased both protein and yield whereas split application of 120 kg N ha$^{-1}$ increased protein concentration but not the yield in comparison to single application of 120 kg N ha$^{-1}$ at sowing, possibly in limited availability of initial N that needed for increasing number of grain per spike (Li et al., 2001).

When Zn and Fe applied in soil plus on foliage, more protein was obtained in comparison to their respective soil application (Annex 2: Table 10) and the highest was for soil plus foliar application of Fe or Zn+Fe. It was also evidenced by positive correlation between Fe- or Zn-concentrations in grain with WGP (Eq. 5) similar to (Cakmak et al., 2010a; Velu et al., 2011). Cakmak et al. (2010a) stated that protein in wheat grain might be a sink for Zn and Fe. Further, Cakmak et al. (2010a) and Kutman et al. (2010) demonstrated the coexistence of Zn, Fe and protein in durum wheat grain. In addition, they advised the existence of some kind of synergetic relation between Zn, Fe and N for co-segregation in grain when higher rates of N provided. The mechanism involved in such co-segregation need to be further explored for the knowledge, why and how the co-segregation happens. Similarly, Peterson et al. (1986) and Peleg et al. (2008) also reported positive correlation between the grain concentration of Fe or Zn with protein in wheat.

Similar to earlier studies (Beres, et al., 2008; Abedi et al., 2011), current work agreed that when N was applied at optimum rate at sowing, it increased the grain yield by increasing the number of grains per spike even if the tillers were missed at early stage and N applied at stem elongation after the period for tillering, and it could raise protein concentration unless other stress prevail. Based on the current study and earlier literatures, the concentration of protein in grain was not controlled by one or two factors and also the relation with yield factors was not straight forward, but a dynamic relation between N availability, grain yield potential of wheat, availability of Zn and Fe, and other environmental factors affecting longevity of grain filling period and photosynthesis was felt.

### 5.5 Iron and zinc concentrations in wheat grain:

In general higher concentrations of Fe and Zn in grain were recorded for the treatments with lower grain yield and lower concentrations when higher grain yields were achieved. Studies in the past have mentioned about the dilution of Zn and Fe concentrations in wheat grain when grain yield increased (Yilmaz et al., 1997; Bänziger and Long 2000; Liu et al., 2006; Gomez-Becerra et al., 2010). Multiple linear regression analysis (Eq. 6 and Eq. 7) presented a decreasing tendency of Fe- and Zn- concentrations in grain with increase in grain yield parameters: TGW and number of grains pot$^{-1}$. As mentioned by Zhao et al. (2009), grain Zn- concentration of wheat correlated negatively with grain yield, but not the Fe- concentration and the correlation was weak with grain size as well as weight. But positive coefficient of Zn- and Fe- uptake (i.e. total uptake in grain) suggested, higher concentration of Zn and Fe in wheat grain was due to the increased uptake from soil or translocation of Zn and Fe from vegetative part to the grain (Cakmak et al., 2010a).

A multiple linear regression insinuated a dynamic relation among grain Zn- and Fe- concentrations, their uptake in to grain and grain yield parameters (Marschner, 1995) suggesting process was governed by sink-source relation. The negative coefficients for grain yield components, suggested that the dilution of Zn and Fe in grain was due to combined effect of grain size and the number of grains pot$^{-1}$ (sink size) as indicated by Sperotto et al. (2013) in rice plant, pointing involvement of factors other than grain (sink strength) only. Other factors could be the availability of metals (Marschner, 1995), for instance Zn and Fe during grain filling (Cakmak, 2008; Cakmak et al., 2010a) or factors contributing dry weight (starch) in grain which increase the size and weight of grain (Marschner, 1995; Pleijel et al., 1999).

A significant positive correlation was observed between Zn and Fe concentrations in grain (Eq. 8) as mentioned by Ortiz-Monasterio et al. (2007) and Velu et al. (2011), suggesting co-segregation of Zn and Fe in wheat grain which was further elucidated by co-localization of Zn and Fe in element distribution maps produced by using MA-XRF (Fig. 4.16 B & C) and plots for isotopic counts of Zn and Fe obtained from LA-ICPMS (Fig. 4.18). As discussed in protein of wheat, Cakmak et al. (2010a) also urged co-segregation of Zn, Fe and protein in durum wheat.

Interaction of N- and Zn-Fe- treatments (Table: 4.1) defined the variation in grain Fe-concentration in better way than the main effects of treatments. For instance, higher grain Fe-concentration was observed when soil applied Zn+Fe at N- treatments: N1N160 and N2N160 but at N- treatments: N1N80 and N2N120, comparatively higher concentration was noticed at soil application of Fe (Annex 2: Table 11). But in case of grain Zn- concentration, the variation was mainly defined by the main effect of N- treatments. Higher Zn- concentration was felt at Zn+Fe when 80 and 160 kg N ha$^{-1}$ was applied in soil at sowing (Annex 2: Table 15). In general, increasing the rate of N up to 120 kg N ha$^{-1}$ at sowing, Fe- and Zn- concentrations reduced in grain but with different proportion in connection to grain yield. For instance, in comparison to 80 kg N ha$^{-1}$ at sowing, the average concentration of Fe and Zn in grain diluted (Yilmaz et al., 1997; Bänziger and Long 2000; Liu et al., 2006; Gomez-Becerra et al., 2010) by 12% and 8% when the grain yield increased more than 35% (Annex 2: Tables 1, 11 & 15). As discussed before it clearly showed that proportionally higher accumulation of assimilate (starch) in grains than Fe and Zn (Marschner, 1995; Pleijel et al., 1999). But this proportion of dilution was higher for grain Fe- concentration. In addition, when the rate of N increased either at sowing or at stem elongation in split N treatments, the concentration of both metals did not increase, which contrasted with results of Shi et al. (2010). Shi et al. (2010) observed that N fertilization increased the concentration of Zn and Fe in wheat grain under field condition. Rather concentrations reduced whenever the yield was high justifying the dilution effect of higher yield.

