Zinc and Iron Concentration as Affected by Nitrogen Fertilization and Their Localization in Wheat Grain

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Nearly half of the world cereal production comes from soils low or marginal in plant available zinc, leading to unsustainable and poor quality grain production. Therefore, the effects of nitrogen (N) rate and application time on zinc (Zn) and iron (Fe) concentration in wheat grain were investigated. Wheat (*Triticum aestivum* var. Krabat) was grown in a growth chamber with 8 and 16 h of day and night periods, respectively. The N rates were 29, 43, and 57 mg N kg^{-1} soil, equivalent to 80, 120, and 160 kg N ha^{-1}. Zinc and Fe were applied at 10 mg kg^{-1} growth media. In one of the N treatments, additional Zn and Fe through foliar spray (6 mg of Zn or Fe in 10 ml water /pot) was applied. Micro-analytical localization of Zn and Fe within grain was performed using scanning macro-X-ray fluorescence (MA-XRF) and laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). The following data were obtained: grain and straw yield pot^{-1}, 1000 grains weight, number of grains pot^{-1}, whole grain protein content, concentration of Zn and Fe in the grains. Grain yield increased from 80 to 120 kg N ha^{-1} rates only and decreased at 160 kg N ha^{-1} g. Relatively higher protein content and Zn and Fe concentration in the grain were recorded with the split N application of 160 kg N ha^{-1}. Soil and foliar supply of Zn and Fe (Zn + Fe_{s+f}), with a single application of 120 kg N ha^{-1} N at sowing, increased the concentration of Zn by 46% and of Fe by 35%, as compared to their growth media application only. Line scans of freshly cut areas of sliced grains showed co-localization of Zn and Fe within germ, crease and aleurone. We thus conclude that split application of N at 160 kg ha^{-1} at sowing and stem elongation, in combination with soil and foliar application of Zn and Fe, can be a good agricultural practice to enhance protein content and the Zn and Fe concentration in grain.

Keywords: nitrogen application, zinc and iron uptake, zinc and iron distribution in grain, wheat, LA-ICP-MS, MA-XRF

INTRODUCTION

Cereals are genetically low in Zn and Fe concentration, with reduced bioavailability (Graham et al., 2001; Cakmak, 2002). About half of the world cereal production come from soils low in plant available Zn (Cakmak, 2002), leading to poor quality of cereal grain with respect to Zn content. The situation is similar, concerning Fe deficiency in cereals. About one third of the developing countries' population and 10% of Americans and Canadians experience Zn deficiency or are at risk

of it (Hotz and Brown, 2004), erasing the geographical and political boundaries. Every year Fe and Zn deficiency causes deaths of about 800000 children and 2.4% of global disability-adjusted life years worldwide (DALYs), while the corresponding value of DALYs for Zn is 1.9% (Mutangadura, 2004). DALYs are calculated as the sum of years of life lost (YLLs) and the years lived with disability (YLDs) based on 291 causes and 20 age groups of both sexes. The consumption of white flour made predominantly from endosperm of wheat grain discarding bran in the milling process has even worsened the degree of Fe and Zn malnutrition. This is because Fe and Zn accumulate in higher concentrations in the embryo and aleurone layer than in endosperm of a wheat grain (Šramková et al., 2009; Cakmak et al., 2010). Hence, the consumption of whole grain wheat rather than white wheat flour has been advocated to increase the daily Fe and Zn intake.

Nitrogen fertilization is known not only to increase wheat grain yield but also to facilitate the uptake of Fe and Zn in wheat grain (Cakmak et al., 2010; Shi et al., 2010). The uptake and transport of Fe and Zn to grain is probably facilitated by metal chelating compounds (Kutman et al., 2010), such as 2-deoxymugineic acid (DMA) mainly for the translocation of Fe and Zn from flag leaves to grain in wheat (Barunawati et al., 2013). Kutman et al. (2011) reported that N nutrition is critical in both the uptake and translocation of Zn and Fe to wheat grain and they showed that at high N rate, nearly 80% and 60% of total shoot Zn and Fe, respectively, were harvested with grain. Improving N status of plants from low to sufficient resulted in threefold increase in shoot Fe content of wheat plants (Aciksoz et al., 2011) Similarly, Erenoglu et al. (2011) demonstrated that N is a critical player in the uptake and accumulation of Zn in plants and thus deserves special attention in biofortification of food crops with Zn. Depending on N supply, Zn remobilization from pre-anthesis sources provided almost all grain Zn, when the Zn supply was withheld at anthesis (Kutman et al., 2012). Cakmak et al. (2010) found co-localization of protein, Fe and Zn in embryo and aleurone layer of wheat grain, indicating that the protein rich grains accumulate higher amount of Zn and Fe in wheat grain. Increasing Zn and N supply had a major impact on Zn accumulation in the endosperm, reaching concentrations higher than the current breeding targets (Persson et al., 2016).

Cakmak et al. (2010) suggested the positive role of soil and foliar applied Zn and Fe in increasing respective metal concentrations in durum wheat grain and also claimed that increased activity of Zn and Fe in the source (flag leaf and stem) during grain filling could be increased by additional Zn and Fe application through soil or by foliar application. Habib (2012) showed that joint Zn-Fe application could increase in grain concentration more than with their separate application. However, the concentrations of Zn and Fe depend on the size of wheat grains (Velu et al., 2011) and number of grains per spike (Nowack et al., 2008). Haslett et al. (2001) and Timsina (2014) demonstrated the role of phloem transport of Zn in wheat plants by performing stem girdling, and they showed that ^{65}Zn supplied on upper leaf was transported to lower leaves and root tip.

