UPTAKE, TRANSPORT AND SEED DEPOSITION OF ZINC IN WHEAT AND MAIZE UNDER VARIED ZINC AND NITROGEN SUPPLY

by

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ABSTRACT

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Molecular Biology, Genetics and Bioengineering, PhD Dissertation, November 2019

Supervised by: Prof. Dr. Levent Ozturk

Keywords: agronomy, biofortification, maize, nitrogen, wheat, zinc

Chronic zinc (Zn) deficiency is a major health issue affecting over two billion people, caused by heavy reliance on staple crops (*i.e.* wheat, rice and maize) which are inherently low in Zn. This project was devoted to reveal the individual and combined effects of genetic and agronomic Zn biofortification in wheat and maize. The first part focused on understanding the mechanisms involved in differences in uptake and translocation of foliar-applied Zn among wheat and maize species. It was shown that wheat has a greater capacity of leaf uptake and translocation of foliar-applied Zn compared to maize. The second part investigated the effect of nitrogen (N) supply on uptake and accumulation of Zn in maize and wheat. Improving N supply significantly enhanced the shoot accumulation as well as leaf uptake of Zn from foliar Zn sprays in wheat and maize. The third part studied the effectiveness of Zn fertilizers in the form of soil, foliar and soil + foliar for improving growth, grain yield and nutrients uptake by genetically biofortified HarvestPlus wheat genotypes. It was demonstrated that the genetically biofortified genotypes have higher capacity to uptake, utilize and translocate Zn from soil and/or foliar applications as compared to conventional cultivars. These results conclude that the most sustainable way of tackling human Zn deficiency would be to improve grain Zn concentration of cereal crops by unifying genetic and agronomic biofortification strategies.

ÖZET

FARKLI ÇİNKO VE AZOT UYGULAMALARI ALTINDA YETİŞEN BUĞDAY VE MISIRDA ÇİNKONUN ALIMI, TAŞINMASI VE TANEDE BİRİKİMİ

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Anahtar sözcükler: agronomik, azot, biyofortifikasyon, buğday, çinko, mısır

Kronik çinko (Zn) eksikliği iki milyardan fazla insanı etkileyen önemli bir sağlık sorunudur ve temelinde Zn bakımından fakir tahıllara (buğday, pirinç ve mısır) olan bağımlılık yatmaktadır. Bu proje, buğday ve mısırda genetik ve agronomik Zn biyofortifikasyonunun bireysel ve kombine etkilerini ortaya çıkarmak için yürütülmüştür. Birinci bölüm, buğday ve mısır türleri arasında yapraktan uygulanan Zn'nun alımı ve taşınmasındaki farklılıkta rol oynayan mekanizmaların anlaşılmasına odaklanmıştır. Yapraktan uygulanan Zn'nun alımı ve taşınması bakımından, buğdayın mısırdan üstün olduğu gösterilmiştir. İkinci bölümde farklı azot (N) uygulamalarının mısır ve buğdayın Zn alımı ve birikimine etkisi araştırılmıştır. Buğday ve mısıra uygulanan N arttıkça yeşil aksamda daha fazla Zn birikmiş ve yapraktan uygulanan Zn'nun alımı önemli oranda artmıştır. Üçüncü bölümde, genetik olarak biyofortifiye edilmiş HarvestPlus genotiplerinin büyüme, tane verimi ve besin alımını iyileştirmek üzere toprak, yaprak ve toprak + yaprak formunda uygulanan Zn gübrelemesinin etkinliği incelemiştir. Biyofortifiye edilmiş genotiplerinin konvansiyonel çeşitlere göre toprak ve/veya yaprağa uygulanan Zn'yu daha etkin bir şekilde alma, kullanma ve taşıma kapasitesine sahip olduğu gösterilmiştir. İnsanda Zn eksikliği ile başa çıkmak üzere kullanılabilecek en sürdürülebilir yöntemin tahılların tane Zn konsantrasyonunu arttırmak üzere genetik ve agronomik biyofortifikasyon stratejilerinin birleştirilmesi olduğu sonucuna varılmıştır.

This work is dedicated to my little boy, *Muhammad Aashir Jilani*, who, with eyes full of dreams and heart full of hope, wished to see his Mom for early years of his life…

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA .....analysis of variance

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

Conc .....concentration

cv .....cultivar

dH_2O .....distilled water

DI ..... deionized

DTPA .....diethylenetriamine penta acetic acid

e.g. .....exempli gratia (for example)

FAO .....Food and Agricultural Organization

Fe .....iron

Fe-EDTA .....iron ethylenediamine tetra acetic acid

H_2O .....water

HNO_3 .....nitric acid

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

ICP-MS.....inductively coupled plasma Mass spectrometry

KCl .....potassium chloride

KH_2PO_4 .....potassium dihydrogen phosphate

K_2SO_4 .....potassium sulfate

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

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

N .....nitrogen

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

P .....phosphorus

ppm .....parts per million

SD .....standard deviation

Std.....standard

UN..... united nations

v/v.....volume per volume

WHO.....World Health Organization

w/v .....weight per volume

Zn .....zinc

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

(A) GENERAL INTRODUCTION

A.1. Functions of Zinc and Zinc-deficiency Related Health Problems

Zinc (Zn) deficiency is one of the most important malnutrition problems affecting over one-third of the world's population (Velu *et al.,* 2014; FAO *et al.,* 2015). Zinc deficiency is more prevalent in the developing world (Hess SY, 2017) with percentage of individuals at risk being highest in the South East Asia (33%), followed by Sub Saharan Africa (28%), South Asia (27%), Latin America and the Caribbean (25%) (Wuehler *et al.,* 2005). In Pakistan, unfortunately, more than 50% of the total population is suffering from micronutrient deficiencies with Zn and Fe deficiency being the most common. The National Nutrition Survey (NNS) report indicated that 37% of 0 to 5-years old children and 48% of pregnant women in Pakistan are Zn deficient (Bhutta *et al.,* 2011).

The importance of Zn as a micronutrient is well known for both humans and plants (Cakmak *et al.,* 1996) where it is practically found in all tissue types and with a variety of metabolic functions. Numerous proteins which are directly involved in structural and regulatory functions in the human body has Zn as a foremost component/element (Andreini and Bertini, 2012, Andreini *et al.,* 2011, Krezel & Maret, 2016). Zn is necessary for cellular functions such as cell growth and division, and it plays a vital role in a wide range of biochemical processes within the cell such as carbohydrates catabolism. It has a crucial role in the proper working of the immune (defensive) system in the body and is important for wound healing. Furthermore, Zn is important for reproductive health and fertility in both males and females because it has a critical role in balancing levels of reproductive hormone including testosterone, estrogen and progesterone. Therefore, low Zn in the body can cause infertility in both men and women (Frassinetti *et al.,* 2006).

Optimal Zn level in the body is essential for appropriate physical performance, energy level, and body configuration because it is required for the proper functioning of red and white blood cells and mainly concentrated in body organs like kidneys, bones, liver, and pancreas (Kaur *et al.,* 2014). Zn deficiency in humans leads to many critical health problems especially related to the immune system. An adequate level of Zn in the body enhances the immune system and hence, prevents many infectious diseases like diarrhea and pneumonia as well as different types of cancers. Recently, researchers related Zn deficiency to various kinds of cancers such as breast, ovaries, colon, lungs, and skin cancer. This deficiency can lead to the accumulation of cholesterol and inflammation, which results to increase the heart diseases risk. It is also required for the proper functioning of insulin and potentially can prevent diabetes (Alam and Kelleher, 2012, Vidyavati *et al.,* 2016, Liu *et al.,* 2017).

The symptoms of Zn deficiency in humans include stunted growth, reduced brain development, mental disability, and increased vulnerability to many infectious diseases such as pneumonia and diarrhea (Black *et al.,* 2008; Gibson, 2012; Krebs *et al.,* 2014; Terrin *et al.,* 2015).

The recommended dietary allowance for Zn generally depends on gender, age and special conditions like pregnancy and lactation period. According to International Zinc Nutrition Consultative Group (IZiNCG) the recommended dietary allowance (RDA) of Zn for adults varies between 9 and 19 mg per day (Gibson *et al.,* 2010). However, the average daily Zn intake of individuals consuming wheat as a major food is estimated to be about 3.2 mg per day, resulting in severe Zn deficiency and related diseases (Cakmak and Kutman, 2017). Zinc deficiency is especially more dangerous for children under 5 years of age due to higher demand to meet rapid growth and development (Wessells & Brown, 2012). It has been reported that annually, around half a million children in the world die because of the diseases related to Zn deficiency. Similarly, pregnant women require high relatively high amount of Zn and a higher miscarriage rate was recorded in Zn deficient pregnant women (Black *et al.,* 2008; Krebs *et al.,* 2014; Vidyavati *et al.,* 2016).

Humans can take up Zn both from animals and plants-based products as a part of their natural diet. Meat-based foods which include beef, pork, lamb, dairy products, chicken and some seafood particularly oysters are considered as a good source of Zn

(Rangan and Samman, 2007). Legumes, whole grains and other plant-based food contain Zn but lower than animal-based food. Cereals (*e.g.* wheat, rice, and maize) are considered as inherently low in Zn (Cakmak *et al.*, 2010a). Moreover, bioavailability of Zn in cereals and legumes is compromised by the existence of high levels of anti-nutrients, mainly in the form of phytate and phenolic compounds (Gibson *et al.*, 2010).

A.2. Agronomic Biofortification: Instant Solution to Zn Deficiency Problem

Zinc biofortification is an approach using multiple strategies to improve the nutritional quality of food by deliberately increasing the Zn concentration in food and provide a public health benefit to reduce Zn deficiency related diseases in humans (White and Broadely, 2011). Genetic manipulations of the plant genome through the integrated approaches of conventional breeding or genetic engineering to increase the Zn concentration in edible plant parts is called "genetic biofortification", whereas the "agronomic biofortification" is the use of soil and/or foliar fertilizer application strategies to enhance the food Zn concentrations (Bouis and Welch, 2010; Velu *et al.*, 2012; Bouis and Saltzman, 2017). Both, genetic and agronomic biofortification are very useful approaches to enhance Zn in food and combat Zn deficiency in vast human populations (Graham *et al.*, 1999, 2001, 2007; White and Broadley, 2005, 2009; Cakmak, 2008; Khoshgoftarmanesh *et al.*, 2009; Bouis and Welch, 2010). However, agronomic biofortification has proved to be an immediate and thus faster solution compared to long-term genetic biofortification (Cakmak, 2008a; Velu *et al.*, 2014; Cakmak *et al.*, 2010 a; Chen *et al.*, 2017). Moreover, genetically biofortified genotypes (i) may not able to express their full potential to uptake, utilize and accumulate Zn from soils in Zn deficient areas of the world and (ii) can result in extensive depletion of Zn in such areas in the long term. It has been reported that more than 50% of the total soils in the world used for cereal cultivation is Zn deficient or Zn is not bio-available to plants due to the distinct chemical or physical properties of soils (Graham & Welch, 1996; Cakmak, 2008a; White and Broadley, 2011; Cakmak and Kutman, 2017).

According to the Food and Agriculture Organization (FAO), maize, rice, and wheat in combination provide more than half (51%) of the caloric requirement of the

world population (FAO *et al.*, 2015). These cereals are not only inherently low in Zn but also, they are high in phytates which bind the minerals including Zn making it unavailable for absorption in human digestive track (Gibson *et al.*, 2010). Moreover, part of Zn is also lost during the grain processing practices (Cakmak *et al.*, 2010b). Agronomic biofortification or fertilizer use strategy provides an instant solution to the problem by applying Zn fertilizer to the soil and plant as a foliar spray (Cakmak, 2008 b).

At first, the use of Zn fertilizers aimed to cure and mitigate visible Zn deficiency symptoms to increase the ultimate yield. No emphasis was given to human Zn requirements or increasing Zn concentration in crops and food. In 2008, International HarvestPlus (www.harvestplus.org) program and its sub-projects HarvestZinc (www.harvestzinc.org) were launched with the objectives of improving the nutritional quality of food crops especially cereals (wheat, rice, and maize) for targeted countries. Numerous soil and foliar Zn treatments were tested on a variety of cereal crops at multiple locations in 12 different countries. The results showed that soil Zn treatment is essential for proper crop stand, plant vigor, and yield enhancement but it does not have significant effect on grain Zn concentrations In contrast, foliar Zn application has a positive impact on increasing the grain Zn concentration in cereals particularly in wheat (Cakmak and Kutman, 2017).

Various field experiments under the HarvestZinc project on cereals (wheat, rice, and maize) revealed a differential response of wheat, rice and maize for the foliar application of Zn fertilizer (Cakmak and Kutman, 2017). Wheat is very responsive to the foliar application of zinc fertilizer as compared to rice and maize. In average, wheat has shown 83% increases in grain Zn with foliar Zn fertilization whereas the effect was much less in rice (27%) and particularly maize (9%) (Cakmak and Kutman, 2017).

A.3. Questions addressed in this project

The first step was to investigate the physiological reasons of differential response of maize and wheat to foliar Zn fertilizer application. In Chapter I, a series of experiments are described which were performed to test different hypotheses of poor response of

maize plants to foliar Zn application as compared to wheat. For a better understanding of Zn uptake and translocation, very sensitive and selective techniques involving stable Zn isotopes and Zn-specific fluorescent dyes were used.

Chapter II concentrates on the characterization of biofortified HarvestPlus (www.harvestplus.com) wheat genotypes developed through long-term conventional breeding activities under the HarvestPlus program in Pakistan and India. Experiments were conducted to study root uptake, shoot translocation, foliar absorption, re-mobilization and seed deposition of Zn in 12 biofortified genotypes developed for the targeted areas of Pakistan and India.

Chapter III involves a study on the effects of increased nitrogen (N) nutrition on root uptake and shoot accumulation of zinc in maize and wheat plants. Experiments were also conducted to illustrate how the increase in N nutrition affects the leaf uptake of Zn from foliar Zn application in these plant species.