Although Fe and Zn concentration did not respond significantly to soil application of Zn and Fe, Zn+Fe tended to increase the concentration of Zn or Fe very often as explained in review article by Cakmak et al (2010a) suggesting positive role of soil and foliar applied Zn and Fe to increase respective metal concentrations in durum wheat grain. As an example, such trend was apparent at N- treatments: N1N80 and N2N120 but lowered at N2N160 when Zn+Fe was applied in soil in comparison with separate application of Zn and Fe in soil (Fig. 4.12 & Fig. 4.8). This variation was due to the interaction effect of N- and Zn-Fe- treatments ($p < 0.05$) (Table 4.1). Yilmaz et al. (1997) reported that Zn and Fe fertilizers can increase Fe and Zn concentration in wheat grain up to 3- or 4- folds.

The application of 30% higher Zn and Fe either separately or together as foliar spray in addition to soil application caused positively significant increase in Zn- (Kutman et al., 2010) and Fe- concentrations in grain (Cakmak et al., 2010a; Habib 2012). For instance, in comparison with soil application soil applied Fe and Zn+Fe, Fe- concentration in grain was raised by 34% and 64% for the supply of Fe and Zn+Fe, respectively on foliage at booting stage. Similarly, the concentration of Zn was increased by 17% and 46% for foliar applied Zn and Zn+Fe. This could be due the increased activity of Zn (Kutman et al., 2012) in sources (flag leaf and stem) during grain filling when additional Zn and Fe was supplied at boot stage and similar phenomenon also prevailed in response of foliar applied Fe. The increment was notably higher for the application of Zn+Fe in case of both metals, similar to the findings of Habib (2012) that Fe and Zn concentration in wheat grain increased by applying Zn and Fe together as foliar spray.

According to contemporary results, better concentration of Zn and Fe in whole grain can be achieved by spraying Zn and Fe together at boot stage ensuring sufficient available N at sowing and stem elongation or late stage of wheat to even to maximize the grain yield and protein content. Mixing of nutrients for foliar application can also reduce the economic burden to achieve more nutritious wheat grains.

### 5.6 Total Fe and Zn uptake in grain:

The total uptake of Fe tend to increase when TGW decreased ($p < 0.001$) (Eq. 9) contrasted with Velu et al. (2011), higher positive correlation for total content of Fe or Zn in wheat grain than for respective concentrations indicating concentration effect due to small seed size. Similarly, total grain Zn- uptake raised with increase in the number of grains pot$^{-1}$ ($p < 0.05$) (Eq. 10) contrasted to the finding of Nowack et al. (2008), when the number of grains per spike decreased, the total content of Fe and Zn reduces as fewer seeds received a later proportion of the available Fe and Zn. In this study, the grain yield was found determine by the combination of TGW and number of grains pot$^{-1}$ (Eq. 1).

The effect of N- and Zn-Fe- treatments over grain uptake of Fe and Zn was not simple, but the interaction of treatments prevailed. In general, uptake of Fe and Zn in grains was proportional to grain yield (Annex 2: Table 1, Table 13 & Table 17). When N application rate was increased either at sowing or at stem elongation, average total uptake of Fe and Zn in grain increased, ensuring the positive role of N- rates (Shi et al., 2010; Cakmak 2008; Cakmak et al., 2010a).

However, the uptake of both elements decreased when N rate at sowing was above or below to 120 kg N ha$^{-1}$. The mechanism of Zn and Fe uptake and transport to grain is still not clear (Cakmak et al., 2010a) but earlier studies suggested the promising role of metal chelating agents (Cakmak 2008; Kutman et al., 2010) called nicotianamine as observed in tobacco plants (Takahashi et al., 2003) to promote the transport of Zn and Fe in phloem and finally into grain (Schmidke and Stephan, 1995 in Cakmak et al., 2010a). In addition, in a comparative study for the effect of ammonium and nitrate fertilizers on wheat, Barunawati et al. (2013) found increase in the total content of Fe, Zn and Cu in wheat grain was not related to the extent of metal translocation from flag leaves even for the increase in NA due to increased N. But mentioned the probable role of 2-deoxymugineic acid (DMA) in translocation of metals from flag leaf to the grain in modern high-yielding wheat as evidenced by higher proportion of DMA than NA in flag leaf and flag leaf exudates. Shi et al. (2010) observed the accumulation of Zn and Fe will be in shorts and bran of wheat grain. In separate study, increasing the rate of Zn application in the form of zinc sulphate, higher accumulation of NPK (nitrogen, phosphorus and potash) was evidenced (Abbas et al., 2009), suggesting synergetic relationship between N and Zn application.

Among treatments with soil application of nutrients, the highest and lowest Fe- uptake in grain was visible at soil application of Fe, respectively when 160 kg N ha$^{-1}$ was split to 70% at sowing and 30% at stem elongation and 80 kg N ha$^{-1}$ was applied in soil at sowing. Similarly, the highest and lowest Zn- uptake in grain was obtained at soil application of Zn, respectively when 160 kg N ha$^{-1}$ was split to 70% at sowing and 30% at stem elongation and 160 kg N ha$^{-1}$ was applied in soil at sowing. In contrast to general trend, at N treatment: N1N160, uptake of Fe raised as equal as other higher grain yielding N- treatments even at lower grain yield but in connection to lower number of grains pot$^{-1}$ as encountered by Nowack et al. (2008), proposed higher availability of Fe for fewer number of grains. Similarly, Sperotto et al. (2013) demonstrated increase in total seed content of Zn, Fe and manganese when half of the grains were removed from a penicle in rice plant but the concentration in grain did not surpass the control with intact penicle partially due to increase in the size of grain.

For the treatments with soil plus foliar application of Zn and Fe, Fe- uptake in grain responded only to the main effects of N- and Zn-Fe- treatments while Zn- uptake in grain responded to the main effect of Zn-Fe- treatments. Total uptake of Zn and Fe in grain was higher in comparison with their soil application for foliar spray of 30% higher Zn and Fe at boot stage of wheat in addition to soil application at sowing. The effect was more when both of them were combined

(Nowack et al., 2008) most probably due to the increase in availability at grain filling period. The increment was about 42% for Fe-uptake in grain and 35% for Zn- uptake in grain when Zn and Fe applied together as soil plus foliar spray.