The concentration of minerals vary within a grain, depending on its portions. For example, wheat endosperm contains about 15 mg kg^{-1} Zn, while germ and aleurone holds about 150 mg kg^{-1} Zn (Šramková et al., 2009). By using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), Wang et al. (2011) depicted higher concentration of Zn in the aleurone layer and crease vascular tissue with decreasing gradient of Zn from crease vascular tissue to endosperm, suggesting that translocation of Zn toward the endosperm occurred through the crease vascular tissue. Moreover, protein rich grain accumulated higher amount of Zn and Fe in wheat than low protein grain (i.e., Fe = 71 mg kg^{-1} and Zn = 57 mg kg^{-1} vs. Fe = 36 mg kg^{-1} and Zn = 30 mg kg^{-1}) (Ozturk et al., 2009). This showed that higher protein or nitrogen content favors the accumulation of Zn and Fe in wheat grain (Peleg et al., 2008; Ozturk et al., 2009; Kutman et al., 2010).

In spite of the available literature on the role of N on Zn and Fe uptake by plants, information on the optimum rate and time of N application, and its effect on Zn and Fe uptake under varying levels of micronutrients in the soil are scanty. Similarly, the localization of these micronutrients in grains is not fully understood. We hypothesized that (i) N fertilization increases protein yield components of wheat and the concentration of Fe and Zn in grain, (ii) foliar Zn and Fe spraying increases their concentration in wheat grain, and (iii) micro-analytical techniques can provide information on the location of Zn and Fe in wheat grain. To test these hypotheses, we investigated the interactive effect of N, Zn, and Fe on grain yield, protein content and nutrient concentration in a pot experiment conducted in an environmentally controlled growth chamber. In addition, we investigated the distribution of Fe and Zn in selected wheat grains by using scanning macro-X-ray fluorescence (MA-XRF) and laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS).

MATERIALS AND METHODS

Growth Media
Artificially prepared growth media, according to the OECD 207 guideline (OECD, 1984), were used for growing plants. They consisted of 80% sand (<2 mm), 10% peat (<4 mm), and 10% kaolin on a dry weight basis. In the absence of sphagnum peat, unfertilized 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 an oven at 105°C for 24 h for the correction of moisture content in peat. Average moisture content varied from 41 to 48% depending on peat delivery bags. To maintain the pH of growth media at 6.5 ± 0.2, CaCO$_3$ was mixed at rates from 0.5 to 5 g per 100 g growth media and the amount of lime required was calculated from the liming curve obtained. Hand mixed growth media and lime were prepared. The homogeneous growth media mixture was filled in 3-l plastic pots containing 2015 g mixture (dry weight) in each pot.

Experimental Design and Fertilizer Rates
The experiment was set up as a complete randomized factorial design (Table 1). and It consisted of two major treatment factors: N treatments and Zn-Fe treatments. The experiment was further

TABLE 1 | Design of experiment and rate of fertilization as per treatment.

Zn–Fe-treatments	N- treatments N1N80	N1N120	N1N160	N2N120	N2N160
Experiments with soil application of treatment factors (N, Zn, and Fe)					
Zn	NlN80Zn	NlN120Zn	NlN160Zn	N2N120Zn	N2N160Zn
Zn + Fe	NlN80 Zn + Fe	NlN120Zn + Fe	NlN160Zn + Fe	N2N120Zn + Fe	N2N160Zn + Fe
Fe	NlN80Fe	NlN120Fe	NlN160Fe	N2N120Fe	N2N160Fe
Experiments with soil plus foliar application of Zn and Fe					
Zn_{s+f}		$NlN120Zn_{s+f}$		$N2N120Zn_{s+f}$	
$Zn + Fe_{s+f}$		$NlN120(Zn+Fe)_{s+f}$		$N2N120(Zn+Fe)_{s+f}$	
Fe_{s+f}		$NlN120Fe_{s+f}$		$N2N120Fe_{s+f}$	

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 wit growth media. N1N120, single application of N at sowing equivalent to 120 kg N ha^{-1} mixed with growth media. N1N160, single application of N equivalent to 160 kg N ha^{-1} mixed with growth media. 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 of 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 of 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 growth media at sowing. Fe, Fe mixed with soil at sowing. Soil plus foliar application of Fe and Zn at booting stage: Zn_{s+f}, application of Zn at sowing plus 30% of growth media applied Zn as foliar spray. $(Zn + Fe)_{s+f}$, application of Zn and Fe at sowing plus 30% of growth media applied zinc and iron as foliar spray. Fe_{s+f}, application of Fe at sowing plus 30% of growth media applied iron as foliar spray.

divided into two groups: (i) experiments with growth media application of all treatment factors (5 N treatments × 3 Zn–Fe treatments = 15 growth media treatments) and (ii) experiments with growth media l plus foliar spray of Zn and Fe (2 N treatments × 3 Zn–Fe treatments = 6 growth media plus foliar treatments) (**Table 1**). Both treatment factors were incorporated into the same experiment to see the combined effect of them. Among the five N-treatments, three were single N application to growth media before sowing at the rates equivalent to 80, 120, and 160 kg N ha^{-1}, and two were split N applications at the rates equivalent to 120 kg N ha^{-1} and 160 kg N ha^{-1}. In split application treatments (N120 and N160 kg N ha^{-1}), 70% N was applied at sowing and 30% at the stem elongation phase. Similarly, the three Zn–Fe treatments included Zn, Fe, and Zn + Fe. The rate of Zn and Fe application at sowing was 10 mg kg^{-1} of growth media. Foliar spray of Zn and Fe (equal to 6 mg pot^{-1}) was made at booting stage in the two N treatments (single application and split application of 120 kg N ha^{-1}. This was done to assess the effect of foliar spray of Zn and Fe on wheat grain yield and Zn and Fe concentration. Since the artificially made growth media used supplied all nutrients required for plant growth, the need of having a control pot was not felt.