(B) GENERAL MATERIALS AND METHODS

In all experiments, wheat and maize plants were grown in soil or solution culture in growth facilities described below:

B.1. Plant Growth Facilities

Experiments describe in this thesis were conducted in either green house or in growth chambers.

B.1.1. Greenhouse

The experiments conducted in greenhouse were under natural daylight in summer or with supplemented light in winters depending upon the day length. The geographic coordinates of the greenhouse are 40^{o} 53' 24.5" N and 029^{o} 22' 46.7" E. The greenhouse is equipped with a heating system and an evaporative cooling system, which keep the temperature inside the greenhouse in the range of 15-25°C depending on the season and day time.

B.1.2. Growth Chamber

Few of the experiments describe in Chapters I and III were carried out in a growth chamber under controlled climatic conditions (light/dark periods: 16/8 h; temperature

(light/dark): 22°C/18°C; relative humidity (light/dark): 60%/70%; photosynthetic flux density: 400 μmol m^{-2} s^{-1}).

B.2. Soil Culture

The soil used in all experiments was transported from a Zn-deficient location (Eskişehir) in Central Anatolia, Turkey. This experimental soil was calcareous (18% $CaCO_3$), alkaline (pH 8.04), organic matter (1.5%), Zn deficient (DTPA-Zn: 0.13 mg kg^{-1} soil) with clay-loam texture. Seeds were sown in plastic pots containing 3 kg of soil. Before potting, the soil was mixed homogeneously with the following nutrients (in mg per kg of soil): 100 P in the form of KH_2PO_4, 30 S in the form of K_2SO_4, 5 Fe in the form of sequestrene. Additionally, different rates of N and Zn were used in the form of Ca $(NO_3)_2.4H_2O$ and $ZnSO_4.7H_2O$ respectively, depending on the experimental design (individual rates are provided in respecting chapters). The pots were watered twice a day with deionized water to ensure the soil was kept at 60-80% water holding capacity.

B.3. Solution Culture

Seeds were germinated in perlite moistened with a saturated $CaSO_4$ solution for 5 days at room temperature. Then, seedlings were transferred to 3-L pots containing a nutrient solution with the following composition: 0.2 mM KH_2PO_4, 1.7 mM K_2SO_4, 1 mM MgSO4.7H2O, 0.1 mM KCl, 100 μM Fe-EDTA, 1 μM H_3BO_3, 1 μM $MnSO_4.H_2O$, 0.2 μM $CuSO_4.5H_2O$ 0.2 μM $NiCl_2.6H_2O$, 0.14 μM $(NH_4)6Mo_7O_{24}.4H_2O$. Zinc in the form of ZnSO4.7H2O, and N in the form of Ca $(NO_3)_2$.4H2O, were supplied according to the respective experimental treatment plan. Lower N pots were supplemented with $CaSO_4.2H_2O$ to compensate the missing Ca. Nutrient solutions were well aerated continuously and replaced after every three days.

B.4. Harvest

Plant age at the time of harvest differed according to the designed experiment and is explained in respective chapters. Green plant shoots harvested before maturity were washed with DI H_2O right after harvesting and placed in labelled paper bags. Roots and the application leaves were sequentially washed in DI H_2O, 10 mM $CaCl_2$ and 10 mM EDTA. All harvested plant samples were dried at 60°C in oven until complete dryness.

Grains from the plants harvested at full maturity were threshed using a laboratory thresher. Dried samples were weighed at room temperature for biomass and yield determination.

B.5. Elemental Analysis

Dried shoot and root samples were ground to fine powder with an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany). For mineral nutrients analysis (other than N), 200 mg (±5) ground plant sample (shoot or root) was subjected to acid-digestion in closed vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA) in the presence of 2 ml of 30% H_2O_2 and 5 ml of 65% HNO_3. For grain samples, 6-12 whole grains of equivalent weight were used in acid-digestion.

Following digestion, the total sample volume was topped up to 20 ml by DI water and filtered through quantitative filter paper. Concentrations of mineral nutrients were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian Pty Ltd, Mulgrave, Australia). The N concentrations in samples were determined by using LECO TruSpec C/N Analyzer (Leco Corp., St Joseph, MI, USA). Measurements were checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

B.6. Calculations

The elemental concentrations other than N in the samples were calculated by multiplying the values measured by ICP-OES with the dilution factor, which is calculated for each sample separately by dividing the total sample volume by the dry weight of the digested sample. For calculating the elemental contents for a given plant part, the calculated elemental concentrations were multiplied by the measured total dry weight of the concerned plant part. Similarly, the grain elemental yield, i.e. the total amounts of elements of interest deposited in the grains, were determined by multiplying the grain yield by the grain elemental concentrations.

B.7. Statistical Analysis

All experiments had factorial designs and 4-6 replicates in each treatment group. The Statistix 10 software was used for statistical analysis. The significance of the effects of treatments and their interactions on the reported traits was evaluated by analysis of variance (ANOVA). Then, Tukey's honestly significant difference (HSD) test ($p < 0.05$) was used to determine significant differences between means.

CHAPTER 1

ABSORPTION AND MOBILIZATION OF ZINC IN MAIZE AND WHEAT DURING EARLY VEGETATIVE STAGE AS EFFECTED BY VARIED ZINC SUPPLY IN SOIL

1.1. Introduction

Micronutrient malnutrition particularly zinc (Zn) deficiency is highly prevalent worldwide, affecting about two billion people, especially children and women. Zinc deficiency in humans causes various health problems (Cakmak, 2000) including retardation in physical growth and brain development, reduced immunity against infectious diseases and poor birth outcomes in pregnant women (Black *et al.,* 2008; Gibson, 2012; Krebsetal., 2014; Terrin *et al.,* 2015).

The application of plant nutrients in the form of spray to the foliage is an important agricultural practice to correct nutrient deficiencies particularly when soil conditions limit the availability of nutrients or to meet the internal plant demands according to its developmental stage (Fernández and Brown, 2013). Soil Zn application was found effective in increasing yield and yield components, however, to increase the Zn concentration in grains, foliar Zn applications were found more effective, particularly in wheat (Cakmak *et al.,* 2010). The effectiveness of foliar treatments varies for different plant species depending upon the plant characteristics as well as environmental factors which influence the uptake and translocation of applied fertilizer (Fernández *et al.,* 2013).

The effect of Zn fertilizer application on crop yield and grain Zn concentration depends upon many factors such as crop variety and methods of Zn fertilizer application. For example, maize was more sensitive to Zn deficiency than wheat, and foliar Zn

application increased Zn concentration of wheat but not much in maize (Wang *et al.*, 2012). In previous activities of the international HarvestZinc project (www.harvestzinc.org), one of the interesting results were the poor response of maize to foliar Zn application (Cakmak and Kutman, 2017). Rice and particularly wheat crops responded to foliar Zn fertilization positively and significantly in terms of increases in grain Zn concentrations whereas the response of maize was very low and insignificant (Zhou *et al.,* 2012; Phattarakul *et al.,* 2012; Cakmak and Kutman, 2017). All these studies reported the lower response of maize to foliar Zn application, however, none of these studies focused on revealing the mechanisms involved in differential responses of wheat and maize to foliar Zn applications. Therefore, there is a dire need of experimentation to investigate the differences in uptake and translocation mechanisms of foliarly applied Zn in maize and wheat.

The effectiveness of foliar application of any nutrient depends upon the absorption and penetration into leaves and translocation of the absorbed nutrients to other plants parts such as sink organs (Fernández and Brown, 2013). The reason behind the poor response of maize to foliar Zn could be inefficient absorption and/or translocation capacity of maize as compared to wheat. Another possible reason can be the "dilution effect". Dry matter production as well as grain yield and thousand grain weight are much higher in maize compared to wheat. Consequently, absorbed Zn is diluted within higher biomass resulting in less deposition of Zn in the maize grain. Moreover, a lower Zn concentration in maize grains can be related with lower protein content as compared to wheat grain. Zn is an important component of grain proteins which is considered as a sink for Zn (Cakmak, 2009). Low protein content of maize grain could be among the reasons for low Zn accumulation in the maize grain compared to wheat.

Due to high sensitivity and ease of sample preparation and handling, use of stable isotopes to trace the movement of mineral elements in plants is an efficient technique (Wang *et al.,* 2011). The uptake and translocation of metals can also be measured using radioactive isotopes (Page *et al*., 2006). Many studies have shown the use of stable and radioactive Zn isotopes (^{68}Zn and ^{65}Zn) as a tracer to study Zn transport in rice and wheat (Wu *et al,* 2010; Haslett *et al.,* 2001, Yilmaz *et al.,* 2017). Stable ^{70}Zn isotope was also used to trace the movement of Zn from culture medium to wheat grain (Wang *et al.,* 2011). Similarly, use of Zinpyr-1 and fluorescence microcopy is another useful addition to the

tools available for studying Zn localization and homeostasis in plant tissues (Sinclair *et al.,* 2007). Zinpyr-1 is membrane-permeable staining dye which is very selective for Zn over other biological metals, therefore very useful for binding intracellular Zn (Burdette *et al.,* 2001).

This study involves a series of experiments to test the different hypotheses for poor response of maize plants to foliar Zn application as compared to wheat. The first experiment was conducted to assess the changes in uptake and translocation of foliar-applied Zn in young wheat and maize plants cultured in soil with low or adequate Zn supply. Foliar Zn was applied on the older leaves of plants by dipping in fertilizer solution and young shoots were analyzed for the translocation of absorbed Zn from foliar application.

The second experiment was aimed to reveal the differences in leaf absorption and translocation of foliar-applied Zn in maize and wheat plants cultured in nutrient solution with low or adequate Zn supply. In order to trace the movement of foliar-applied Zn within the plant tissues, stable isotope ^{70}Zn was included in the foliar application solution. Second experiment was consisted of two sub experiments 2-A and 2-B to overcome the "dilution effect" due to biomass differences among maize and wheat. In 2-A, different aged maize and wheat plants were subjected to same volume of fertilizer solution application, while in 2-B same age plants were treated with different volume of fertilizer solution (for example maize was applied double volume of fertilizer solution compared to wheat in 2-B experiment).

In the third experiment, results from the first and second experiments were confirmed by fluoresce microscopy and using a Zn-responsive fluorescent dye 'Zinpyr'. The fluoresce microscopic images provides a visual demonstration of Zn localization in maize and wheat leaves after foliar Zn application.

1.2. Experiment 1: Absorption and Translocation of Foliar Zinc (as ZnSO4.7H2O) in Maize and Wheat during early vegetative stage

1.2.1. Materials and Methods

Plants were grown in marginal Zn (0.5 mg kg^{-1}) and sufficient (2 mg kg^{-1} Zn) Zn levels in soil under greenhouse conditions. Preparation of soils and planting method is described in "General Material and Methodology". When the plants were two weeks old, the oldest leaves of wheat and second oldest leaf of maize plants were dipped into solutions containing Zn (0.2 % $ZnSO_4.7H_2O$ + 0.02 % Tween-20) for 10-15 seconds twice a day for four days. The surfactant Tween-20 was added in the application solution to facilitate leaf penetration and absorption of foliar-applied Zn. Plants were harvested 24 h after the final leaf treatment. Maize and wheat plants were harvested in two fractions namely F-I (upper portion of the plant including stem and young leaves) and F-II (application leaf and the stem parts below). Plants were dried in the oven and their dry weight were determined. Only uncontaminated young plant shoot (fraction-I) was analyzed for Zn concentration.

Zn concentrations were measured by ICP-OES after digesting the ground leaf samples in a closed vessel microwave digestion system in the presence of concentrated HNO_3 and H_2O_2 (details of the procedure are described in the "General Material and Methodology" section)

Figure 1.1: Immersion of second oldest leaf of maize plant in fertilizer solution (0.2 % $ZnSO_4.7H_2O$ + 0.02 % Tween-20) for 10-15 seconds at room temperature.

1.2.2. Results

Adequate soil Zn supply increased the shoot biomass in both maize ad wheat, however this effect was not statistically significant. The results showed that soil Zn treatment was effective in increasing the shoot Zn concentration significantly in both maize and wheat. Shoot Zn concentration of the plants grown in adequate soil Zn was about two-folds of the low-Zn plants (Table 1.1).

In maize, under low and adequate soil Zn supply shoot Zn concentration increased with foliar Zn treatment however, the effect was not significant. In case of wheat, with low or adequate soil-Zn supply, foliar Zn treatments significantly increased leaf Zn concentrations compared to their respective control (*i.e.* no foliar treatment) (Table 1.1). Moreover, wheat showed higher extent of increase in Zn concentration as compared to maize plants. Wheat plants absorbed and translocated more Zn from the treatment leaf

compared to maize, particularly when grown under low Zn conditions. There was no significant effect of foliar treatments on plant biomass production (Table 1.1).

Analysis of variance showed that Zn concentration was significantly ($p<0.01$) affected by soil Zn level as well as foliar Zn application (Table 1.2). Young shoot Zn concentrations were significantly ($p<0.01$) higher in wheat as compared to that of maize. Crop species interacted with soil Zn and foliar Zn significantly but the interaction among all other variables had no significant effects on shoot Zn concentration (Table 1.2).

Table 1.1: Effect of foliar Zn application to oldest leaf on shoot Zn concentration and shoot biomass in maize and wheat grown under low (0.5 mg kg-1) and adequate Zn supply (2.0 mg kg-1) in soil.

Plants	Soil Zn supply*	Foliar treatments**	Biomass (mg plant^{-1})		Zn Concentration (mg kg^{-1})	
Maize	Low Zn	No Foliar Zn	397 ± 57	A	12.9 ± 1.2	F
		With Foliar Zn	363 ± 91	A	15.8 ± 0.9	F
	Adequate Zn	No Foliar Zn	463 ± 27	A	23.3 ± 2.5	DE
		With Foliar Zn	463 ± 27	A	25.7 ± 1.8	CD
Wheat	Low Zn	No Foliar Zn	112 ± 24	B	19.9 ± 1.4	E
		With Foliar Zn	125 ± 11	B	27.7 ± 1.4	C
	Adequate Zn	No Foliar Zn	127 ± 17	B	37.8 ± 1.2	B
		With Foliar Zn	117 ± 6	B	43.0 ± 1.1	A

*Low Zn: 0.5 mg Zn / kg and Adequate Zn: 0.5 mg Zn / kg of soil supplied as $ZnSO_4.7H_2O$

**"No foliar Zn" plants were treated with Tween-20 (0.02%, w/v) only, whereas "With foliar Zn" plants were treated with 0.2 % $ZnSO_4.7H_2O$ + 0.02 % Tween-20 (w/v). See Materials and Methods section for treatment details.