Putting together the relations among studied variables and treatment effects, Fe- and Zn- uptake as well as concentrations in wheat grain can be increased by proper management (at least rate and timing of application) of N and Zn+Fe supplement on foliage at boot stage or around grain filling period without loss in final grain yield.

### 5.7 Localization of Fe and Zn in wheat grain:

Concentration map of Zn and Fe obtained by MA-XRF and normalized plot of counts from LA-ICPMS, divulged the coexistence of these metals especially at embryo and just outside the endosperm most probably indicating the aleurone layer. This was in accordance with the results obtained using staining technique Cakmak et al (2010b). Further they claimed the co-localization of protein and Zn and Fe in embryo due to the co-segregation. Similarly in another study by Kutman et al. (2010) staining of Zn and protein suggested similar place of existence for Zn and protein in a durum wheat grain. Tsuji et al. (2006) used μ-XRF technique for the elemental mapping of biological materials and found μ-XRF was useful for the analysis of element distribution in grain samples. In elemental map of black wheat and buck or soba wheat, Zn and Fe were located either at embryo and/or coat of grains. Current LA-ICPMS revealed relatively higher concentration of both Zn and Fe at embryo and bran portion including aleurone and crease area and the concentrations raised relatively when the laser scan pass through the germ area (Fig. 4.19 ). These results are similar to the earlier research about the distribution of Zn in wheat grain and its translocation to the endosperm (Cakmak et al. 2010b). Based on decreasing concentration gradient of Zn from crease area of a grain towards endosperm, they claim the translocation and distribution of these elements happen through the crease and then pass into the endosperm. Wang et al. (2011) also aggress with this phenomenon of Zn supply to the endosperm from crease. In their LA-ICPMS analysis of grains, clear gradient of Zn concentration was observed from crease to the endosperm. This study also supported the earlier claims regarding the distribution of Zn and Fe and their translocation into the endosperm. In addition, during this study a relatively higher accumulation of Zn than Fe was obtained at endosperm, while higher concentration of Fe than Zn was noticed in bran or aleurone (Fig. 4.18).

To clearly define the location of Zn and Fe and their gradient for instance from bran to endosperm, crease area to endosperm and embryo to endosperm was felt essential for the identification of direction of element supply to endosperm. The scale of measurements needs to be even more detailed than used in this study. Finally, depth profiles at the Zn, Fe hotspots would also be needed to clarify the extent of the metal accumulation.

## 6 CONCLUSIONS:

Based on current results and discussions, the following conclusions are drawn.

- The role of N-, Zn- and Fe- fertilization seems vital in vegetative as well as generative development of wheat, grain quality (protein, Zn and Fe concentrations) and uptake of Zn and Fe in grain. Often, soil applied N interacted with soil applied Zn and Fe at sowing resulting differences in number of grains pot$^{-1}$, straw yield, grain Fe- concentration and uptake of Zn and Fe in grain. For instance, results showed higher number of grains pot$^{-1}$ and straw yields at higher rate of soil applied N and Zn at sowing.
- Based on current results and discussion, concentrations of protein, Zn and Fe in grain may be determined by the availability of N, Zn and Fe, respectively during the grain filling period.
- Increasing N rate may promote the total uptake of Zn and Fe in grain. It is probably due to larger sink size created by higher number of grains per spike as signified by the number of grains pot$^{-1}$ in response of increasing N rate at sowing. According to literatures, N may promotes the root uptake and translocation of Zn and Fe to grain by affecting the levels of nitrogenous chelating compounds involved in the transport of Zn and Fe within plant and transporter proteins for root uptake and phloem loading of Zn and Fe (Cakmak 2010).
- Increasing the rate of N application at sowing increases grain and straw yields and number of grains pot$^{-1}$ but only up to a certain level of N (120 kg N ha$^{-1}$) then decreases if the N rate continued to increase. Initial dose of N in soil applied at sowing increase the number of grains pot$^{-1}$ (number of grains spike$^{-1}$) which certainly increase the grain yield.
- In general, the dilution of grain protein, Zn- and Fe- concentrations were found related to both the number of grains per pot and 1000 grains weight which was tried to explain with the help of source-sink relation as described by (Zhang et al., 2010; Sperotto et al., 2013) in rice plant. The concentration of Zn and Fe in grain cannot be defined just considering the yield components but also the other factors like source activity (supply of assimilate to grain) and environmental factors affecting source and sink activity should be considered. At lower N rates at sowing resulted in reduced grain yield with higher concentrations of protein, Zn and Fe.
- MA-XRD and LA-ICPMS results indicated the co-localization of Zn and Fe in grain especially at germ and aleurone layer. LA-ICPMS results also indicated higher concentration of Zn and Fe at crease area and their concentrations decreased at

endosperm indicating the direction of Zn and Fe translocation from crease tissue into endosperm. In addition to earlier studies, during this study a relatively higher accumulation of Zn than Fe was obtained at endosperm, while higher concentration of Fe than Zn was noticed in bran or aleurone.

## 7 RECOMMENDATIONS AND FUTURE PERSPECTIVES:

For optimum yield and better quality of grain, proper management of N, Zn and Fe supplement should be guaranteed. Timing of fertilizer application (soil as well as foliar) should be taken in to account. For instance, this study showed the application rate of N at sowing determined the number of grains pot$^{-1}$ and late applied N increased the protein content in grain. Similarly, foliar applied Zn and Fe at booting stage of wheat offered higher uptake and concentration of Zn and Fe in grain, particularly when Zn and Fe applied together on foliage. While planning fertilizer and its application time, the climatic factors also should be equally priorities to assure better and quality yield.

Study on the physiological barriers between the crease vascular tissue and endosperm should be focused along with studies on root uptake and translocation of Zn and Fe from stem or flag leaf to grain. Such study could set the basis for future study to invade such barriers by means of genetic engineering or cultivation practices like the manipulation of growth condition and Zn and Fe regulating hormones.