All basic nutrients and the treatment factors (N, Zn, and Fe) were applied in deionized water solution. Powdered calcium carbonate was mixed with growth media to maintain soil pH at 6.5 ± 0.2. The treatment combinations and rates and sources of N fertilizers and micronutrients are presented in **Tables 1**, **2**, respectively. The solution volume of all nutrients was fixed to 25 ml, which was later taken into account while watering the growth media after sowing. All added nutrients and lime were mixed manually to the growth media to get a homogeneous distribution. The second dose of nitrogen in split nitrogen treatments (N2N120 and N2N160), amounting to 30% of the total N, was added at the beginning of stem elongation and watered immediately, so that N could spread properly. A hand-held sprayer was used. The sprayed solution of 10 ml water per pot contained 6.0 mg of Zn as zinc sulfate and 6.0 mg of Fe Fe-EDTA mixed with DP-Klebemiddel surfactant with a concentration of 0.5 ml per one liter solution. Spraying was done after complete emergence of flag leaf at booting stage and 10 mL solution was sprayed several times to ensure that the whole solution was effectively sprayed on plant leaves.

Plant Growth and Harvesting

Wheat plants were grown in a control growth chamber at about 21°C. The duration of day and night length was 8 and 16 h, respectively. The source of light was halogen metal halide lamps by POWERSTAR HQI-BT 400W/D. The test plant was a hard red winter wheat variety "Krabat" used by farmers since 2011 which is claimed to be medium early in growth period, high yielding with good agronomical characteristics, medium protein, relatively good disease resistance and baking quality. Twenty seeds were sowed in each pot, which after 1 week were thinned to eight plants. While watering for the first time, the amounts of water contributed by peat and other liquid nutrients were taken into consideration to maintain moisture at 60% of field capacity throughout the growth period. It was achieved by weighing the pots with soil mixture and plants regularly and adding water to compensate the weight loss.

Plant were harvested at maturity by cutting each spike separately, and these were kept in bags for each pot. After removal of spikes, straw was cut just at the base of first node. Grain and straw yields were recorded after oven drying at 75°C for 48 h. Wheat grain and straw were ground in a ball mill (Retsch MM301), with ball and container walls of zirconium to avoid sample contamination. However, for the sake of brevity, only grain yield, protein, Zn, and Fe concentration in grain are reported in this paper.

Chemical Analysis

Total N in grain nitrogen was analyzed by dry combustion as described by Bremner and Mulvaney (1982). The whole grain protein (WGP) was obtained by multiplying the total N by a factor of 5.70 (ISO, 2009).

TABLE 2 | 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 and 36 kg N ha^{-1}) and 160 (i.e., 112 and 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**

All solutions were added to growth media before sowing at volume 25 ml. Rows with bold letters represent for treatment factors.

About 0.2 g of ground wheat flour was digested in 5 ml conc. HNO_3 for about 2 h in ultra clave microwave reactor (MLS-MILESTONE, ultra-CLAVE III) at 250°C and at 160 bar pressure. The digested samples were diluted to 50 ml by adding double de-ionized water (B-pure, Barnstead). Three Standard Reference Materials (SRM) (SRM1567a wheat flour) and 5 method blanks (5 ml HNO_3 solution) were also digested along with grain samples. Concentrations of Fe and Zn were analyzed by an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 5300 DV) in wheat samples, SRM, and method blanks.

The lower detection limits (LOD's) and lower quantification limits (LOQ's) were determined for the concentration of Fe and Zn in the method. 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 with the certified concentration limits and the RSD was <5%.

Localization of Fe and Zn in Wheat Grain

Six selected wheat grains from different treatments containing relatively high Zn (42–99 mg/kg) and Fe (44–115 mg/kg) concentration were used. These grain samples were collected from different pots showing higher Zn and Fe concentration and mostly from foliar experiment and thus here the effect of N on Zn and Fe location was not clearly investigated. At first, the Environmental scanning electron microscope with dispersive X-ray spectrometry (ESEM-EDS) technique, available at the Norwegian University of Life Sciences (NMBU), Norway, was used for observation of grain morphology and element distribution. However, the limit of detection of ESEM-EDS (0.1% w/w) was not sufficiently low to detect and quantify Zn and Fe in the expected concentrations. For trace element 2D distribution analysis, Scanning macro- X-ray fluorescence (MA-XRF) and laser ablation-inductively coupled plasma-mass

spectrometry (LA-ICP-MS) were performed at the University of Antwerp, Belgium.

Each seed was sliced in two parts. Slicing of grains was done with a razor blade that was cleaned with ethanol prior to use. The half grains were measured with no additional sample preparation. In the case of MA-XRF, they were mounted by means of adhesive tape on a diapositive frame positioned vertically on sample holder. The measurement, after verifying the sample-source-detector distance, was performed in an entirely non-invasive way.

In the case of LA-ICP-MS, they were introduced mounted horizontally on plasticine on the floor of the sample chamber, which was closed and purged. Detailed photographs of the surface were acquired by means of the instrument software, and the line profiles were drawn and ablated on the basis of those.

MA-XRF was performed using a non-commercial self-assembled Scanning macro- X-ray fluorescence (XRF), with the setup named instrument C (Alfeld et al., 2011). 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. XRF maps were obtained for each element detectable in the grains. The most relevant elements were Fe, Zn, and K, whose distribution is shown in **Figure 6**. For a more detailed analysis of these elements and their co-occurrence in the grain, the following technique was used.