Table 1.2: Analysis of variance (ANOVA) for Zn concentration in young shoots.

Source of variation	DF	SS	MS	F	P
Species (Maize, Wheat)	1	1280.43	1280.43	555.34	<0.0001
Soil Zn level	1	1430.59	1430.59	620.47	<0.0001
Foliar Zn	1	167.99	167.99	72.86	<0.0001
Species x soil Zn	1	82.88	82.88	35.95	<0.0001
Species x foliar Zn	1	29.15	29.15	12.64	0.0016
Soil x foliar Zn	1	4.59	4.59	1.99	0.1711
Species x soil x foliar Zn	1	2.41	2.41	1.04	0.3169
Error	24	55.34	2.31		
Total	31	3053.38			

1.3. Experiment 2-A and 2-B: Absorption and Translocation of Foliar-applied Zinc (^{70}Zn) in Maize and Wheat grown with low or adequate Zn supply

1.3.1. Materials and Methods

Experiments 2-A and 2-B were designed to understand how maize and wheat species differ from each other in terms of leaf uptake and translocation of foliar-applied Zn to shoot and root. The movement of Zn from the point of application on the leaf to younger parts of the shoot and root was investigated by using the stable isotope ^{70}Zn. To overcome the possible "concentration" and "dilution" effects two separate nutrient solution culture experiments (i.e. Experiment 2-A and 2-B) were conducted sequentially.

In Experiment 2-A, considering the fact that maize grows faster and produces more biomass compared to wheat, the interspecies difference in biomass production at the time of foliar Zn application was compensated by using younger maize plants. For this, wheat was sown nine days earlier than maize. Cultivars of maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) were grown in nutrient solution supplied with low (10^{-2} µM) and adequate (1 µM) Zn in the form of $ZnSO_4 \cdot 7H_2O$. Composition of the nutrient solution, planting and growth conditions were described in the "General Material and Methodology" section. When maize plants were 9 days old, and wheat plants were 18 days old, the second leaf of each species was treated with a solution of ^{70}Zn (Trace Sciences International Corp., Canada) at an equivalent rate of 0.05% $ZnSO_4 \cdot 7H_2O$ along with the non-ionic surfactant Plantacare (0.02 %, w/v). Each leaf was applied with a total of 50 µl (20 x 2.5 µl = 50µl) of application solution on the abaxial surface using a fixed-volume (*i.e.*, 2.5 µL) microliter pipet. Twenty droplets of 2.5 µL were placed on the middle part of the application leaf with about 2 mm distance from each other (see illustrations below).

In Experiment 2-B, the effect of varied biomass production between the two species was compensated by using twice the volume of foliar application solution on maize plants compared to wheat. Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum*

aestivum L. cv. Tahirova) plants were grown in nutrient solution as described above with low (10-2 µM) and adequate (1 µM) Zn supply in the form of $ZnSO_4.7H_2O$. When both maize and wheat plants were 18 days old, ^{70}Zn at an equivalent rate of 0.05% $ZnSO_4.7H_2O$ mixed with Plantacare (0.02 % w/v) was applied on abaxial surface of the second leaf. Considering the "dilution factor", maize plants with larger biomass were applied with 60 µl (24 x 2.5 µl), whereas the wheat plants with smaller biomass received 30 µl (12 x 2.5 µl) of the application solution.

Fig 1.2. Application of 50µl (20 x 2.5 µl= 50µl) of ^{70}Zn at an equivalent rate of 0.05% $ZnSO_4{\cdot}7H_2O$ mixed with Plantacare® (0.02 % w/v) on 2nd leaf of 9-days old maize and 18-days old wheat plants grown in nutrient medium solution.

In both Experiments 2-A and 2-B, plants were misted every two hours with DI-H_2O to extend the contact duration of the leaf with application solution. Following 36 hours after foliar application, plants were harvested in three fractions *viz.* application leaf, remaining shoot and root. Root and shoot fractions were washed with DI-H_2O whereas the application leaves were sequentially washed in DI-H_2O, 10 mM $CaCl_2$ and 10 mM EDTA solution for three min to remove the residual ^{70}Zn on the leaf surface. The harvested plant parts were dried at 60°C until a constant weight grain for determination of biomass. Dried samples were ground and digested in a closed vessel microwave

digestion system in the presence of concentrated HNO_3 and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) for determination of ^{70}Zn.

In both Experiments 2-A and 2-B, each treatment consisted of four independent (pots) replicates. The ^{70}Zn contents per plant (e.g., total amounts of ^{70}Zn) were calculated by multiplying the shoot and root dry weights by the shoot and root ^{70}Zn concentrations respectively. The significance of the effects of treatments and their interactions on the reported traits were evaluated by analysis of variance (ANOVA). Significant differences among means were determined by Tukey's HSD test at the 5% level ($P \leq 0.05$).

1.3.2. Results

1.3.2.1. Experiment 2-A

In Experiment 2-A, there was a significant increase in shoot, root and total biomass production with adequate supply of Zn in nutrient solution in 18 days old wheat plants (Table 1.3) whereas, shoot, root, and total biomass was not affected significantly in 9 days old maize plants. Similarly, shoot:root ratio was increased significantly with adequate Zn supply in wheat but not in maize. Foliar ^{70}Zn treatment had no significant effect on biomass production or shoot:root ratio in low and adequate Zn maize and wheat plants (Table 1.3).

^{70}Zn concentrations in shoot and root increased significantly in response to the foliar applied ^{70}Zn solution in low (10^{-8} M) and adequate Zn (10^{-6} M) maize and wheat plants (Table 1.4). The magnitude of increase varied between the plant species and with Zn supply in nutrient solution. The results showed that wheat performed better in uptake of leaf-applied Zn as compared to maize. Generally, under both low (10^{-8} M) or adequate Zn supply (10^{-6} M), ^{70}Zn concentrations in shoot, root and application leaf increased more dramatic in wheat compared to maize (Table 1.4).

In low Zn maize, shoot dry weight increased 7.9 % with foliar ^{70}Zn from the control plants as compared to 9.8 % in adequate Zn plants. Root dry weight decreased by

2.8 % in low Zn plants but increased by 4.32% in adequate Zn with ^{70}Zn treatment. Total biomass was increased 4.5% in low Zn and 7.4% in adequate Zn. Shoot: root ratio also increased by 13.6% and 5% in low and adequate Zn treated maize plants respectively. However, these differences were not statistically significant. In case of wheat, foliar ^{70}Zn application resulted in 5.9% and 11.8% decreases in shoot dry weights under low and adequate Zn supply respectively. Root dry weight in low Zn plants was also reduced by 1.4% however, it increased in adequate Zn plants. Total biomass was reduced by 3.5% and 5.7% in low and adequate Zn supplied wheat respectively. Shoot:root ratio was also decreased in wheat, but all these effects were statistically non-significant (Table 1.3).

Relative change in ^{70}Zn concentration was calculated as percent increase in ^{70}Zn concentration (in shoot, root and application leaf) with foliar ^{70}Zn application as compared to non-treated control plants. In maize shoot and root ^{70}Zn concentration were doubled in adequate Zn plants whereas, there was 4.5 and 5.2 foldincrease in low Zn plants respectively (Table 1.4). In case of wheat, shoot and root ^{70}Zn concentration were increased around 5-fold in adequate Zn conditions. Shoot ^{70}Zn concentration was increased by 6.2 folds whereas, root showed a marked increase of 27 folds under low Zn conditions in wheat. Analysis of application leaf showed that ^{70}Zn concentration was increased significantly with foliar application of ^{70}Zn in both maize and wheat under low and adequate Zn supply, but ^{70}Zn concentration was three folds higher in low Zn maize and wheat application leaves as compared to adequate Zn plant application leaves (Table 1.4). Generally, the results showed that low Zn maize and wheat plants tended to absorb and translocate more ^{70}Zn from foliar spray as compared to adequate Zn plants. Under low Zn supply, the major portion of absorbed Zn was translocated to roots in wheat as compared to shoots (Table 1.4).

Similarly, shoot and root ^{70}Zn content was increased in maize as well as in wheat, but with a significantly higher rate in wheat particularly under low Zn conditions (Table 1.5). Relative change in total ^{70}Zn contents (root, shoot, application leaf) were higher in low Zn plants as compared to adequate Zn plants. Under low Zn supply, maize shoot and root ^{70}Zn contents were increased by five folds, whereas the wheat shoot and root contents were increased up to seven and 27 folds respectively (Table 1.5). At adequate Zn supply, shoot and root ^{70}Zn content were doubled in maize and increased five times in wheat. In low Zn-supplied wheat, root ^{70}Zn content was found markedly higher, indicating higher

translocation rate of absorbed Zn towards roots under low Zn supply (Table 1.5). Overall, total ^{70}Zn contents of wheat were 4.4 and 3 times higher as compared to that of maize under low and adequate Zn conditions respectively.

Total Zn concentration (including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) was significantly affected with the Zn supply in nutrient medium solution (Table 1.6). Both maize and wheat plants showed significant increase in total Zn concentration with adequate supply of Zn in nutrient solution as $ZnSO_4.7H_2O$. Foliar ^{70}Zn application had no significant effect on total Zn concentration of maize and wheat shoots and root, however, increased significantly in application leaves particularly due to higher ^{70}Zn uptake (Table 1.6).

Table 1.3. Dry matter production of 9-days-old maize and 18-days-old wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Biomass															
Zn supply in nutrient solution*	Foliar treatments**	Maize								Wheat							
		Shoot		Root		Total		Shoot : Root		Shoot		Root		Total		Shoot : Root	
		(mg plant^{-1})								(mg plant^{-1})							
Low Zn	No foliar ^{70}Zn	305 ± 11	ab	143 ± 3	ab	448 ± 13	bc	2.13 ± 0.06	b	236 ± 60	cd	125 ± 9	abc	339 ± 27	d	1.92 ± 0.62	b
	With foliar ^{70}Zn	329 ± 7	a	139 ± 23	ab	468 ± 23	ab	2.42 ± 0.48	ab	211 ± 8	d	123 ± 13	abc	325 ± 21	d	1.73 ± 0.20	b
	Relative change (%)	7.88		-2.80		4.46		13.6		-5.93		-1.39		-3.49		-9.89	
Adequate Zn	No foliar ^{70}Zn	297 ± 8	ab	139 ± 8	ab	474 ± 14	ab	2.14 ± 0.12	bc	308 ± 10	ab	107 ± 4	c	437 ± 9	bc	2.87 ± 0.16	a
	With foliar ^{70}Zn	326 ± 4	ab	145 ± 1	a	509 ± 6	a	2.24 ± 0.02	ab	271 ± 23	bc	118 ± 7	bc	411 ± 28	c	2.30 ± 0.18	a
	Relative change (%)	9.76		4.32		7.38		4.67		-11.8		9.81		-5.75		-19.9	

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.4. ^{70}Zn concentration in 9-days-old maize and 18-days-old wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		^{70}Zn Concentration in Plant Tissue											
Zn supply in nutrient solution*	Foliar treatments**	Maize						Wheat					
		Shoot		Root		Application leaf		Shoot		Root		Application leaf	
		($\mu g\ kg^{-1}$)											
Low Zn	No foliar ^{70}Zn	120 ± 23	e	160 ± 24	de	124 ± 28	d	50.1 ± 8.6	e	61.2 ± 3.9	e	76.6 ± 13.6	d
	With foliar ^{70}Zn	665 ± 43	c	988 ± 74	b	67433 ± 5248	c	364 ± 75	d	1750 ± 64	a	137556 ± 5124	b
	Relative change (%)	455		519		54418		623		2757		179385	
Adequate Zn	No foliar ^{70}Zn	442 ± 33	d	442 ± 21	c	450 ± 22	d	174 ± 10	e	317 ± 15	cd	206 ± 19	d
	With foliar ^{70}Zn	871 ± 75	b	886 ± 104	b	69855 ± 5332	c	1136 ± 123	a	1871 ± 209	a	153752 ± 14846	a
	Relative change (%)	97		100		15428		552		490		74475	

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.5. ^{70}Zn contents in 9-days-old maize and 18-days-old wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		^{70}Zn content per tissue (ng/tissue/plant)							
Zn supply in nutrient solution*	Foliar treatments**	Maize				Wheat			
		Shoot	Root	Application leaf	Total	Shoot	Root	Application leaf	Total
		(ng/tissue/plant)							
Low Zn	No foliar ^{70}Zn	31.9 ± 5.1 de	22.9 ± 3.5 cd	4.69 ± 0.95 c	59.4 ± 7.2 c	8.00 ± 1.07 e	7.63 ± 0.53 d	1.90 ± 0.31 c	17.5 ± 0.7 c
	With foliar ^{70}Zn	193 ± 17 b	137 ± 21 b	2592 ± 105 b	2922 ± 92 b	64.3 ± 12.3 d	215 ± 25 a	3467 ± 303 a	3746 ± 321 a
	Relative change (%)	507	500	55139	4819	704	2713	182627	21275
Adequate Zn	No foliar ^{70}Zn	115 ± 11 c	61.5 ± 2.5 c	16.6 ± 1.1 c	193.3 ± 13.8 c	49.9 ± 2.6 de	34.0 ± 2.3 cd	4.44 ± 0.44 c	88.3 ± 2.0 c
	With foliar ^{70}Zn	250 ± 20 a	129 ± 15 b	2678 ± 100 b	3056 ± 125 b	283.6 ± 48.8 a	221 ± 37 a	3429 ± 179 a	3934 ± 137 a
	Relative change (%)	117	109	15985	1481	468	551	77147	4353