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# ANNEXES:

## Annexes 1:

Table 1: Moisture content of air dried peat before mixing with other constituent of growth media.

| Bag no./container no. | Wt. of container, g | Wt. of container + moist peat, g | Wt. of container + dry peat, g | Moisture, g | Total weight of peat, g | Moisture % | Average moisture % |
|---|---|---|---|---|---|---|---|
| 1/129 | 18.56 | 29.66 | 24.9 | 4.76 | 11.1 | 42.88 | |
| 1/136 | 18.97 | 31.16 | 25.94 | 5.22 | 12.19 | 42.82 | 42.87 |
| 1/143 | 17.73 | 28.8 | 24.05 | 4.75 | 11.07 | 42.91 | |
| 2/162 | 18.65 | 31.62 | 25.37 | 6.25 | 12.97 | 48.19 | |
| 2/228 | 19.21 | 32.04 | 25.84 | 6.2 | 12.83 | 48.32 | 48.06 |
| 2/241 | 17.6 | 28.95 | 23.54 | 5.41 | 11.35 | 47.67 | |
| 3/146 | 18.07 | 28.98 | 24.51 | 4.47 | 10.91 | 40.97 | 40.93 |
| 3/169 | 18.01 | 27.9 | 23.85 | 4.05 | 9.89 | 40.95 | |
| 3/177 | 17.75 | 28.86 | 24.32 | 4.54 | 11.11 | 40.86 | |

Note: Peat was produced by Weibells AB, Sweden. For more information: www.weibulls.com

Table 2: Liming rates and their response for the selection of liming rate prepare growth media with pH 6.5±0.2.

| Observation No./replicate | $CaCO_3$ per 100 soil, g | pH | Observation No./replicate | $CaCO_3$ per 100 soil, g | pH | Remarks |
|---|---|---|---|---|---|---|
| 1.1 | 0 | 3.93 | 5.1 | 3 | 6.76 | Soil:water =1:2.5 |
| 1.2 | 0 | 3.92 | 5.2 | 3 | 6.94 | |
| 1.3 | 0 | 3.82 | 5.3 | 3 | 6.74 | |
| 2.1 | 0.5 | 6.4 | 6.1 | 4 | 6.9 | Finally, 0.5 g $CaCO_3$ powder per 100 g soil mixture was selected as liming rate to achieve 6.5 ± 0.2 pH. |
| 2.2 | 0.5 | 6.33 | 6.2 | 4 | 6.87 | |
| 2.3 | 0.5 | 6.36 | 6.3 | 4 | 7.01 | |
| 3.1 | 1 | 6.6 | 7.1 | 5 | 6.81 | |
| 3.2 | 1 | 6.71 | 7.2 | 5 | 7.01 | |
| 3.3 | 1 | 6.56 | 7.3 | 5 | 6.95 | |
| 4.1 | 2 | 6.8 | | | | |
| 4.2 | 2 | 6.85 | | | | |
| 4.3 | 2 | 6.77 | | | | |

Table 3: Rate of treatment factors and basic nutrients applied in soil.

| Nutrients | Source | Rate | State of application |
|---|---|---|---|
| Calcium (Ca) | $CaCO_3$ | 0.5 g/kg soil mixture | powder |
| **Nitrogen (N)** | **Ca $(NO_3)_2$** | **80, 120 and 160 kg N $ha^{-1}$** | **Solution** |
| | | **70% and 30% of 120 (i.e. 84 & 36 kg N $ha^{-1}$) and 160 (i.e. 112 & 48 kg N $ha^{-1}$) respectively at sowing and stem elongation.** | |
| Phosphorus (P) | Ca $(H_2PO_4)2.aq$ | 3 g P/l | Solution |
| Potash (K) | $K_2SO_4$ | 12 g K/l | Solution |
| Magnesium (Mg) | $MgSO_4.7H_2O$ | 12.5 g st/l | Solution |
| **Iron (Fe)** | **Fe-EDTA ($C_1H_{12}FeN_2NaO_8.H_2O$)** | **10 mg Fe/kg soil** | **Solution** |
| Manganese (Mn) | $MnSO_4.\ 4H_2O$ | 2.50 g st/l | Solution |
| Copper (Cu) | $CuSO_4.2H_2O$ | 2.50 g st/l | Solution |
| Molybdenium (Mo) | $(NH_4)6Mo7O_{22}.4H_2O$ | 0.05 g st/l | Solution |
| Boron (B) | $Na2B_4O7.10H_2O$ | 0.25 g st/l | Solution |
| **Zinc (Zn)** | **$ZnSO_4.7H_2O$** | **10 mg Zn/ kg soil** | **Solution** |

Note: all solutions were added in soil before sowing at volume 25 ml. Rows with bold letters represent for treatment factors.

Table 4: Germination rate of test plant grains: wheat (var. Krabat).

| Petridis no. | Germination stage | 48 hours | | 72 hours | | 96 hours | |
|---|---|---|---|---|---|---|---|
| 1 | With primary root | 6 | 17 | 13 | 17 | 15 | 18 |
| | Sprouted | 11 | | 4 | | 3 | |
| | Non sprouted/decayed | 3 | | 3 | | 2 | |
| 2 | With primary root | 10 | 19 | 17 | 19 | 17 | 17 |
| | Sprouted | 9 | | 2 | | - | |
| | Non sprouted/decayed | 1 | | 1 | | 3 | |
| 3 | With primary root | 9 | 19 | 16 | 20 | 17 | 20 |
| | Sprouted | 10 | | 4 | | 3 | |
| | Non sprouted/decayed | 1 | | 0 | | - | |
| Average germination rate | | | | | | (55/60) × 100% = 91.66% | |

Table 5: Spray calibration for foliar treatments of Zn and Fe.

| S.N. | Number of full spray until consumed 10 ml water | |
|---|---|---|
| | Spray 1 | Spray 2 |
| 1 | 9 | 9 |
| 2 | 10 | 10 |
| 3 | 11 | 10 |
| 4 | 11 | 10 |
| 5 | 10 | 9 |
| 6 | 10 | 10 |
| 7 | 10 | 10 |
| 8 | 10 | 9 |
| 9 | 10 | 9 |
| 10 | 9 | 10 |
| | Mode: 10* | Mode: 10* |

* Zn and Fe solutions sprayed two times mode value as average water retention on wheat plant was about 50% (see in Annex 1 Table: 5).