LA-ICP-MS was performed with a New Wave NWR193 ArF excimer laser and a Varian 7700 quadrupole ICP-MS. 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 μm/s with dimension more than 3 mm length and 100 μm 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 until 20 s and washout begin after about 290 s and lasting until 350 s. This generated gross

element counts, which were further refined in the following way. Background counts were collected along the scan line before and after the wheat grain (which was located in the middle of a scan line). Net counts were determined by subtracting from the gross counts the average background counts (after removal of outliers) for each element (K, Fe, Zn). 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 since it occurs in a more evenly distributed way throughout the grain, and particularly in the crease, as made visible by MA-XRF in **Figure 6D**. The use of K-normalized counts for Fe and Zn, helps evidencing any real increase of either element in spots/areas of the grain, independently from total ion count and surface/positioning effects.

Statistical Analysis

The analysis of variance (ANOVA) was performed by two-way ANOVA and the relations 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 the respective variable. The comparison between all treatments, considering the interaction of treatment factors and main effects of N- and Zn–Fe- treatments, was carried out by Tukey comparison. In all cases, data were analyzed considering 5% level of significance ($p = 0.05$).

RESULTS

Grain Yield

The grain yield pot^{-1} increased while increasing N rate from 80 to 120 kg N ha^{-1}, but decreased when N was increased to 160 kg N ha^{-1}. A similar trend was also observed for split application of N from 120 to 160 kg N ha^{-1}. The single application of 120 kg N ha^{-1} at sowing (N1N120) and split application of 160 kg N ha^{-1} at sowing and stem elongation (N2N160) resulted in the highest yield (**Figure 1**). Likewise, the growth media application of Zn produced higher yield than growth media applied Fe and Zn + Fe, particularly at the application rate of 160 kg N ha^{-1} at sowing (**Figure 1**).

The growth media plus foliar application (Zn$_{s+f}$ or Fe$_{s+f}$) for Zn and Fe, single or together, increased the grain yield in comparison to their growth media application at sowing. The single application (N1N120) of 120 kg N ha^{-1} resulted in significantly higher yield (3.02 ± 0.04 g pot^{-1}) than the split application (N2N120) of 120 kg N ha^{-1} (2.87 ± 0.04 g pot^{-1}) (**Table 3**) in all combinations of Zn and Fe, except for growth meida plus foliar spray of Zn. The Zn-Fe treatment (Zn + Fe) did not show a significant difference in mean grain yield for experiments with growth media l plus foliar application (Zn + Fe$_{s + f}$) of Zn and Fe ($p = 0.107$).

Whole Grain Protein (WGP)

The growth media and foliar application (Zn$_{s+f}$ or Fe$_{s+f}$) resulted in significantly higher wheat grain protein (WGP) as compared to growth media application (Zn or Fe) ($p = 0.021$) at single as well as split N equivalent to 120 kg N ha^{-1} (**Table 4**). Irrespective of N treatments, average increase of WGP for growth media l plus foliar application of Zn + Fe$_{s + f}$, Zn$_{s + f}$ and Fs + fe was 8.4, 6.5, and 7% as compared to their growth media - applied rate (Zn + Fe, Zn, Fe). Among all treatments, the growth media plus foliar application of Zn + Fe at split application of 120 kg N ha^{-1} (Zn + Fe$_{s + f}$ at N2N120) produced the highest protein rich grains (8.96 ± 0.133%), suggesting that split N application is a better method to achieve higher protein in wheat grain. Zinc in combination with single growth media application of 120 kg N ha^{-1} (Zn at N1N120) gave the lowest protein content (7.95 ± 0.06%) (**Table 4**).

Iron Concentration in Wheat Grain

Iron concentration in grain was significantly affected by growth media supplied N ($p < 0.001$). The increasing rate of N from N1N80 to N1N160) resulted in significantly higher Fe concentration in grain when Fe was applied alone (**Figure 2**).

The growth media and foliar application (Fe$_{s + f}$ and Zn + Fe$_{s + f}$) of Fe increased the Fe— concentration in wheat grain significantly ($p < 0.001$) as compared to their growth media applied rate (Fe and Zn + Fe). Both growth media (Zn + Fe) or growth media plus foliar application (Zn + Fe$_{s + f}$) of Zn + Fe showed higher Fe- concentration in wheat grain, but a significantly higher Fe-concentration was achieved only with Zn + Fe$_{s + f}$ at N1N120 (**Figure 3**).

Zinc Concentration in Wheat Grain

Grain Zn-concentration responded significantly to the main effect of N-treatment ($p < 0.001$). When N rate at sowing increases from 80 to 120 kg N ha^{-1} (N1N80 to N1N120), the concentration of Zn in grain tends to decrease (**Figure 4**). For instance, single application of 120 kg N ha^{-1} (N1N120) and split application of 160 kg N ha^{-1} (N2N120) showed about 10% less Zn as compared to single application of 80 and 160 kg N ha^{-1} (N1N80 or N1N160). All other combinations of treatments showed generally the same Zn concentration.

In the experiments with growth media plus foliar spray of Zn and Fe (Zn$_{s + f}$ and Zn + Fe$_{s + f}$), Zn- concentration in wheat grain increased significantly ($p < 0.01$), but was not affected by N- treatments and the interaction between N- and Zn–Fe- treatments. The increase in the grain Zn-concentration in foliar sprayed treatments (Zn$_{s + f}$ and Zn + Fe$_{s + f}$) was higher than with Zn or Zn + Fe treatments at N1N120 or N2N120, but a significantly higher Zn- concentration was achieved only with Zn + Fe$_{s + f}$ at N1N120 (**Figure 5**).