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of 70Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.6. Total Zn concentration (all isotopes including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) in 9-days-old maize and 18-days-old wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Total Zn Concentration in Plant Tissue					
Zn supply in nutrient solution*	Foliar treatments**	Maize			Wheat		
		Shoot	Root	Application leaf	Shoot	Root	Application leaf
		(mg kg^{-1})					
Low Zn	No foliar ^{70}Zn	15.9 ± 1.4 cd	26.7 ± 3.6 c	17.6 ± 1.9 fg	7.84 ± 1.29 de	11.7 ± 0.7 d	10.9 ± 1.1 g
	With foliar ^{70}Zn	23.0 ± 4.1 bc	25.6 ± 3.1 c	90.6 ± 9.4 d	7.09 ± 0.55 e	12.9 ± 0.5 d	155 ± 5 b
	Relative change (%)	44.9	-4.47	416	-9.56	10.0	1320
Adequate Zn	No foliar ^{70}Zn	70.7 ± 1.6 a	72.4 ± 3.8 a	70.7 ± 2.8 e	28.0 ± 1.4 b	52.8 ± 2.8 b	31.2 ± 1.4 f
	With foliar ^{70}Zn	63.4 ± 8.5 a	71.3 ± 7.8 a	132 ± 8 c	28.5 ± 0.8 b	52.8 ± 4.0 b	186 ± 16 a
	Relative change (%)	-10.3	-1.58	187	1.85	-0.15	498

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.7. Total Zn contents (all isotopes including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) in 9-days-old maize and 18-days-old wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Total Zn content per tissue (µg/tissue/plant)							
Zn supply in nutrient solution*	Foliar treatments**	Maize				Wheat			
		Shoot	Root	Application leaf	Total	Shoot	Root	Application leaf	Total
		(µg/tissue/plant)							
Low Zn	No foliar ^{70}Zn	4.25 ± 0.47 c	3.83 ± 0.54 c	0.67 ± 0.08 e	8.75 ± 0.44 d	1.49 ± 0.49 d	1.46 ± 0.08 d	0.27 ± 0.02 e	3.21 ± 0.52 e
	With foliar ^{70}Zn	6.70 ± 1.28 bc	3.55 ± 0.65 c	3.48 ± 0.22 c	13.7 ± 1.1 c	1.26 ± 0.16 d	1.57 ± 0.10 d	3.90 ± 0.35 bc	6.74 ± 0.39 d
	Relative change (%)	57.57	-7.36	420	56.9	-15.2	7.89	1345	110
Adequate Zn	No foliar ^{70}Zn	18.4 ± 0.8 a	10.1 ± 0.4 a	2.62 ± 0.16 d	31.1 ± 1.3 a	8.01 ± 0.37 b	5.67 ± 0.41 b	0.67 ± 0.04 e	14.4 ± 0.2 c
	With foliar ^{70}Zn	18.2 ± 2.4 a	10.3 ± 1.1 a	5.08 ± 0.37 a	33.6 ± 3.1 a	7.09 ± 0.67 b	6.21 ± 0.45 b	4.16 ± 0.17 b	17.5 ± 0.6 b
	Relative change (%)	-1.15	2.64	93.9	8.08	-11.5	9.49	520	21.6

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.8. Relative distribution of absorbed ^{70}Zn in shoot, root and application leaf of maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Zn^{70} distribution per tissue (%)					
Zn supply in nutrient solution*	Foliar treatments**	Maize			Wheat		
		Shoot	Root	Application leaf	Shoot	Root	Application leaf
		(%)					
Low Zn	No foliar ^{70}Zn	53.6 ± 4.1 b	38.5 ± 4.9 a	7.92 ± 1.43 d	45.5 ± 4.3 c	43.6 ± 3.9 a	10.9 ± 2.1 c
	With foliar ^{70}Zn	6.62 ± 0.6 de	4.71 ± 0.81 c	88.7 ± 1.2 b	1.74 ± 0.44 e	5.72 ± 0.18 c	92.5 ± 0.3 a
Adequate Zn	No foliar ^{70}Zn	59.5 ± 1.2 a	31.9 ± 1.1 b	8.62 ± 0.40 cd	56.5 ± 2.6 ab	38.5 ± 2.6 a	5.02 ± 0.40 e
	With foliar ^{70}Zn	8.17 ± 0.41 c	4.20 ± 0.39 c	87.62 ± 0.79 b	7.23 ± 1.34 de	5.64 ± 1.04 c	87.1 ± 1.8 b

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, wheras "With foliar ^{70}Zn plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Similarly, total Zn contents were improved significantly with adequate Zn supply in maize and wheat plants (Table 1.7). Foliar applied ^{70}Zn had no significant effect on total Zn contents of shoots and roots in both low and adequate supplied plants. Total Zn contents of application leaf were increased in maize and wheat under both low and adequate Zn supply and consequently total Zn contents per plant (including application leaf) were increased significantly with foliar ^{70}Zn application (Table 1.7).

Evidently, most of the Zn taken up by the application leaf was retained (*i.e.* > 87%) in the application leaf in both plant species (Table 1.8). In low-Zn supplied wheat, Zn taken up by the application leaf was preferentially translocated to the root as compared to the shoot tissue (Table 1.8). Conversely, in low-Zn supplied maize, a larger portion of Zn was retained in the shoot compared to root. In adequate Zn plants, a significantly higher ratio of Zn was distributed in shoots than in roots irrespective of the plant species. For example, in maize with adequate-Zn supply, shoot tissue maintained 8.17% of the Zn taken up by the application leaf while the root maintained only 4.2%. The same figures for wheat shoot and root were 7.23% and 5.64% respectively (Table 1.8).

Leaf relative Zn uptake was calculated by the ratio of total ^{70}Zn in the whole plant biomass (including shoot, root and application leaf) to that of total ^{70}Zn applied on the application leaf. Leaf relative Zn uptake ranged from 12.9% in low Zn-supplied maize to 17.1% in adequate Zn-supplied wheat (Table 1.9) and was significantly (p<0.05) higher in wheat than maize at both low and adequate Zn supply. Both maize and wheat had higher leaf relative Zn uptake when grown with adequate Zn, however this effect was non-significant (Table 1.9).

Table 1.9 Leaf relative Zn uptake in maize and wheat plants grown in nutrient solution with low (10^{-8} M) or adequate Zn (10^{-6} M) supply.

Treatments	^{70}Zn Uptake ratio (%)	
Zn supply in nutrient solution*	Maize	Wheat
	(%)	
Low Zn	12.9 ± 0.4 b	16.5 ± 1.4 a
Adequate Zn	13.4 ± 0.5 b	17.1 ± 0.6 a

*Low Zn: 10^{-8} M Zn, Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4$
Statistical letters show the comparison between the species

1.3.2.2. Experiment 2-B

Experiment 2-B investigated response to foliar Zn application in maize and wheat plants sown at the same time and cultured for 20 days. There was a significant difference in biomass production among maize and wheat and with low and adequate Zn supply (Table 1.10). As expected, maize produced significantly higher biomass compared to wheat. Adequate Zn supply significantly affected the shoot:root and total biomass production in twenty days old maize plants, whereas this effect was non-significant in wheat (Table 1.10). Foliar Zn application resulted in enhanced biomass production in both low and adequate Zn maize and wheat plants, however, the increase was statistically non-significant (Table 1.10).

The large differences in biomass at the time of foliar application was compensated by doubling the foliar fertilizer rate (*i.e.* doubling the volume applied) in maize compared to wheat. Result showed maize and wheat were able to absorb and translocate the foliar ^{70}Zn significantly, but the magnitude of Zn uptake and translocation was significantly higher in wheat as compared to maize (in the same way as in experiment 2-A).

^{70}Zn concentration in low-Zn maize shoots and roots were increased by 4.5 and 6.7 folds with foliar application respectively (Table 1.11). Adequate Zn-maize shoot ^{70}Zn concentration was doubled whereas root showed 2.4 folds increase compared to the non-treated control plants. In case of low-Zn wheat, there was 7.8-folds and 16.3-folds increase in shoot and root ^{70}Zn concentration respectively whereas, at adequate Zn supply, the increase was only up to three folds. The ^{70}Zn concentration in roots were found higher than shoot in both plant species particularly in low-Zn wheat roots (Table 1.11). Generally, wheat showed two times higher ^{70}Zn concentration in shoot and root under low and adequate Zn supply. The amount of ^{70}Zn absorbed and translocated by wheat was not affected by the applying 50% less volume of fertilizer solution compared to maize (Table 1.11).

Similarly, shoot and root ^{70}Zn content increased in maize as well as in wheat, but with a higher rate in wheat particularly under low Zn conditions (Table 1.12). Relative change in total ^{70}Zn contents (root, shoot, application leaf) were higher in low Zn maize

and wheat plants as compared to adequate Zn plants. Under low Zn supply, maize shoot and root ^{70}Zn contents were increased by 5.7 and 6.5 folds respectively, whereas the wheat shoot and root contents increased up to 9.6 and 18.7 folds respectively (Table 1.12). At adequate Zn supply, maize shoot ^{70}Zn content were doubled while root showed more than three times increase as compared to non treated contro plants. Adequate Zn wheat plants also showed three times increase in shoot and root ^{70}Zn contents. In low Zn-supplied wheat, root ^{70}Zn content was strikingly higher, same as experiment 2-A, indicating higher translocation rate of absorbed Zn towards roots under low Zn supply (Table 1.12). Overall, total ^{70}Zn contents were improved 4.4 times higher in wheat compared to that of maize under low Zn conditions and 3 folds higher under adequate Zn condition.

Total Zn (including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) concentration and contents were significantly affected with the Zn supply in nutrient medium solution (Table 1.13, Table 1.14). Both maize and wheat plants showed significant increase in total Zn concentration and contents with adequate supply of Zn in nutrient solution as $ZnSO_4.7H_2O$. Foliar ^{70}Zn application improved the total Zn concentration and contents in all plant parts however the effect was only significant in application leaves mainly due to higher ^{70}Zn uptake (Table 1.13, Table 1.14).

Table 1.10. Biomass production of 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Biomass							
Zn supply in nutrient solution*	Foliar treatments**	Maize				Wheat			
		Shoot	Root	Total	Shoot : Root	Shoot	Root	Total	Shoot : Root
		(mg plant^{-1})				(mg plant^{-1})			
Low Zn	No foliar ^{70}Zn	281 ± 51 b	202 ± 28 c	483 ± 78 b	1.39 ± 0.11 b	76.6 ± 15.7 c	73.1 ± 15.1 d	150 ± 30 c	1.05 ± 0.07 c
	With foliar ^{70}Zn	279 ± 28 b	198 ± 8 c	477 ± 35 b	1.41 ± 0.11 b	86.9 ± 12.3 c	74.4 ± 10.8 d	161 ± 22 c	1.17 ± 0.10 bc
	Relative change (%)	-0.61	-1.86	-1.13	1.50	13.4	1.83	7.76	11.7
Adequate Zn	No foliar ^{70}Zn	541 ± 11 a	294 ± 18 b	835 ± 7 a	1.85 ± 0.1 a	92.0 ± 7.0 c	69.8 ± 6.6 d	162 ± 13 c	1.32 ± 0.09 b
	With foliar ^{70}Zn	578 ± 50 a	344 ± 28 a	922 ± 69 a	1.68 ± 0.1 a	110 ± 5 c	81.9 ± 3.1 d	192 ± 8 c	1.34 ± 0.02 b
	Relative change (%)	6.85	17.0	10.4	-8.81	19.4	17.3	18.5	1.52

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.11. ^{70}Zn concentration in 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		^{70}Zn Concentration in Plant Tissue											
Zn supply in nutrient solution*	Foliar treatments**	Maize						Wheat					
		Shoot		Root		Application leaf		Shoot		Root		Application leaf	
		($\mu g\ kg^{-1}$)											
Low Zn	No foliar ^{70}Zn	61.0 ± 1.5	f	87.5 ± 7.9	ef	60.9 ± 8.1	d	71.4 ± 13.7	ef	72.0 ± 9.6	f	69.6 ± 9.9	d
	With foliar ^{70}Zn	336 ± 61	cd	678 ± 42	c	24321 ± 4064	c	632 ± 116	b	1246 ± 109	b	47489 ± 4231	b
	Relative change (%)	450		675		39818		785		1630		68120	
Adequate Zn	No foliar ^{70}Zn	209 ± 10	de	207 ± 18	e	149 ± 10	d	283 ± 28	d	382 ± 22	d	241 ± 19	d
	With foliar ^{70}Zn	435 ± 44	c	713 ± 31	c	24074 ± 4361	c	1135 ± 90	a	1442 ± 74	a	57707 ± 9930	a
	Relative change (%)	108		245		16023		301		277		23809	

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.12. ^{70}Zn contents in 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		^{70}Zn content per tissue (ng/tissue/plant)											
Zn supply in nutrient solution*	Foliar treatments**	Maize				Wheat			
		Shoot	Root	Application leaf	Total	Shoot	Root	Application leaf	Total
		(ng/tissue/plant)							
Low Zn	No foliar ^{70}Zn	13.4 ± 2.7 e	17.8 ± 3.7 e	3.41 ± 0.56 c	34.5 ± 8.64 d	4.91 ± 0.66 e	4.72 ± 0.52 e	0.95 ± 0.11 c	10.9 ± 1.7 d
	With foliar ^{70}Zn	75.9 ± 10 bc	134 ± 8 b	1237 ± 194 a	1448 ± 202 ab	52.5 ± 10 cd	92.9 ± 17 c	685 ± 101 b	831 ± 112 b
	Relative change (%)	576	656	36231	4095	968	1867	72037	7517
Adequate Zn	No foliar ^{70}Zn	103 ± 5.7 b	60.6 ± 5.1 d	7.5 ± 0.4 c	171 ± 6.2 d	22.4 ± 3.5 de	26.8 ± 4.1 e	3.2 ± 0.5 c	52.3 ± 7.9 d
	With foliar ^{70}Zn	231 ± 43 a	245 ± 18 a	1216 ± 156 a	1692 ± 128 a	105 ± 7 b	118 ± 8 b	1002 ± 174 a	1225 ± 179 b
	Relative change (%)	125	304	16157	892	368	341	31686	2240

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.13. Total Zn concentration (all isotopes including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) in 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Total Zn Concentration in Plant Tissue					
Zn supply in nutrient solution*	Foliar treatments**	Maize			Wheat		
		Shoot	Root	Application leaf	Shoot	Root	Application leaf
		(mg kg^{-1})					
Low Zn	No foliar ^{70}Zn	9.06 ± 0.2 d	14.0 ± 1.2 c	8.66 ± 0.32 f	10.1 ± 1.79 d	12.4 ± 0.14 c	9.72 ± 1.75 ef
	With foliar ^{70}Zn	10.0 ± 0.7 d	14.5 ± 0.7 c	34.2 ± 5 cd	11.0 ± 1 d	13.5 ± 0 c	58.6 ± 5 b
	Relative change (%)	10.2	3.04	295	8.99	8.58	504
Adequate Zn	No foliar ^{70}Zn	34.9 ± 1.4 c	31.5 ± 2.5 b	22.7 ± 1.7 de	41.2 ± 2.7 a	48.2 ± 9.1 a	34.7 ± 2.4 cd
	With foliar ^{70}Zn	36.4 ± 2.6 bc	33.3 ± 6.2 b	43.6 ± 4.8 c	40.6 ± 2.4 ab	47.0 ± 4.2 a	92.6 ± 13.1 a
	Relative change (%)	4.55	5.66	91.9	-1.52	-2.61	167

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.14. Total Zn contents (all isotopes including ^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) in 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) and adequate Zn (10^{-6} M) supply. Foliar treatments were applied 36 hours before harvesting the plant tissues.