Note: The weight of distilled water was considered 1 ml = 1 gm at room temperature. Pot number represents the number of pot used in growing plants.

Table 6: Spray solution retention % on wheat plant.

| Pot No. | Wt. of water sprayed , g | Wt. of surrounding paper and bag, g | Wt. of wet paper and bag after spray, g | Wt. of water fallen out of plant parts, g | Wt. of water retained on plant, g | Water retained on plant, % |
|---|---|---|---|---|---|---|
| 65 | 10 | 43.28 | 48.09 | 4.81 | 5.19 | 51.9 |
| 66 | 10 | 43.53 | 48.80 | 5.27 | 4.73 | 47.3 |
| 67 | 10 | 43.64 | 49.08 | 5.44 | 4.56 | 45.6 |
| 68 | 10 | 43.57 | 48.72 | 5.15 | 4.85 | 48.5 |
| 77 | 10 | 43.84 | 48.62 | 4.78 | 5.22 | 52.2 |
| 78 | 10 | 43.86 | 48.15 | 4.29 | 5.71 | 57.1 |
| 79 | 10 | 44.06 | 49.10 | 5.04 | 4.96 | 49.6 |
| | | | | | Average water retained on plant, % | 50.31 |

Note: Water was used as pilot spray on wheat plant in place of real solution used for foliar treatments of Zn and Fe. Pot No. in the table was retained from the experiment.

## Annexes 2:

Table 1: Mean ± 1SE for grain yield (g pot$^{-1}$) at experiment with soil application of nutrients.

| | N-treatment | | | | | |
|---|---|---|---|---|---|---|
| **Zn-Fe treatment** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | **Average* ****<br>**(n=20)** |
| **Zn** | 2.41±0.074 DEF | 3.14±0.052A | 2.63±0.034 CDE | 2.88±0.12 ABC | 3.26±0.031A | **2.87±0.077a** |
| **Zn+Fe** | 2.27±0.095 DEF | 3.16±0.092 A | 2.15±0.073 F | 2.85±0.001 ABC | 2.93±0.08 ABC | **2.67±0.095 b** |
| **Fe** | 2.22±0.099 EF | 3.1 ±0.117 AB | 2.14±0.051 F | 2.69±0.107 BCD | 3.11±0.111 AB | **2.65±0.103b** |
| **Avg.*** (n=12)** | **2.30±0.053 c** | **3.13±0.048 a** | **2.31±0.075 c** | **2.81±0.059 b** | **3.1±0.059 a** | |

*** Significant at p = 0.001; Avg. = Average; Treatment means ± 1SE (n = 4) followed by same upper case alphabet are not significantly different for N- × Zn-Fe- treatments. Average means followed by same lower case alphabet at the left hand side of table and at the bottom are not significantly different for Zn-Fe- treatments and N- treatments respectively. Tukey comparison was performed at 5% level of significance. N1 and N2 stand for single and split application of N. 80, 120 and 160 stands for soil applied rate of N in kg ha$^{-1}$. Zn, Zn+Fe and Fe represent the soil application of Zn-Fe- treatments.

Table 2: Mean ± 1SE grain yield (g pot$^{-1}$) at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average**** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | **(n = 24)** |
| **N1N120** | 3.14±0.052 A | 3.16±0.092 A | 3.1±0.117A | 2.9±0.066 AB | 2.79±0.058 AB | 3.045±0.058 AB | **3.02±0.04a** |
| **N2N120** | 2.88±0.119 AB | 2.85±0.007 AB | 2.69±0.107B | 3.1±0.072 A | 2.84±0.042 AB | 2.85±0.091AB | **2.87±0.04b** |
| **Avg. # (n=8)** | **3.01±0.077a** | **3.01±0.204a** | **2.89±0.107a** | **3.0±0.0591a** | **2.81±0.035a** | **2.95±0.177a** | |

****** Significant at p = 0.01; # Not significant at p = 0.05; Avg. = Average; Treatment means ± 1SE (n = 4) followed by same upper case alphabet are not significantly different for N- × Zn-Fe-treatments. Average means followed by same lower case alphabet are not significantly different for N- treatments and Zn-Fe- treatments respectively. Tukey comparison was performed at 5% level of significance. N1 and N2 stand for single and split application of N. N120 stands for soil application of 120 kg N ha$^{-1}$. Zn, Zn+Fe and Fe without suffix for soil application of Zn-Fe-treatments and with suffix 's+f' for soil plus foliar application.

Table 3: Mean ± 1SE for number of grains pot$^{-1}$ at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average **** |
|---|---|---|---|---|---|---|
| **Zn-Fe- treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | **(n=20)** |
| **Zn** | 90.50±4.17DEF | 122.0±2.20 A | 95.75±0.479 CDE | 108.75±4.25ABC | 124.25±0.946 A | **108.25 ± 3.31a** |
| **Zn+Fe** | 88.25±4.48 DEF | 124.0±2.48A | 77.75±1.49 F | 109.50±2.10 ABC | 114.25±4.05AB | **102.75 ± 4.12b** |
| **Fe** | 86.25±4.31 EF | 118.5±3.84AB | 76.75±2.39F | 103.50±3.84 BCD | 120.25±2.84 A | **101.05 ± 4.20b** |
| **Avg.*** (n = 12)** | **88.33 ± 2.32 c** | **121.50 ±1.68 a** | **83.42 ± 2.77c** | **107.25 ± 2.01b** | **119.58 ± 1.96a** | |