Relationship Between Fe- and Zn- Concentration 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 the total grain weight (TGW), number
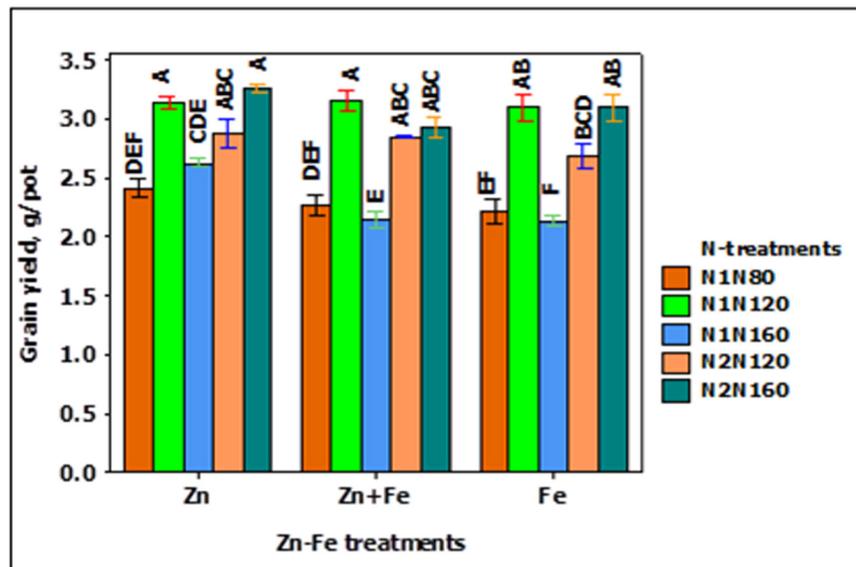
FIGURE 1 | Grain yield at N when Zn, Zn + Fe, and Fe applied to growth media at sowing. N1 and N2 for single and split N. 80, 120, and 160 stand for rate of N in kg ha^{-1}. Bars with same alphabet at head are not significantly different at 5% level of significance.

TABLE 3 | Mean ± 1 SE grain yield (g pot^{-1}) at experiment with growth media plus foliar application of Zn and Fe.

N-treatments	Zn	Zn + Fe	Fe	Zn$_{s + f}$	(Zn + Fe)$_{s+f}$	Fe$_{s+f}$	Average** (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 ± 0.044a**
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**	

Zn–Fe treatments spans the Zn through Fe$_{s+f}$ columns.

**Significant at p = 0.01; #Not significant at p = 0.05; Avg., Average; Treatment means ± 1 SE (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 growth media application of 120 kg N ha^{-1}. Zn, Zn + Fe and Fe without suffix for growth media application of Zn–Fe- treatments and with suffix's + f' for growth media plus foliar application.

TABLE 4 | Mean ± 1 SE (n = 4) whole grain protein (%) in wheat grains at experiment with growth media plus foliar application of Zn and Fe.

N-treatments	Zn	Zn + Fe	Fe	Zn$_{s+f}$	(Zn + Fe)$_{s+f}$	Fe$_{s+f}$	Average * (n = 24)
N1N120	7.9 ± 0.108 C	8.13 ± 0.101BC	8.0 ± 0.042BC	3.51. ± 0.1 (BAB	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	S. 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 ± 0.07Scd**	**8.ll ± 0.084d**	**8.48 ± 0.073bc**	**8.92 ± 0.085a**	**8.67 ± 0.108ab**	

Zn–Fe treatments spans the Zn through Fe$_{s+f}$ columns.

****Significant at p = 0.001; *significant at p = 0.05; Avg., Average. The significance of letters after means and treatments are explained in Table 3.*

of grains pot^{-1} and grain Fe-uptake (Equation 1) rather than with a 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}.

$$\text{Grain Fe} - \text{concentration (mg kg}^{-1}) = 69.89 - 0.96 \text{ TGW}$$
$$\text{(g)} - 0.321 \text{ No. of grains pot}^{-1} + 261.46 \text{ Grain Fe} - \text{uptake}$$
$$\text{grain (mg pot}^{-1}) \qquad (1)$$

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

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 ($p < 0.01$) (Equation 2). 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
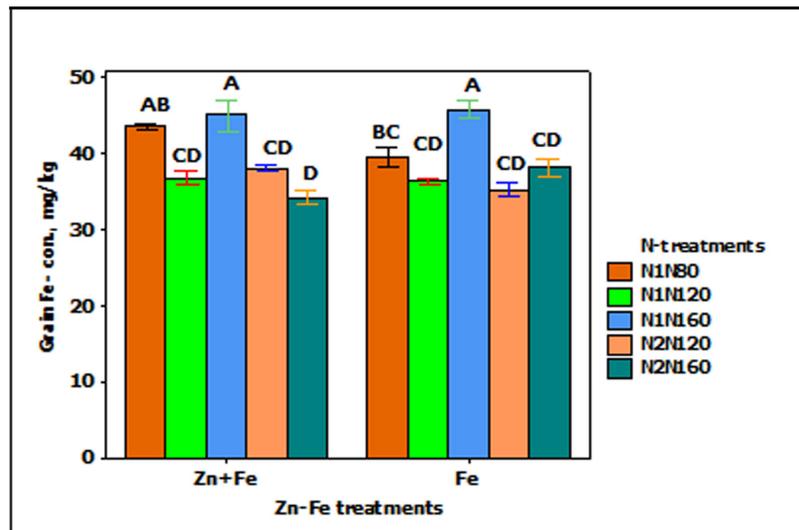
FIGURE 2 | Mean $\pm$ 1 SE ($n = 4$) bar plot for the responses of grain Fe- concentration at N-treatments when Zn + Fe and Fe applied to growth media at sowing. N1 and N2 for single and split N. 80, 120, and 160 stand for rate of N in kg ha^{-1}. Bars with same alphabet at head are not significantly different at 5% level of significance.
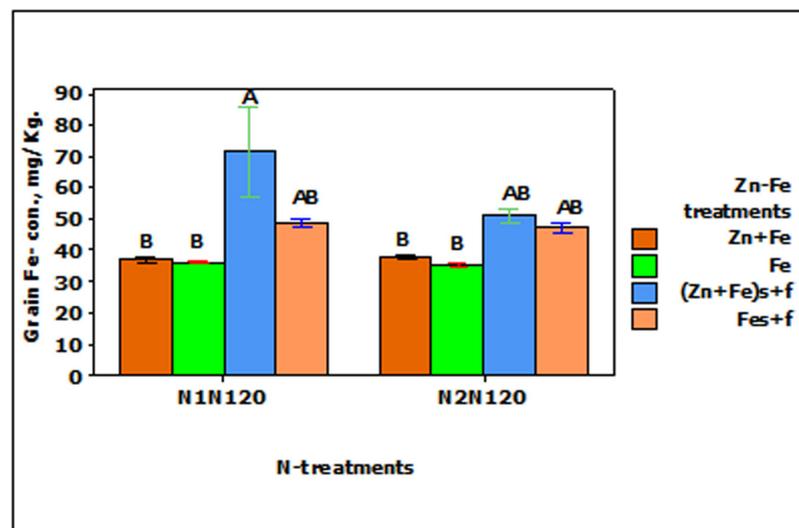
FIGURE 3 | Mean $\pm$ 1 SE ($n = 4$) bar plot of grain Fe- concentration for the effect of Zn–Fe- treatments. N1 and N2 stand for single and split application of N. N120 stands for 120 kg N ha^{-1}. Zn + Fe and Fe without suffix for growth media application and with suffix s + f for growth media plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