Treatments		Total Zn content per tissue (µg/tissue/plant)							
Zn supply in nutrient solution*	Foliar treatments**	Maize				Wheat			
		Shoot	Root	Application leaf	Total	Shoot	Root	Application leaf	Total
		(µg/tissue/plant)							
Low Zn	No foliar ^{70}Zn	1.98 ± 0.37 de	2.84 ± 0.50 b	0.59 ± 0.08 de	5.42 ± 0.84 d	0.72 ± 0.07 e	0.99 ± 0.10 c	0.13 ± 0.02 f	1.84 ± 0.09 e
	With foliar ^{70}Zn	2.27 ± 0.14 d	2.87 ± 0.22 b	1.74 ± 0.24 b	6.88 ± 0.58 d	0.80 ± 0.15 e	1.00 ± 0.12 c	0.85 ± 0.13 cd	2.65 ± 0.33 e
	Relative change (%)	14.5	1.02	195	27.1	11.5	0.71	542	44
Adequate Zn	No foliar ^{70}Zn	17.1 ± 0.8 b	9.25 ± 0.76 a	1.14 ± 0.09 c	27.5 ± 0.7 b	3.25 ± 0.31 cd	3.35 ± 0.60 b	0.45 ± 0.06 ef	7.05 ± 0.66 d
	With foliar ^{70}Zn	19.2 ± 1.4 a	10.2 ± 0.3 a	2.22 ± 0.25 a	31.6 ± 1.3 a	3.76 ± 0.35 c	3.85 ± 0.42 b	1.61 ± 0.23 b	9.22 ± 0.57 c
	Relative change (%)	12.0	10.2	94.7	14.8	15.6	14.9	255	30.6

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Table 1.15. Relative distribution of absorbed ^{70}Zn in shoot, root and application leaf of 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) or adequate Zn (10^{-6} M) supply.

Treatments		^{70}Zn distribution per tissue (%)					
		Maize			Wheat		
Zn supply in nutrient solution*	Foliar treatments**	Shoot	Root	Application leaf	Shoot	Root	Application leaf
		(%)					
Low Zn	No foliar ^{70}Zn	38.7 ± 3.0 c	51.4 ± 3.3 a	9.92 ± 0.84 c	46.3 ± 3.4 b	44.7 ± 3.4 b	9.00 ± 0.99 cd
	With foliar ^{70}Zn	5.30 ± 0.88 e	9.37 ± 1.06 e	85.33 ± 1.82 a	6.96 ± 1.16 e	11.15 ± 1.11 de	82.42 ± 1.86 a
Adequate Zn	No foliar ^{70}Zn	60.1 ± 2.4 a	35.5 ± 2.5 c	4.40 ± 0.42 d	42.8 ± 0.8 bc	51.1 ± 0.1 a	6.05 ± 0.71 cd
	With foliar ^{70}Zn	13.7 ± 3.0 d	14.6 ± 2.1 d	71.7 ± 4.1 b	8.67 ± 1.13 de	9.77 ± 1.35 de	81.6 ± 2.4 a

*Low Zn: 10^{-8} M Zn and Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4.7H_2O$

**"No foliar ^{70}Zn" plants were treated with Plantacare (0.02%, w/v) only, whereas "With foliar ^{70}Zn" plants were treated with 0.05% of ^{70}Zn dissolved in Plantacare (0.02%, w/v). See Materials and Methods section for treatment details.

Statistical letters show the comparison among the organs between the species, e.g., Maize shoot is compared with Wheat shoot, and so on.

Clearly, most of the Zn taken up by leaf after the foliar Zn application was retained in the application leaf in both maize and wheat ranging from 71.7% to 85.3% in low and adequate-Zn supplied maize respectively (Table 1.15). As a consequence, the lowest (i.e., 5.30% in shoot of low-Zn maize) and highest (i.e., 14.6% in root of adequate-Zn) Zn translocation rates from the application leaf towards shoot and root were recorded in low- and adequate-Zn supplied maize plants respectively (Table 1.15). The absorbed Zn was evidently distributed to the root than the shoot tissue in both species, particularly when supplied with low Zn. For example, in low-Zn supplied wheat, roots received almost double the amount of Zn compared to shoot (Table 1.15).

The uptake ratio of leaf-applied Zn ranged from 10.6% in low Zn-supplied maize to 17.9% in adequate Zn-supplied wheat (Table 1.16). Leaf Zn uptake rate was higher in wheat than maize in both low and adequate Zn supplied plants. Both maize and wheat had higher Zn uptake when grown with adequate Zn, however this effect was significant (p<0.05) in wheat but not maize (Table 1.16). The relative absorption of leaf-applied ^{70}Zn was 10.6 % and 12.2% in 20-days-old low Zn maize and wheat plants respectively. At adequate Zn supply, the relative absorption of ^{70}Zn applied on maize second leaf was 12.4%. while significantly higher in wheat i-e 17.9 % (Table 1.16).

Table 1.16. Relative absorption of leaf-applied ^{70}Zn in 20-days-old maize and wheat plants grown in nutrient solution with low (10^{-8} M) or adequate Zn (10^{-6} M) supply.

Treatments	^{70}Zn Uptake ratio (%)	
Zn supply in nutrient solution*	Maize	Wheat
	(%)	
Low Zn	10.6 ± 1.5 b	12.2 ± 1.6 b
Adequate Zn	12.4 ± 0.9 b	17.9 ± 2.6 a

*Low Zn: 10^{-8} M Zn, Adequate Zn: 10^{-6} M Zn supplied as $ZnSO_4$
Statistical letters show the comparison between the species

1.4. Experiment 3: Studying leaf Uptake of Zinc by using a Zinc-responsive fluorescent dye 'Zinpyr' in Maize and Wheat

1.4.1. Material and Methods

This experiment was performed to visualize Zn localization and mobilization in maize and wheat leaf tissues using the fluorescent dye 'Zinpyr-1' and a fluorescence microscopy.

Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) were grown in low Zn soil (0.5 mg Kg^{-1}) under greenhouse conditions. Planting procedures and soil nutrients composition (other than Zn) described in the "General Material and Methodology" section was followed.

When the plants were 13 days old, the first leaf of each wheat plant and second leaf of maize plants were dipped in 0.25% $ZnSO_4$ mixed with Tween-20 as a surfactant (0.02 % w/v) for twice a day, and it was repeated for four consecutive days. The plants were allowed to grow in green house for another week after the last treatment.

Three leaves from each plant were used for staining and visualization. The application leaf, the second younger leaf after application leaf and the 3rd younger leaf. For microscopic studies, a transverse leaf sections of ~0.1 mm were cut by scalpel and washed with running water first and then with Saline solution (0.9% NaCl) twice. Leaf sections were then transferred into 10 µM Zinpyr solution prepared in 0.9 % NaCl from a 2 mM Zinpyr stock solution prepared in dimethyl sulphoxide (DMSO) and incubated at room temperature for 2 h in darkness. Leaf sections were washed again in saline solution and mounted on microscopy slides. Images were taken by using a fluorescent microscope on 10X magnification. Filter S484/15 and S517/30 were used for excitation and emission (NIB) respectively for visualization of Zinpyr fluorescence and the $ZnSO_4$ treated plants were compared with the control plants.

1.4.2. Results

We have compared the intensity of Zinpyr fluorescence under florescence microscope in the maize and wheat leaf sections following the Zn fertilizer application. In both maize and wheat plants, Zinpyr fluorescence showed an enhanced accumulation of Zn localized in the Zn treated leaf cross section particularly in xylem and phloem tissues as compared to non-Zn treated control plants (Fig 1.3, 1.4). Although the 2nd and 3rd younger leaves from treated plants showed less fluorescence intensity compared to treatment leaf itself but still the fluorescence was much higher than the non-Zn treated control plants in both maize and wheat (Fig 1.3, 1.4). This provides an evidence that foliarly applied Zn was absorbed and translocated to the younger shoots. The intensity of Zinpyr fluorescence continued to decrease from the application leaf to the 2nd and 3rd younger leaves in maize and wheat indicating the upward translocation/remobilization of absorbed Zn to the younger plant parts (Fig 1.3, 1.4).

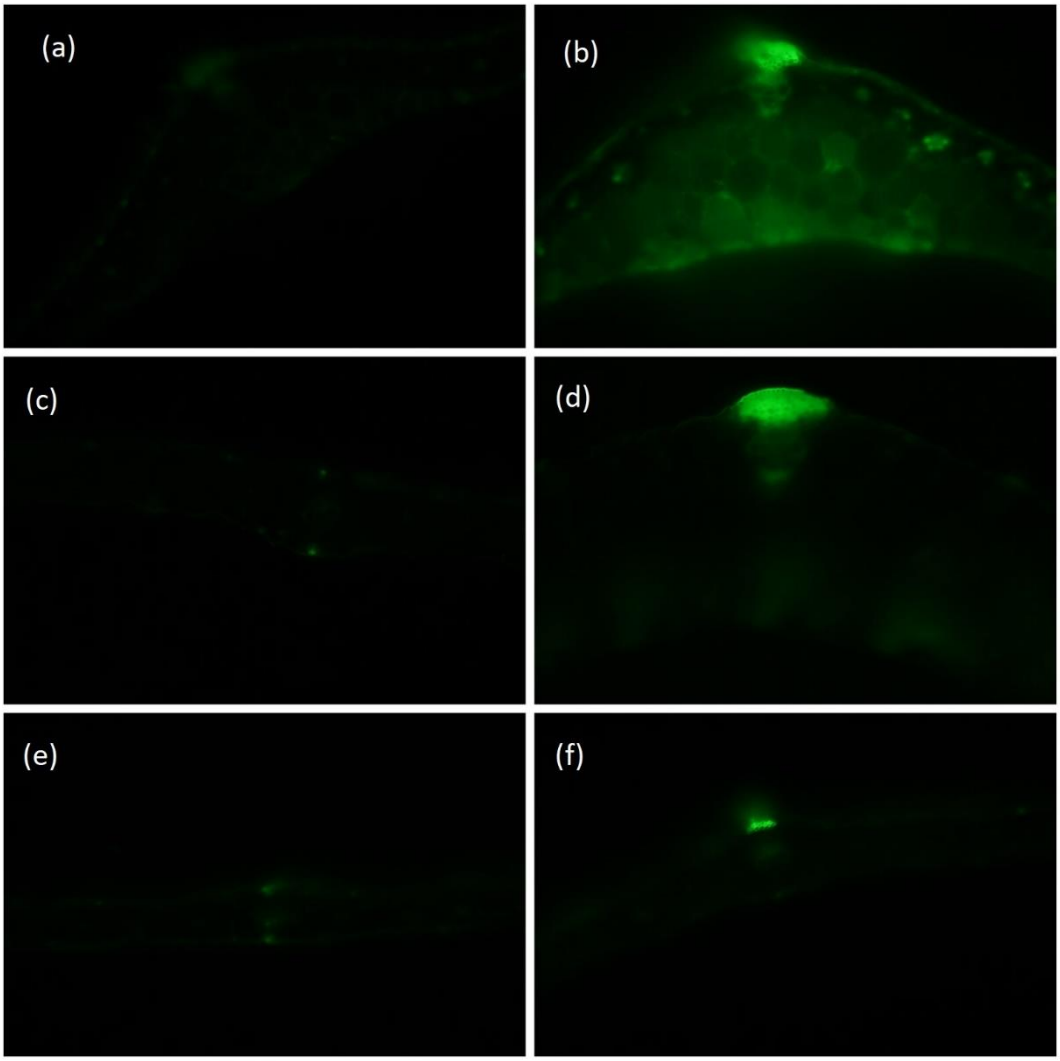

Fig 1.3. Microscopic images (10X) of maize leaf cross sections (a) application leaf, (c) 2nd younger leaf and (e) 3rd younger leaf of untreated control plant in comparison with the cross section of (b) application leaf, (d) 2nd younger leaf and (f) 3rd younger leaf of treated plant with 0.25% $ZnSO_4 .7H_2O$ and exposed to 10 µM zinpyr for 2 h.

Zinpyr florescence provided a visual evidence of the fact that wheat can absorb and translocate the leaf applied Zn with a significantly higher rate than maize. A comparison of maize and wheat treatment leaf cross section (Fig 1.5 a) shows a higher intensity of Zinpyr fluorescence in wheat leaf than maize leaf representing more absorption of leaf applied Zn.