*** Significant at p = 0.001; ****** significant at p = 0.01; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 4: Mean ± 1SE (n = 4) number of grains pot$^{-1}$ obtained at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average **** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | **(n=24)** |
| **N1N120** | 122.0±2.20A | 124.0±2.48A | 118.50±3.84AB | 112.0±3.19AB | 111.75±4.50AB | 122.0±2.16A | **118.38±1.53a** |
| **N2N120** | 108.75±4.25AB | 109.50±2.10AB | 103.50±3.84B | 118.50±1.71AB | 114.50±2.6AB | 118.±4.86AB | **112.21±1.68b** |
| **Avg. # (n=8)** | **115.38±3.34a** | **116.75±3.133a** | **111±3.79a** | **115.25±2.08a** | **113.13±2.46a** | **120.25±2.55a** | |

** Significant at p = 0.01; # Not significant at α = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 5: Mean ± 1SE for 1000 grains weight (g) obtained at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average#**<br>**(n=20)** |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | |
| **Zn** | 26.72±0.463AB | 25.743±0.257AB | 27.5±0.443 AB | 26.51±0.351AB | 26.26±0.150AB | **26.544±0.193a** |
| **Zn+Fe** | 25.80±0.521AB | 25.48±0.517B | 27.61±0.641AB | 26.08±0.491AB | 25.67±0.408B | **26.129±0.271a** |
| **Fe** | 25.73±0.261B | 26.13±0.397AB | 27.88±0.503A | 25.94±0.332AB | 25.8±0.328AB | **26.298±0.237a** |
| **Avg. *** (n=12)** | **26.083±0.262b** | **25.787±0.226 b** | **27.663±0.284a** | **26.175±0.220b** | **25.910±0.181b** | |

*** Significant at p = 0.001; **#** Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 6: Mean ± 1SE (n = 4) 1000 grains weight (g) obtained at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average#**<br>**(n=24)** |
|---|---|---|---|---|---|---|---|
| **N-_treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | |
| **N1N120** | 25.74±0.257 AB | 25.48±0.517 AB | 26.13±0.397A | 25.9.±0.568AB | 24.97±0.632AB | 24.96±0.182AB | **25.531±0.926a** |
| **N2N120** | 26.51±0.351A | 26.08±0.491AB | 25.94±0.332 AB | 26.16±0.423A | 24.84±0.311AB | 24.04±0.372B | **25.594±0.227a** |
| **Avg. ** (n=8)** | **26.124±0.247a** | **25.78±0.349 a** | **26.038±0.242a** | **26.029±0.332a** | **24.906±0.327ab** | **24.501±0.258b** | |

** Significant at p = 0.01; **#** Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 7: Mean ± 1SE for straw yield (g pot$^{-1}$) obtained at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average***<br>**(n=20)** |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | |
| **Zn** | 3.1±0.94 DE | 3.91±0.022A | 3.16±0.045 CDE | 3.19±0.033 CD | 3.63±0.035AB | **3.397±0.0758a** |
| **Zn+Fe** | 3.0±0.096 DEF | 3.94±0.065A | 2.7±0.103 F | 3.18±0.050 CD | 3.532±0.087 BC | **3.271±0.104a** |
| **Fe** | 2.96±0.104 DEF | 3.89±0.121AB | 2.8±0.062 EF | 3.23±.0.064 CD | 3.812±0.014 AB | **3.336±0.106a** |
| **Avg.*** (N=12)** | **3.02±0.054 d** | **3.91±0.043 a** | **2.887±0.071d** | **3.2±0.027c** | **3.66±0.045b** | |

*** Significant at p = 0.001; ***** significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 8: Mean ± 1SE (n = 4) straw yield (g $pot^{-1}$) obtained at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average*** (n=24)** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | |
| **N1N120** | 3.91±0.022 A | 3.94±0.065 A | 3.89±0.121 AB | 3.757±0.061AB | 3.75±0.032 AB | 3.87±0.036 AB | **3.852±0.0280a** |
| **N2N120** | 3.19±0.033 C | 3.18±0.050 C | 3.23±0.064C | 3.56±0.08 ABC | 3.5±0.048 BC | 3.21±0.201C | **3.3096±0.0479b** |
| **Avg. # (n=8)** | **3.547±0.137a** | **3.561±0.149a** | **3.558±0.139a** | **3.658±0.0600a** | **3.624±0.0547a** | **3.538±0.157a** | |

*** Significant at p = 0.001; **#** Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 9: Mean ± 1SE for whole grain protein (%) in wheat grains obtained at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average*** (n=20)** |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | |
| **Zn** | 8.09±0.083BCDE | 7.9±0.108DE | 7.64±0.133E | 8.01±0.061CDE | 8.28±0.045ABCD | **7.984±0.0611b** |
| **Zn+Fe** | 8.56±0.075 ABC | 8.13±0.101BCDE | 8.02±0.148CDE | 8.32±0.108ABCD | 8.62±0.09AB | **8.331±0.0681a** |
| **Fe** | 8.35±0.172 ABCD | 8.0±0.503CDE | 7.91±0.086DE | 8.21±0.154ABCD | 8.75±0.164A | **8.245±0.0860a** |
| **Avg. *** (n=12)** | **8.333±0.0842ab** | **8.012±0.0542cd** | **7.857±0.0817d** | **8.180±0.0717 bc** | **8.550±0.0828 a** | |

*** Significant at p = 0.001; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 10: Mean ± 1SE (n = 4) whole grain protein (%) in wheat grains obtained at experiment with soil plus foliar application of Zn and Fe.

| | **Zn-Fe treatments** | | | | | | **Average * (n=24)** |
|---|---|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **Fe s+f** | |
| **N1N120** | 7.9±0.108 C | 8.13±0.101BC | 8.0±0.042BC | 8.51.±0.103AB | 8.88±0.0.122A | 8.46±0.096AB | **8.315±0.0788 b** |
| **N2N120** | 8.01±0.061BC | 8.326±0.108BC | 8.21±0.154BC | 8.44±0.114ABC | 8.956±0.133A | 8.87±0.131A | **8.468±0.0836 a** |
| **Avg.*** (n=8)** | **7.95±0.061d** | **8.23±0.078cd** | **8.11±0.084d** | **8.48±0.073bc** | **8.92±0.085a** | **8.67±0.108ab** | |