grain Zn- concentration when number of grains pot^{-1} tended to increase.

$$\text{Grain Zn} - \text{concentration (mg kg}^{-1}) = 45.625 - 0.646 \, \text{TGW}$$
$$\text{(g)} - 0.211 \, \text{No. of grains pot}^{-1} + 120.98 \, \text{Grain Zn} - \text{uptake}$$
$$\text{(content) in grain (mg pot}^{-1}) \qquad (2)$$

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

Localization of Zn and Fe in Wheat Grain

Element distribution map of half wheat grains generated by the MA-XRF are shown in **Figure 6**. Relatively bright spots in the maps represent higher X- ray signal from the respective elements. This signal is influenced by the element concentration in the sample, among other factors. However, the analyzed surfaces of grains were slightly irregular and this gives rise to somewhat hazy depiction of the element distribution.

In the MA-XRF maps, it is obvious that Fe and Zn concentrations are rather variable inside the wheat grain.
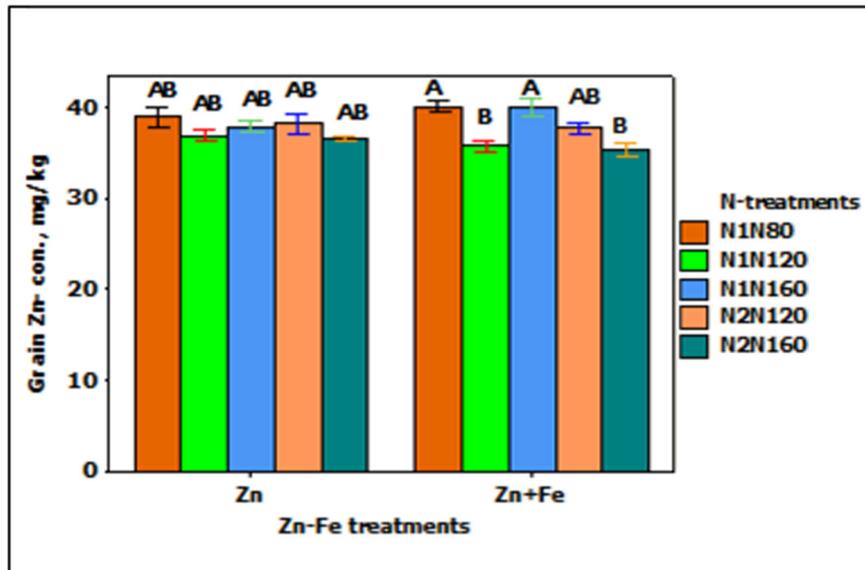
FIGURE 4 | Mean ± 1 SE (*n* = 4) bar plot for the responses of grain Zn- concentration at N-treatments when Zn and Zn + Fe applied to growth media at sowing. N1 and N2 for single and split N. 80, 120, and 160 stand for rate of N in kg ha^{-1}. Bars with same alphabet at head are not significantly different at 5% level of significance.
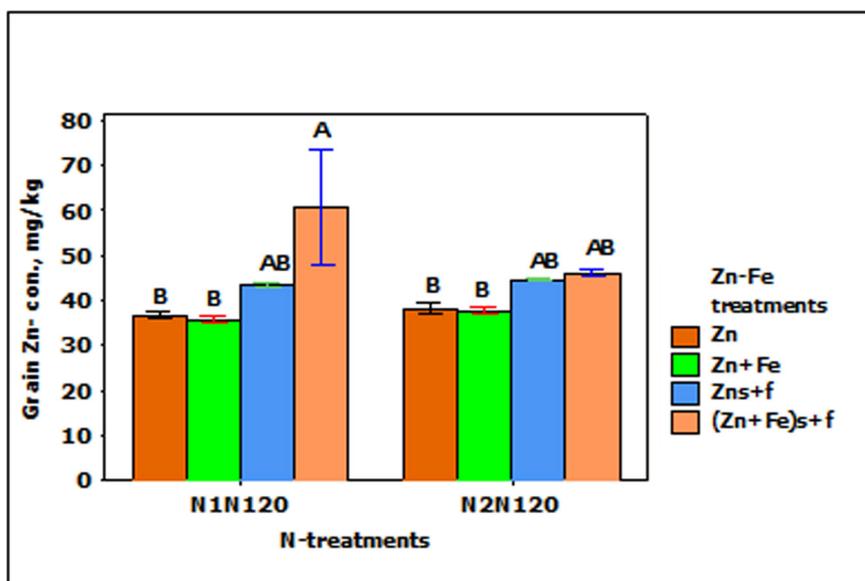
FIGURE 5 | Mean ± 1 SE (*n* = 4) bar plot of grain Zn- concentration for the effect of Zn–Fe- treatments. N1 and N2, respectively stand for single and split application of N. N120 stands for 120 kg N ha^{-1}. Zn and Zn + Fe without suffix for growth media application and with suffix s + f for growth media plus foliar application. Bars with same alphabet at head are not significantly different at 5% level of significance.