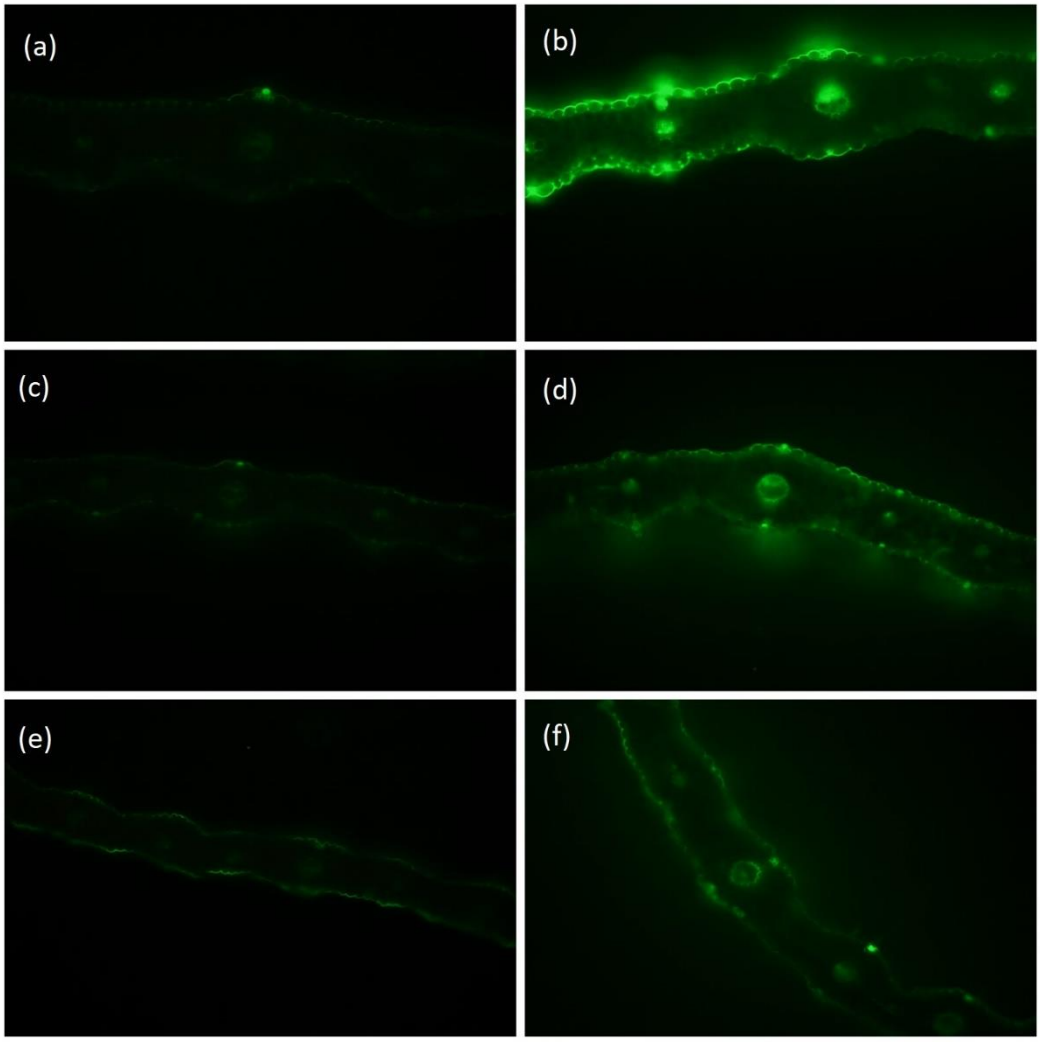

Fig 1.4. Microscopic images (10X) of wheat leaf cross section (a) application leaf, (c) 2nd younger leaf and (e) 3rd younger leaf of untreated control plant in comparison with the cross section of (b) application leaf, (d) 2nd younger leaf and (f) 3rd younger leaf of treated plant with 0.25% $ZnSO_4 .7H_2O$ and exposed to 10 µM zinpyr for 2 h.

Evidently there is a translocation of absorbed Zn to the younger plan parts in both plant species, however, wheat remobilize and transfer the absorbed Zn more efficiently to the younger parts than maize (1.5) It appears that one of the plausible reasons for the poor response of maize plants to foliar Zn spray regarding the grain Zn accumulation might be related to lower Zn penetration and absorption through leaf cells.

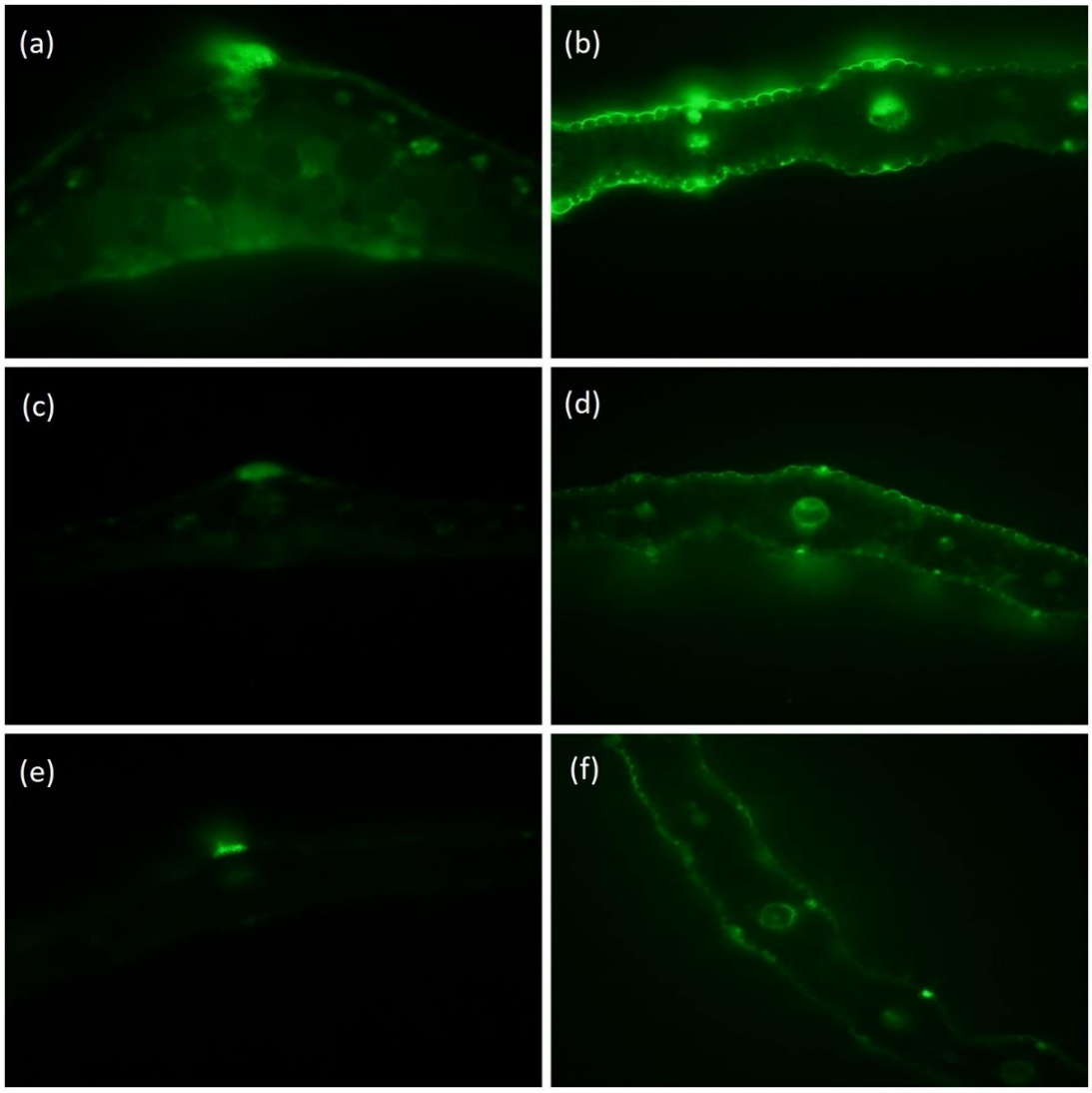

Fig 1.5 Microscopic images (10X) cross section of (a) maize application leaf (b) wheat application leaf (c) maize 2nd younger leaf (d) wheat 2nd younger leaf (e) maize 3rd younger leaf (f) wheat 3rd younger leaf. Zinpyr inflorescence intensity indicates the translocation/remobilization of absorbed Zn from foliar fertilizer application.

1.5. Discussion

In the present studies, foliar application of Zn fertilizer was found effective in increasing Zn concentration of root and shoot tissues in wheat plants but not much effective in maize (Table 1.1, 1.4) suggesting wheat is more capable of uptake and translocation of foliar Zn to the developing shoots and roots as compared to maize. Wang

et al., 2012 reported similar results where foliar Zn spray increased the Zn concentration up to 89% in wheat compared to only 37% in maize (Wang *et al.*, 2012). Cakmak and Kutman (2017) reported that in international HarvestZinc project (www.harvestzinc.org) several soil and foliar fertilizer application were studied on cereal crops under field conditions for the past 8 years in 12 different countries. Foliar Zn fertilization was found significantly effective in wheat but not in maize. Similar results were reported by Zhou *et al.*,2012, wherein data from four different locations in Turkey showed that wheat was most responsive to foliar Zn spray in terms of increased grain Zn (up to 83%), rice showed intermediate response (up to 27%) whereas maize appeared to be less responsive (9%), however, the reasons remained unclear (Cakmak and Kutman, 2017).

In present studies, main question was to elucidate the physiological factors behind the poor response maize plant to foliar Zn application as compared to wheat. Maize and wheat responded in different ways in terms of effectiveness of foliar Zn application (Table 1.3, 14, 1.5, 1.9). Previous studies reported number of factors influencing the performance of foliar nutrient sprays (Fernández *et al.*, 2013). These may include the physicochemical properties of the fertilizer formulation, the environmental conditions under which foliar fertilizers are applied and most importantly characteristics of the target plant. In experiment 2-A maize and wheat plants were grown under same controlled environment and supplied with same fertilizer formulation with exact calculated volume. Therefore, the resulting different behavior is attributed to plant species specifically. The efficiency of the leaf applied nutrients in plant species is a complex process, consisting of series of steps including foliar adsorption, cuticular penetration, diffusion into apoplastic and symplastic spaces, phloem loading into vascular veins and remobilization from application leaf into other actively growing parts of the plants (Du *et al.*, 2014; Alshaal and El-Ramady, 2017).

The possible physiological barriers reducing the rate of uptake and translocation of foliar-applied Zn in maize is provided in the following paragraphs.

1.5.1. Leaf uptake of foliar-applied Zn

In the present studies, in all experiments (including soil and nutrient culture) wheat absorbed significantly higher amount of leaf applied Zn as compared to maize (Table1.9, 1.16). Rate of Zn uptake by maize plants did not increase even when the maize plants were supplied with double volume of fertilizer solution (in experiment 2-B) as compared to wheat (Table 1.12, 1.13, 1.16, 1.17). Based on the results from all the experiments reported in this chapter, different response among maize and wheat to foliar Zn application is attributed mainly to less uptake capacity of maize. Current studies confirmed that the reason for poor response of maize plant to foliar applied Zn is limited uptake capacity of maize compared to wheat.

In experiment 2-A, maize was able to uptake 12.9% and 13.4% of applied Zn solution compared to 16.5% and 17.1% in wheat at low and adequate Zn condition respectively (Table 1.9). In experiment 2-B, maize could only absorb 10.6% and 12.4% while wheat absorbed 12.2% and 17.9 % at low and adequate Zn condition respectively (Table 1.16). Uptake rate was enhanced in both maize and wheat, at adequate Zn supply, but still the difference between both species remained same. Uptake capacity of foliar Zn is influenced by several factors including leaf shape, leaf chemistry, and physical attributes like cuticle composition, surface wax architecture, the presence of leaf trichomes, stomatal density, leaf surface architecture, leaf apoplastic space and/or leaf age. All of these factors interact to alter the absorption and translocation of foliar-applied nutrient and ultimately the plant response (Fernández *et al.*, 2013, Du *et al.*, 2014). In foliar application of nutrients, the leaf cuticle is the first obstacle in nutrient absorption (Kannan, 1990). Therefore, use of surfactants/adjuvants can increase penetration of many substances through the waxy cuticle layer on leaf surface (Stock and Holloway, 1993).

In current studies difference in uptake rate of foliar Zn between maize and wheat is attributed to different plant characteristics and leaf physiology of both species as the plants were grown under same experimental conditions and were supplied with same fertilizer formulation. There are number of factors influencing the uptake rate e.g. structure and thickness of lipid rich protecting cuticle layer on the leaf surface mainly influence the penetration of leaf applied nutrients (Fernández and Brown, 2013, Du *et al.*, 2015). The heterogeneity in plant cuticle structure exists among the species and even

within different plant organs in same species (Fernández *et al.,* 2017). The ultra-structure of the leaf cuticle of three different plant species were compared in which wheat leaf cuticle was found much thinner (~40 nm) than those of the poplar (Populus bolleana, ~300 nm) and chiefly the pear leaf cuticle (~800 nm) (Fernández *et al.,* 2016). In another study by Ristic and Jenks, maize genotypes were studied for epidermis outer cell wall and cuticle thickness in abaxial and adaxial leaf surface. Transmission electron microscopy showed variation among genotypes however cuticle layer thickness was found ~100 nm on maize leaf surface (Ristic and Jenks, 2002). Thus, cuticle layer on maize leaf surface being much thicker and consequently less permeable for penetration appeared to be the first barrier to reduce the penetration rate of foliar applied Zn fertilizer.