*** Significant at p = 0.001; ***** significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 11: Mean ± 1SE for grain Fe- concentration (mg kg$^{-1}$) obtained at experiment with soil application of nutrients.

| | N-treatments | | | | | Average # |
|---|---|---|---|---|---|---|
| Zn-Fe treatments | N1N80 | N1N120 | N1N160 | N2N120 | N2N160 | (n=20) |
| Zn+Fe | 43.64±0.463AB | 36.82±1.06CD | 45.08±2.07A | 38.05±0.456 CD | 34.153±0.938D | **39.55±1.06a** |
| Fe | 39.58±1.11BC | 36.31±0.457CD | 45.87±1.18A | 35.27±0.930 CD | 38.13±1.24 CD | **39.03±0.947a** |
| **Average*** (n=8)** | **41.61±0.947 b** | **36.57±0.542 c** | **45.48±1.12a** | **36.66±0.711c** | **36.14±1.04c** | |

*** Significant at p = 0.001; # Not significant at p = 0.05. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 12: Mean ± 1SE (n = 4) Fe- concentration (mg kg$^{-1}$) in wheat grains at experiment with soil plus foliar application of Zn and Fe.

| | Zn-Fe treatments | | | | Average# |
|---|---|---|---|---|---|
| N-treatments | Zn+Fe | Fe | (Zn+Fe ) s+f | Fe s+f | (n=16) |
| **N1N120** | 36.82±1.06 B | 36.31±0.457 B | 71.7±14.3 A | 48.80±1.0 AB | **48.40±4.91a** |
| **N2N120** | 38.05±0.456 B | 35.27±0.930 B | 51.16±2.05 AB | 47.37±1.69 AB | **42.96±1.80a** |
| **Average*** (n=8)** | **37.44±0.581b** | **35.79±0.519b** | **61.42±7.75 a** | **48.09±0.948ab** | |

*** Significant at p = 0.001; # Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 13: Mean ± 1SE for total Fe- uptake in grain (mg pot$^{-1}$) obtained at experiment with soil application of nutrients.

| | N-treatments | | | | | Average # |
|---|---|---|---|---|---|---|
| Zn-Fe treatments | N1N80 | N1N120 | N1N160 | N2N120 | N2N160 | (n=20) |
| Zn+Fe | 0.0992±0.0045ABCD | 0.1162±0.0031AB | 0.0972±0.0074BCD | 0.1085±0.0015ABCD | 0.1±0.0039ABCD | **0.1042±0.0024a** |
| Fe | 0.0881±0.0062 D | 0.1124±0.0036 ABC | 0.0980±0.0032 ABCD | 0.0944±0.0014CD | 0.1182±0.0047A | **0.1022±0.0031a** |
| **Avg. *** (n=8)** | **0.0936±0.0041c** | **0.1143±0.0023 a** | **0.0976±0.0037 bc** | **0.1015±0.0028 bc** | **0.1092±0.0044ab** | |

*** Significant at p = 0.001; # Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 14: Mean ± 1SE (n = 4) Fe- uptake (mg pot$^{-1}$) in wheat grains obtained at experiment with soil plus foliar application of Zn and Fe.

| | Zn-Fe treatments | | | | Average* (n=16) |
|---|---|---|---|---|---|
| **N-treatments** | **Zn+Fe** | **Fe** | **(Zn+Fe ) s+f** | **Fe s+f** | |
| **N1N120** | 0.1162±0.0031 B | 0.1124±0.0036 B | 0.1912±0.0.0374 A | 0.1499±0.0047 AB | **0.1424±0.0118a** |
| **N2N120** | 0.1085±0.0015 B | 0.0944±0.0014 B | 0.1282±0.0172AB | 0.1401±0.0047AB | **0.1178±0.0061b** |
| **Average ** (n=8)** | **0.1124±0.0021c** | **0.1034±0.0038bc** | **0.1597±0.0038a** | **0.1450±0.0036ab** | |

** Significant at p = 0.01; * significant at p = 0.05. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 15: Mean ± 1SE for grain Zn- concentration (mg kg$^{-1}$) obtained at experiment with soil application of nutrients.

| | N-treatments | | | | | Average # (n=20) |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | |
| **Zn** | 38.94±1.09AB | 36.87±0.674AB | 37.88±0.635AB | 38.23±1.16AB | 36.62±0.290 AB | **37.706±0.386a** |
| **Zn+Fe** | 40.23±0.532A | 35.68±0.563B | 40.0±0.960 A | 37.69±0.604AB | 35.34±0.667B | **37.786±0.546a** |
| **Average *** (n=8)** | **39.587±0.612 a** | **36.272±0.465 b** | **38.939±0.668 a** | **37.959±0.613ab** | **35.978±0.414b** | |

*** Significant at p = 0.001; # Not significant at p = 0.05. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

Table 16: Mean ± 1SE (n = 4) Zn- concentration (mg kg$^{-1}$) in wheat grains obtained at experiment with soil plus foliar application of Zn and Fe.

| | Zn-Fe treatments | | | | Average# (n=16) |
|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | |
| **N1N120** | 36.87±0.674 B | 35.68±0.563B | 43.51±0.586AB | 60.70±12.70A | **44.19±3.85a** |
| **N2N120** | 38.23±1.16B | 37.69±0.604B | 44.67±0.342 AB | 46.14±0.883AB | **41.68±1.04a** |
| **Avg. ** (n=8)** | **37.549±0.671b** | **36.683±0.383b** | **44.087±0.383ab** | **53.43±6.52a** | |

** Significant at p = 0.01; # Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

Table 17: Mean ± 1SE for total Zn- uptake in grain (mg $pot^{-1}$) obtained at experiment with soil application of nutrients.