However, Fe and Zn seem to have similar patterns throughout all grains. Iron concentrated mainly in embryo and to some extent along the aleurone layer in the crease area (**Figure 6B**). Zn appears to be present in the embryo and along crease just outside the endosperm (**Figure 6C**).

LA-ICP-MS was performed on three out of the six samples that were analyzed by MA-XRF. LA-ICP-MS was used to obtain normalized signals for Fe and Zn along a well-defined line profile transversally across the grains (**Figure 7**). Generally, the profiles for both elements appear to vary in a similar fashion, confirming their co-localization. The normalized Zn and Fe counts clearly peaked at well-defined points. Higher signals of both elements were found at both ends of the grain and, for Zn only, at the embryo-aleurone interface in the middle of the grain. The

FIGURE 6 | Half wheat grains analyzed under MA-XRF Instrument C. **(A)** Position of wheat grains subjected to MA-XRF. **(B–D)** Element distribution maps (1400 × 400 pixels) of wheat grains. **(B)** Iron, **(C)** Zinc and **(D)** Potassium.
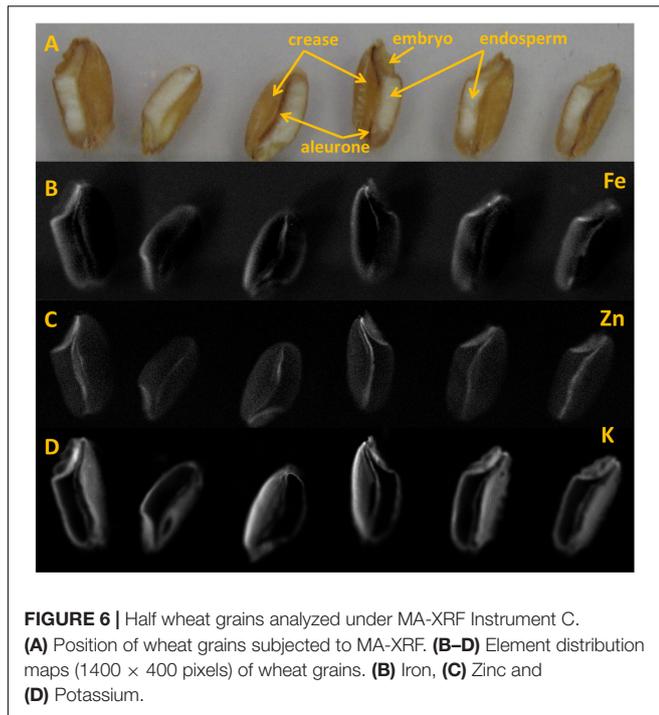
lowest signals for both elements were encountered within the endosperm. Higher normalized signals can be seen for Fe than for Zn at both ends of the grain, generally associated with bran.

DISCUSSION

Grain Yield

Among N treatments, grain yield increased from the N rate of 80 kg N ha^{-1} (N1N80) to the N rate of 120 kg N ha^{-1} (N-treatments: N1N120 and N2N120) but it decreased at the highest N rate of 160 kg N ha^{-1}. This suggested positive yield response to increasing N application rate up to a definite rate only (Marino et al., 2009; Abedi et al., 2011) and decline beyond this level. Split application of 160 kg N ha^{-1} (70% at sowing and 30% at stem elongation) produced higher grain yield than at split application of 120 kg N ha^{-1}. Reduction in grain yield at higher N rate may be associated to dilution of Zn and Fe, thus limiting their supply. As pointed out by Kutman et al. (2011), significant reduction in grain yield was caused by the high N treatment under the discontinued Zn regime. Dilution of Zn may have affected grain yield by impairing the reproductive development (Cakmak and Engels, 1999). It is likely that the reproductive development in later spikes was negatively affected by poor supply of Zn (Kutman et al., 2011).

Grain yield was significantly higher for growth media application of Zn than growth media applied Fe or Fe + Zn. The results are in line with Silspour (2007) and Nadim et al. (2012). Nadim et al. (2012) recorded significant increase in grain yield with soil applied Zn (in the form of ZnSO$_4$) in comparison with soil applied Fe. The effect of N on grain yield and the increase in grain yield for growth media applied Zn was also associated with the increased number of grain pot^{-1} (data not

FIGURE 7 | LA-ICP-MS Normalized counts along scan profiles. Normalized counts represent the relative count intensity of Zn and Fe with respect to K. The signals for Zn and Fe for two scan lines are shown. The arrows indicate approximate location for elevated Zn and Fe signals in the grain fall on scan line. The white part in the picture of the wheat grain is the endosperm and bran is on either sides of endosperm. The embryo is at the top the grain. The bran at right hand side of endosperm is the crease.

shown). Kutman et al. (2012) showed that the increase in the grain yield due to improved N and Zn supply was parallel to the increase in spike number and eventually grain amount. Nadim et al. (2012) and Jiang et al. (2013) in their respective studies pointed out higher leaf area index and photosynthetic rate in connection with soil applied Zn at sowing time. 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.