In addition to cuticle, the epidermis of plants contains specialized cells including stomata or trichomes that may influence foliar nutrient uptake. Many studies have shown that a high density of stomata (such as in the case of abaxial surface) significantly increase the rate of foliar uptake, mainly under the conditions which favor the stomatal opening. (Schlegel and Schönherr, 2002; Fernàndez *et al.*, 2005; Schlegel *et al.,* 2006; Du *et al.,* 2014). Liao *et al.,* 2005 reported the stomatal density in different wheat genotypes ranging between 43-52 mm^{-2}. In another study Zheng *at al.,* 2013 examined the effect of high temperature on stomatal density and reported the maize SD ranging between 56-77 mm^{-2} under ambient temperature. Based on these reports, less Zn uptake by maize in current studies cannot be explained with the stomatal density per the unit area of leaf as both species have more or less same number of stomata. However, stomatal size and functionality also affect the solutes penetration and may vary with species and cultivars as well as with growth conditions of the plants (Liao *et al.,* 2005, Zhao *et al.,* 2015). There was a significant increase in foliar uptake through the open stomata compared to the uptake via cuticle (Eichert *et al.,*2008).

In present study, the increased uptake of Zn from foliar spray in wheat can be better explained with the presence density of trichomes on wheat leaf surface and other aerial organs. Trichomes are the leaf hair like appendages extending from the epidermis and mostly they are not connected to the vascular system of the plant (Schilmiller *et al.,* 2008). Fernández *et al.,* 2017 compared the adaxial and abaxial leaf surface of orange, olive, maize and wheat by SEM. Wheat adaxial surface (upper leaf surface) appeared rough because of presence of dense trichomes. Maize adaxial surface was found to have

very few trichomes compared to wheat (Fernández *et al.*, 2017). Current results suggesting higher Zn uptake by wheat leaves having more trichomes compared to maize coincides with some previous studies. For example Schlegel and Schönherr (2001, 2002) reported the uptake of $CaCl_2$ by leaves of several species and apple fruits of different developmental stages when trichomes were present in the epidermis. Furthermore, function of trichomes to absorb water and nutrients in some species of *Bromeliaceae* was also reported in some studies (Pierce *et al.* 2001; Papini *et al.* 2010). Presence of trichomes on leaf surface had increased the uptake of leaf-applied nutrients. Trichomes have role to facilitate the foliar nutrient uptake due to lower cuticle thickness over the trichomes, and also the occurrence of cracks and discontinuities in the trichome base. Trichomes density also increase the surface area for absorption of foliar-applied fertilizer (Fernández *et al.*, 2014 a).

Thus, foliar-applied nutrients to wheat leaves may penetrate via the cuticle (including the occurrence of cuticular cracks and imperfections), and also through stomata and trichomes (Fernández *et al.*, 2014 a). The reported studies provide evidence that wheat leaf surface have more permeable cuticle layer and more trichomes than maize leaf consequently capable of more absorption of foliar-applied solution than maize. More Zn uptake rate in wheat compared to maize was also confirmed by microscopic images with high zinpyr fluorescence indicating high Zn accumulation in wheat treatment leaf (Fig 1.5).

In present studies, low Zn supplied wheat plants in experiment 2-B showed less uptake of foliar applied Zn as compared to adequate Zn supplied wheat plants (Table 1.16). The reduced absorption rate of the wheat plants grown with low Zn plants is better explained with poor nutritional status of the low Zn wheat leaf. However, in experiment 2-A the uptake rate of low Zn-wheat plants reached almost equal to that of adequate Zn plants where 50ul of fertilizer solution was applied (Table 1.9). Hence, confirming that efficacy of the foliar nutrient formulation can be improved with better coverage of the applied formulation (Fernandez and Eichert T, 2009). Overall low uptake rate by low Zn-maize and wheat plants in all experiments is because of poor nutritional status of the application leaf which influences the absorption rate of leaf-applied solutions. Nutrient deficiency induces the structural and functional changes of leaf surfaces, which may influence the penetration process depending on the severity of the deficiency and nutrients

concerned (Brian, 2008). For examples, boron and zinc deficiencies can cause malformation of leaf surface structure (e.g. stomata size) which directly affect the absorption of foliar applied solutions (Marschner, 1995). Apart from the limited leaf expansion, reduced stomata density and stomata aperture is also associated with Zn deficiency in plants. The stomata density on both sides of the leaf decreased under Zn deficiency conditions (Shi and Cai, 2009) which could result in decreased nutrient uptake through leaf surfaces.

1.5.2. Translocation of absorbed Zn to other plant parts

As in present studies, leaf applied ^{70}Zn was translocated to root and shoot of the plants in both maize and wheat, but major portion of absorbed Zn remained in the application leaf (Table 1.8, 1.15) even after 36 h of treatment. Although, generally both experiments (2-A and 2-B) suggest that under adequate Zn supply wheat and maize distribute the absorbed Zn to roots and shoots with almost an equal ratio. However, under low Zn supply, major portion of absorbed Zn was transported to roots as compared to shoots. This is probably due to large demand for root growth under low Zn supply. Under Zn deficient condition, roots grow longer and more efficiently compared to the Zn sufficient conditions.

In the experiment 2-A, less translocation from the application leaf is observed as compares 2-B which can be explained easily with the developmental stage of the application leaf. In experiment 2-A when relatively younger plants were treated, on average 89.2% portion of absorbed Zn remained inside the application leaf as compared to 80.6% in experiment 2-B where relatively older plants were subjected to foliar Zn treatment. Previous studies reported that developmental stage of the application leaf is an important factor influencing the movement of nutrients (Turgeon, 2006). Immature or young leaves are sink organs that are entirely dependent upon imported assimilate from the older developed leaves. Hence, the young developing leaves are physiologically incapable of exporting nutrients (even if they absorb from the foliar spray) until they have matured. Similarly, once the leaf has reached full maturity, it become incapable of

exporting nutrients to the sink organs (Koontz and Biddulph, 1957; Fernández and Brown, 2013).

In current studies, the plant Zn status was found to influence the movement of Zn within the leaf tissue, Zn is more easily mobilized in Zn-sufficient leaves than in Zn-deficient leaves. Other authors also reported that the Zn status of the plant have impact upon the subsequent redistribution of the foliar-absorbed Zn (Longnecker and Robson, 1993; Du *et al*, 2015).

In experiment 3, microscopic images also confirmed the translocation of absorbed Zn towards younger leaves in both maize and wheat plants tissues (Fig 1.3, 1.4). Comparison of maize and wheat leaves (Fig1.5) suggests better translocation in wheat plants compared to maize. Although the complete knowledge of all the factors influencing the translocation of absorbed Zn is not available, application leaf characteristics e.g. leaf age and leaf apoplastic composition seems to have obvious role in nutrient translocation to the sink organs (Fernández *et al.,* 2013). After penetrating through cuticle layer and/or diffusing through the stomata or trichomes, leaf apoplastic space can act as another barrier for the applied Zn. Apoplastic composition in leaf varies from specie to specie and can play the role in hindering the remobilization of absorbed Zn to the other plant organs (Fernández and Brown, 2013). Several studies reported that leaf apoplast may restrict the mobility of elements supplied as cations such as Zn by accumulating cations and repelling anions (Speer and Kaiser, 1991; Sattelmacher, 2001; White and Broadley, 2011). The composition of apoplastic spaces with high abundance of negative charges reduce the movement of Zn^{+2} and therefore, limit the Zn translocation to other plant organs from the application leaf. In relation to phloem mobility, Zn is classified as intermediate or conditionally mobile (Fernández and Brown, 2013) and it takes 1-2 days for 50 % absorption as compared to N, P, K which are considered as highly mobile nutrients and take ½-10 h for 50% nutrient absorption (Alshaal and El-Ramady, 2017). Being conditionally mobile nutrient, Zn is reported to have a relatively small remobilization out of the application leaf to the sink organ.

1.5.3. Dilution effect

Large increases in yield causes the considerable decreases in the concentrations of essential nutrients such as Zn and this is called dilution effect (Cakmak and Kutman, 2017). Generally maize plants produce significantly big biomass and grain yield, it was hypothesized that low Zn concentration in maize grain is due to the dilution effect of nutrients. In present studies, dilution effect was minimized by applying double volume of Zn solution on maize (2-B) plants as compared to wheat or by growing the wheat plants earlier than maize (2-A), so that they are equal in biomass. In both situations, maize absorption was significantly less than wheat (Table 1.9, 1.16) regardless of more volume of solution supplied or managing to equalize the biomass by using younger maize plants. The results suggested that poor response of maize plants to foliar Zn application is not specifically because of dilution effect but maize plant literally absorb less Zn from foliar spray.

1.6. Conclusion

It can be concluded that the main reason for poor response of maize is low absorption or penetration rate of foliar-applied Zn than wheat. Plant species have a considerable effect on the uptake and translocation of foliar-applied Zn. Zn absorption and translocation capacity of wheat is significantly higher than maize specially when grown in Zn-sufficient medium. In maize, the Zn absorption rate was not increased despite of supplying double volume of foliar Zn fertilizer to leaf. This difference in uptake rate of leaf- applied Zn is related with different morphology and physiological characteristics of both plant species. Although, the plant Zn nutritional status affect the initial absorption and penetration of foliar applied Zn in wheat and also influence the subsequent redistribution of Zn within the plants. One of the reasons for the poor response of maize plants to foliar Zn spray regarding the grain Zn accumulation is related to lower Zn uptake through leaf cells and translocation to other plant parts. The results advance our understanding of the factors that influence the efficacy of foliar zinc fertilizers in maize and wheat crops. Further investigation is needed to better understand and get the insights about the factors that can increase the efficacy of foliar Zn in cereal crops especially in maize.

CHAPTER 2

UPTAKE OF ZN BY WHEAT AND MAIZE DURING AS AFFECTED BY N RATE

2.1. Introduction

Zinc deficiency is a major health challenge, caused due to high dependence on food containing less bioavailable Zn such as cereals (wheat, rice and maize). Cereals are considered as not only inherently low in Zn concentration, but also with less bioavailability of Zn (Graham *et al.,* 2001; Cakmak *et al.,* 2010). Therefore, it is the need of time to increase the Zn concentrations in wheat and maize grains as well as in the edible portions of other staple crops. Food supplementation, food fortification and food diversification are some applicable interventions to reduce the widespread Zn deficiency problems. These strategies are being applied in some countries with positive results, but these are costly and out of the reach of the people living in rural areas of developing countries. People living below the poverty line are unable to afford expensive fortified food or supplements (Pfeiffer & McClafferty, 2007; Stein *et al.,* 2014). The most effective strategy for reducing the global malnutrition problem is the biofortification, that is biologically increasing the micronutrients in edible parts of staple food.

Agronomic biofortification along with breeding for high Zn contents and bioavailability in staple foods is considered as the most suitable and cost-effective approach (White & Broadley, 2005; Cakmak, 2008; Cakmak *et al.,* 2010). Adequate nitrogen nutrition increases wheat grain yield as well as improves the nutritional quality of wheat grains by enhancing the uptake and accumulation of Zn in grain (Shi *et al.,*

2010). Kutman *et al*. 2011 harvested nearly 80% of the shoot Zn within wheat grain by increasing the N supply, suggesting that N nutrition is important in uptake and remobilization of Zn. Nitrogen has a crucial role in Zn uptake and its accumulation in grain, therefore, a special consideration should be given to N while biofortifying food with Zn and Fe (Erenoglu *et al.*, 2011). Nitrogen nutrition influence the molecular and physiological mechanism involve in uptake and remobilization of Zn (Cakmak *et al.,* 2010). Increased N supply enhance the levels of metal-chelating nitrogenous contributes and hence, facilitates the Zn and Fe uptake and transport to the grain (Kutman *et al*. 2011).

Zinc and proteins are closely linked in biological systems as Zn is an important part of a large number of structural, regulatory and functional proteins. Nearly 10% of the all proteins in human body contains Zn as an integral part (Krezel & Maret, 2016). Many previous studies in literature suggested that proteins are a sink for Zn. A high positive correlation between the grain proteins and grain Zn concentration is reported by previous studies (Morgounov *et al.*, 2007; Peleg *et al*., 2008) suggesting that the protein contents in grain may contribute to the Zn accumulation by increasing the sink strength of the grain for Zn.

In cereal grains, major portion of Zn is believed to be confined in the form of protein-Zn-phytate complexes (Lott *et al.*, 1995). The Zn concentration within a grain Is not uniform but vary depending upon the part of the grain. For example, aleurone layer in wheat grain contains up to 150 mg kg^{-1} Zn whereas endosperm holds only 15 mg kg^{-1} Zn (Sramkova *et al.*, 2009). The embryo and aleurone layer of wheat grains are rich in proteins and Zn whereas, endosperm appears low in protein and phytate as well as low in Zn (Welch & Graham, 1999). High accumulation of Zn in embryo and aleurone portions of seeds were shown with the help of a Zn-staining method by Ozturk *et al.* (2006). Moreover, Ozturk *et al.* 2009 also reported that wheat grains rich in protein accumulate higher Zn contents than low protein wheat grains. Thus, the available literature suggests that there is a close relationship between N and Zn and higher proteins or N contents facilitates the Zn uptake and accumulation in grain (Peleg *et al.*, 2008; Kutman *et al.*, 2010)

Studying the effect of N fertilization on uptake and accumulation of Zn in maize and wheat shoot grown at different Zn level in growth medium will help to understand the physiological relationship between N and Zn in these crops. Moreover, considering

the fact that maize is very sensitive to Zn deficiency, any influence of N fertilization to improving Zn nutrition of maize will be beneficial for yield as well as nutritional quality of grains (Elias & Manthey, 2005).

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

i. How does increasing soil N fertilization affect the shoot Zn concentrations in maize and wheat when grown at low or adequate availability?

ii. How does increasing N helps to uptake/absorb Zn from soil in maize and wheat?

iii. How does increasing N nutrition helps to uptake/absorbs and translocate Zn from foliar Zn spray in maize and wheat?

2.1. Experiment (I) Absorption and Translocation of Zinc ($ZnSO_4.7H_2O$) in Maize and Wheat at vegetative growth stage as affected by low and adequate soil N supply

2.1.1. Materials and Methods

This experiment was conducted to assess the changes in Zn uptake from soil by maize and wheat plants at low or adequate supply of N. Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) were grown in plastic pots under greenhouse conditions (Details are described in "General Material and Methods" Section). Zinc in the form $ZnSO_4.7H_2O$ was supplied at the rate of 2 mg kg^{-1} in all pots. Nitrogen was supplied as $Ca[NO_3]_2.4H_2O$ mixed in the soil at the time of sowing. Two N levels viz: low and adequate were established (N1: 100 mg N kg^{-1}, N2: 200 mg N kg^{-1}). After germination, 8 seedling per pot were maintained and plants were allowed to grow under normal conditions in greenhouse. Pots were irrigated with DI-water every day and randomized every two weeks for uniform exposure to possible climatic variations.