| | **N-treatments** | | | | | **Average #** |
|---|---|---|---|---|---|---|
| **Zn-Fe treatments** | **N1N80** | **N1N120** | **N1N160** | **N2N120** | **N2N160** | **(n=20)** |
| **Zn** | 0.0937±0.0012 CD | 0.1157±0.0005 A | 0.0532±0.002 E | 0.1103±0.006 ABC | 0.1195±0.0016 A | **0.0985±0.0057a** |
| **Zn+Fe** | 0.0914±0.0037 D | 0.1126±0.0027 AB | 0.0972±0.007 BCD | 0.1075±0.002 ABCD | 0.1034±0.0018 ABCD | **0.1024±0.0024a** |
| **Avg. *** (n=8)** | **0.0926±0.0019b** | **0.1142±0.0014a** | **0.0752±0.0090 c** | **0.1089±0.0030a** | **0.1114±0.0032a** | |

*** Significant at p = 0.001; # Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 1.

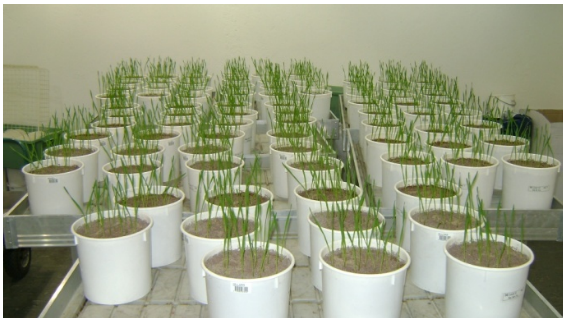
Table 18: Mean ± 1SE (n = 4) Zn- uptake (mg $pot^{-1}$) in wheat grains obtained at experiment with soil plus foliar application of Zn and Fe.

| | Zn-Fe **treatments** | | | | **Average#** |
|---|---|---|---|---|---|
| **N-treatments** | **Zn** | **Zn+Fe** | **Zn s+f** | **(Zn+Fe ) s+f** | **(n=20)** |
| **N1N120** | 0.1157±0.0005 AB | 0.1126±0.0027 B | 0.1260±0.0.0027 AB | 0.1669±0.031 A | **0.1303±0.00896a** |
| **N2N120** | 0.1103±0.0061 B | 0.1075±0.0018 B | 0.1384±0.0023AB | 0.1311±0.0014AB | **0.1218±0.00375a** |
| **Avg. ** (n=8)** | **0.113±0.0030b** | **0.1101±0.0018b** | **0.1322±0.0029 ab** | **0.149±0.0159a** | |

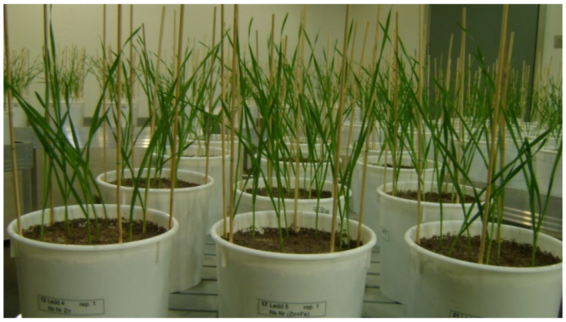
** Significant at p = 0.01; # Not significant at p = 0.05; Avg. = Average. The significance of letters after mean and details of treatments are explained in Annex 2: Table 2.

# Annexes 3:

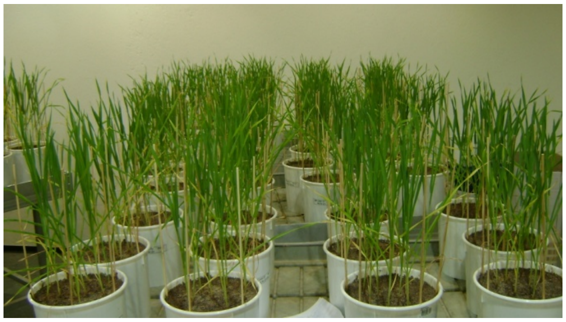
**Some photographs from growth of plants and laboratory works**: (Test plant: wheat var. Krabat, sowing date: 11 May 2012)



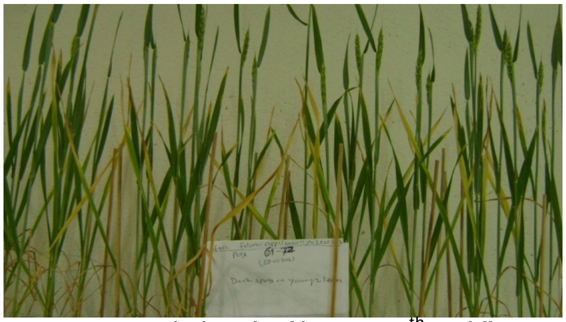
18 May 2012 (1 week seedlings)



25 May 2012 (3rd leaf stage)



15 June 2012 (6th leaf stage)



27 June 2012 (Minor leaf burn on 6th and flag leaves after foliar spray dominantly for Fe spray)

04 July 2012 (flowering stage)

21 July 2012 (Yellowing flag leaf but green spikes)

august (ready for spike harvest)

17

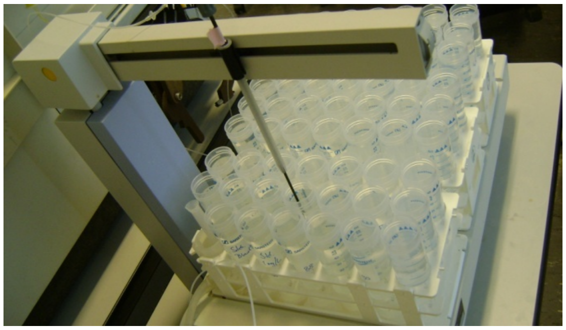
17 August 2012 (spikes collected in sample bags after harvest)

Wheat Sample grinding mill

(Mixture Mill; Retsch MM301)

Double deionization unit for water purification

Sample hanging in Ultra-clave after digestion



Measurement of Zn and Fe in optical emission spectrometer