Whole Grain Protein

In the experiment with soil application of nutrients, N-treatments N2N160 and N1N80, resulted in comparatively higher WGP than other N treatments. Each of these N- treatments represented different grain yield groups in this study. At N2N160, the grain yield was highest but N1N80 treatment produced lowest yield. At N2N160, the increase in protein with increasing grain yield 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 on 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 surplus firstly and N limitation secondly. Similarly, for N1N80, higher protein was associated with lower yield, suggesting the concentration effect (Marschner, 1995). The higher protein concentration may not be the result of sufficient N but it could be due to the reduction in grain yield by limited available N and environmental limitation (Fowler, 2003) leading to less dilution.

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; Abedi et al., 2011). When initial N rate at sowing was sufficient, the split N applied at stem elongation period assured the increase in both yield and protein. For example, split application of 160 kg N ha^{-1} (70% at sowing and 30% at stem elongation) increased both protein and grain yield. However, 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 because of limited availability of initial N needed for an increase of number of grains per spike (Li et al., 2001).

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 that dilution of Zn and Fe in wheat grain occurs at increased grain yields (Liu et al., 2006; Gomez-Becerra et al., 2010). Multiple linear regression analysis (Equation 1 and Equation 2) presented a decreasing tendency of Fe- and Zn- concentrations in grain with increase in grain yield parameters: TGW and number of grains pot^{-1}. Similarly, Zhao et al. (2009) reported that Zn- concentration of wheat

grain correlated negatively with grain yield, but the correlation with grain weight was weak. A positive correlation of Zn- and Fe- uptake (i.e., total uptake in grain) suggested that higher concentrations of Zn and Fe in wheat grain were due to the increased uptake from soil or translocation of Zn and Fe from vegetative parts to the grain (Cakmak et al., 2010; Kutman et al., 2011).

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 that the 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 et al., 2004, 2010; Kutman et al., 2011) or factors contributing dry weight (starch) in grain, which increases the size, and weight of grain (Marschner, 1995; Pleijel et al., 1999).

The application of 30% higher Zn and Fe, either separately (Zn$_{s + f}$ and Fe$_{s + f}$) or together (Zn + Fe$_{s + f}$), 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., 2010; Habib, 2012). For instance, foliar applied Fe (F$_{s + f}$) and Zn + Fe (Zn + Fe$_{s + f}$) increased the Fe- concentration in grain by 34 and 64% in comparison to growth media applied Fe and Zn + Fe, respectively. The respective increases for Zn were 17 and 46% for foliar applied Zn (Zn$_{s + f}$) and Zn + Fe (Zn + Fe$_{s + f}$). This could be explained by the increased activity of Zn and Fe in sources (flag leaf and stem) during grain filling (Cakmak et al., 2010) when additional Zn and Fe was supplied at booting. The increase was notably higher for the application of Zn + Fe together, similar to the finding of Habib (2012), where Fe and Zn concentration in wheat grain increased by applying Zn and Fe together as foliar spray.

Localization of Zn and Fe in Wheat Grain

In this study, concentration map of Zn and Fe, obtained by MA-XRF and normalized count plots provided by LA-ICP-MS, evidenced the co-existence of both elements, especially at embryo stage and just outside the endosperm and the aleurone layer. This is in accordance with the results obtained using staining technique developed by Cakmak et al. (2010), where the co-localization of protein, Zn, and Fe in embryo was claimed to be due to the co-segregation. Similarly, Kutman et al. (2010) also showed the co- existence of Zn and protein in a durum wheat grain. Tsuji et al. (2006) used a 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 by μ-XRF technique, Zn and Fe were found to be located at either embryo and/or coat of grains (Tsuji et al., 2006). In this study, LA-ICP-MS revealed higher signals for both Zn and Fe in the embryo and bran portions, including the aleurone and crease area, with low signals

in the endosperm (**Figure 7**). These results are similar to those reported by Cakmak et al. (2010) and Wang et al. (2011), where the distribution of Zn in wheat grain and its translocation to the endosperm were shown. Based on the decreasing concentration gradient of Zn from crease area toward endosperm, Cakmak et al. (2010) suggested that Zn and Fe are translocated and distributed through the crease and then pass in to the endosperm.

To clearly define the location of Zn and Fe and their gradients, from bran to endosperm, crease area to endosperm and embryo to endosperm, for instance, the identification of the direction of element supply is essential. For this, the spatial resolution should be higher than in this study. Besides, higher sample numbers and improved sample preparation should ensure improved results, allowing for instance to avoid possible topography/surface effects on element signals.

CONCLUSION

The rate of N application at sowing caused an increase in grain and straw yield up to the N rate of 120 kg N ha^{-1} and a decrease at higher rate of N. The increase in grain yield was primarily determined by the increase in the number of grains pot^{-1} or number of grains spike^{-1}. The split application of 160 kg N/ha increased the grain and straw yield more than split application of 120 kg N/ha. The growth media application of Fe and Zn interacted with N to increase protein, Zn and Fe concentration in wheat grain. The foliar sprayed Zn and Fe at booting stage of wheat significantly increased the whole grain protein, total uptake and concentration of Fe and Zn in grain.

MA-XRF and LA-ICP-MS results indicated the co-localization of Zn and Fe in grain especially in the embryo and the aleurone. LA-ICP-MS also indicated higher concentration of Zn and Fe in the crease area and lower in the endosperm, indicating that Zn and Fe could translocate into the endosperm (the common source of flour in daily food) via crease tissue.

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