Plants were harvested at two different developmental stages for elemental analysis. Half of the plants (4 plants per pot) were harvested at 50 days after germination and remaining were harvested at 78 days. Harvested shoot samples were washed with DI water, dried in oven and analyzed for shoot dry matter, Zn and N concentration as described in "General Materials and Methods Section".

2.1.2. Results

Low N plants showed the deficiency symptoms e.g. chlorosis in lower leaves and suppressed growth particularly in maize plants. At young age wheat did not show any symptoms of Zn or N deficiency. N deficiency symptom were observed at later stage of plant development in wheat. At the age of 11 weeks old, clear biomass reduction and Zn and N deficiency symptoms were visible in maize plants (Fig 2.1). Wheat also showed the chlorosis at low N plants. The positive effect of adequate N supply on the shoot growth and development of plants was also observed in this experiment. Plants were sampled for elemental analysis at the two developmental stages i.e. seven weeks and eleven weeks old.

2.1.2.1. Analysis of plants harvested at the age of seven weeks

Shoot biomass of maize and wheat plants were not affected significantly by soil N level, at the age of seven weeks (Table 2.1). As expected, N concentration was significantly ($p<0.001$) improved with adequate N supply in both maize and wheat plants. The maize and wheat plants grown with adequate N supply had greater concentrations of Zn in the shoot as compared to the plants grown with low N supply (Table 2.1). However, this effect was not statistically significant for maize. Results showed N nutrition improved the soil root Zn absorption both crops particularly in wheat. Analysis of variance (ANOVA) revealed that interaction between crops and soil N level effected the N concentration significantly ($p<0.05$) but it did not affect the dry matter production or Zn concentration (Table 2.1).

Table 2.1: Shoot dry matter (g plant^{-1}), leaf N concentration (%) and shoot Zn concentration (mg kg^{-1}) in 50 days old maize and wheat plants grown with low (100 mg kg^{-1}) and adequate (200 mg kg^{-1}) N supply under greenhouse conditions. The soil was supplied with 2 mg kg^{-1} Zn in the form of $ZnSO_4.7H_2O$. The data represents the mean of 4 replicates

Crop	Soil N level (mg kg^{-1})	Dry matter (g plant^{-1})	N (%)	Zn (mg kg^{-1})
Maize	100	0.97 ± 0.05 a	3.01 ± 0.13 d	7.73 ± 0.72 c
Maize	200	0.97 ± 0.09 a	3.76 ± 0.19 c	8.38 ± 0.67 c
Wheat	100	0.57 ± 0.02 b	4.48 ± 0.11 b	15.9 ± 1.0 b
Wheat	200	0.63 ± 0.03 b	4.87 ± 0.10 a	18.0 ± 0.8 a

*Dry matter HSD 0.05 (Soil N, Crop, Soil N x Crop) = NS, 0.06***, N.S*

*N Concentration HSD 0.05 (Soil N, Crop, Soil N x Crop) = 0.15***, 0.15***, 0.29**

*Zn Concentration HSD 0.05 (Soil N, Crop, Soil N X Crop) = 0.90***, 0.90***, N.S*

2.1.2.2. Analysis of eleven weeks old plants

Shoot biomass was increased with increased N rate in 11 weeks old maize and wheat plants however, the effect was not significant for wheat (Table 2.2 Fig 2.1). Analysis of variation showed a significant ($p<0.001$) effect of soil N and its interaction with crop on dry matter production. Low N maize plants showed sever Zn and N deficiency symptoms. However, adequate N supply reduced the Zn deficiency symptoms (Fig 2.1). Nitrogen concentration increased significantly ($p<0.001$) at adequate N supply in both maize and wheat plants (Table 2.2).

N supply in also affected the Zn concentration significantly ($p<0.001$) in both maize and wheat plants. The plants grown with adequate N supply had significantly greater concentrations of Zn in the shoot as compared to the plants grown with low N application (Table 2.2).

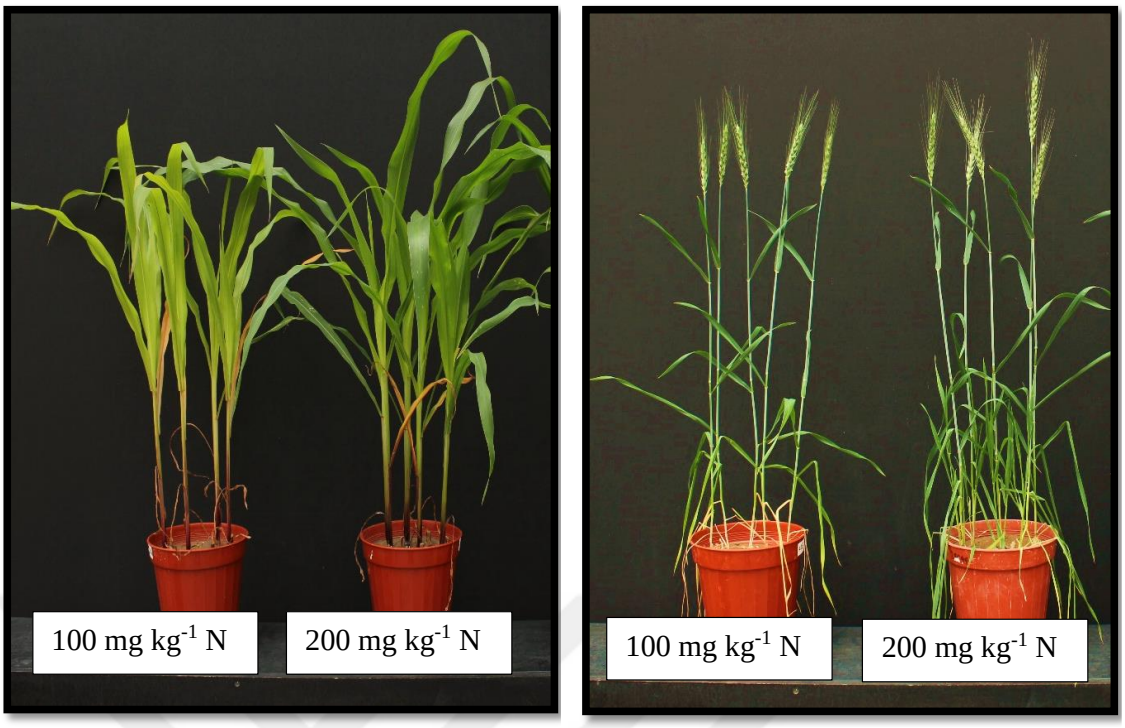

Fig 2.1: Effect of low (100 mg kg^{-1}) and adequate (200 mg kg^{-1}) soil N applications on growth of 11-weeks-old maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) plants. The soil was supplied with 2 mg kg^{-1} Zn in the form of $ZnSO_4.7H_2O$.

Table 2.2: Shoot dry matter (g $plant^{-1}$), leaf N concentration (%) and shoot Zn concentration (mg kg^{-1}) in 79 days old maize and wheat plants grown with low (100 mg kg^{-1}) and adequate (200 mg kg^{-1}) N supply under greenhouse conditions. The soil was supplied with 2 mg kg^{-1} Zn in the form of $ZnSO_4.7H_2O$. The data represents the mean of 4 replicates.

Crop	Soil N level (mg kg^{-1})	Dry matter (g $plant^{-1}$)	N (%)	Zn (mg kg^{-1})
Maize	100	2.89 ± 0.48 b	1.0 ± 0.1 c	5.2 ± 0.1 c
Maize	200	4.51 ± 0.34 a	1.8 ± 0.2 b	6.6 ± 0.3 b
Wheat	100	1.88 ± 0.06 b	1.8 ± 0.3 b	7.6 ± 0.9 b
Wheat	200	2.21 ± 0.12 b	2.8 ± 0.1 a	12.7 ± 0.8 a

*Dry matter HSD 0.05 (Soil N, Crop, Soil N x Crop) = 0.67***, 0.67***, 1.32***

*N Conc. HSD 0.05 (Soil N, Crop, Soil N x Crop) = 0.19***, 0.19***, N.S*

*Zn Conc. HSD 0.05 (Soil N, Crop, Soil N x Crop) =0.75***, 0.75***, 1.45**

N nutrition increased the shoot Zn concenntration significantly in both maize and wheat plants (Table 2.2). Wheat plants had significantly ($p<0.001$) higher Zn concentration as compared to maize. Adequate N grown wheat plants showed the highest Zn concentration in shoots. Interaction between soil N level and crop also significantly ($p<0.05$) affected the shoot Zn concentration (Table 2.2).

2.2. EXPERIMENT II: Absorption and Mobilization of Zinc ($ZnSO_4.7H_2O$) from foliar Zn application in Maize and Wheat at vegetative growth stage as affected by variable soil N supply

2.2.1. Materials and Methods

This experiment was performed to assess the Zn uptake from foliar Zn treatment by maize and wheat plants grown at variable soil N supply.

Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) were grown in marginal (0.5 mg Zn kg^{-1}) soil Zn levels (supplied as $ZnSO_4.7H_2O$) under greenhouse conditions (Details are described in "General Material and Methods" Section). Initially N (as Ca $[NO_3]_2.4H_2O$) was mixed in the soil at the time of sowing at three different rates *viz:* low (N1: 25 mg N kg^{-1} soil), medium (N2: 50 mg N kg^{-1} soil) and adequate N (N3: 100 mg N kg^{-1} soil). After germination, 9 seedlings in each wheat pot and 6 in each maize pot were maintained and plants were allowed to grow under normal conditions in greenhouse.

When the maize plants were at the 4-leaf stage, and wheat plants at 3-leaf stage, the foliar Zn was applied. The control group of pots containing maize and wheat plants were not sprayed with Zn, while the treatment group was sprayed with a 0.25% (w/v) $ZnSO_4.7H_2O$ solution containing 0.02% Tween-20® as surfactant. The soil was covered with towel papers to avoid soil contamination. Fertilizer solution was sprayed with the help of hand sprayer. The foliar applications were repeated after two days and for three

times. At the end of the spraying period, maize plants were at 5 leaf stage and wheat at 4 leaf stage. Plants were allowed to grow in greenhouse condition until to produce new younger uncontaminated fresh leaves.

Three days after the final spray, plants were supplied with another N dose with same ratio (as in beginning) to avoid sever N deficiency. Finally, three N levels were achieved as low (N1: 50 mg kg^{-1}), medium (N2: 100 mg kg^{-1}) and adequate N (N3: 200 mg kg^{-1}).

Ten days after the final spray, maize and wheat plants developed new younger uncontaminated leaves which were harvested separately. Overall maize and wheat plants were harvested in three fractions viz

Fraction I: Young (un-sprayed) leaves
Fraction II: Remaining plant parts (sprayed)
Fraction I was analyzed for Zn concentrations and N concentration. Fraction II (sprayed plant portion) was used to determine dry weight of plants.

2.2.2. Results

Table 2.3 illustrates the dry matter production (mg $plant^{-1}$) and leaf N concentration (%) in younger leaves grown at low (50 mg N kg^{-1} soil), medium (100 mg N kg^{-1} soil) or high (200 mg N kg^{-1} soil) N under low Zn supply in greenhouse. Low N maize plants developed N deficiency symptoms, for example, chlorosis and necrosis on older leaves, however, wheat plants did not show N deficiency symptoms (Fig 2.2, 2.3). A visible biomass reduction can easily be observed in low N maize but not in wheat (Fig 2.2, 2.3).

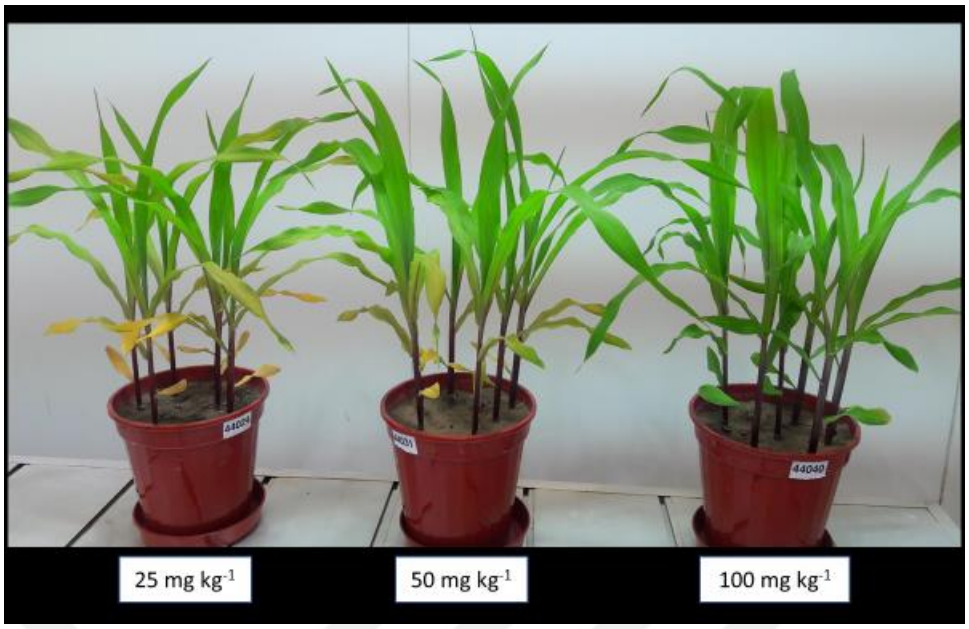

Fig 2.2 Maize (*Zea mays* L. cv. Shemal) grown at low (50 mg N kg^{-1} soil), adequate (100 mg N kg^{-1} soil) or high (200 mg N kg^{-1} soil) N supply on a Zn-deficient soil and supplied with foliar Zn treatment of 0.25% $ZnSO_4.7H_2O$ was applied

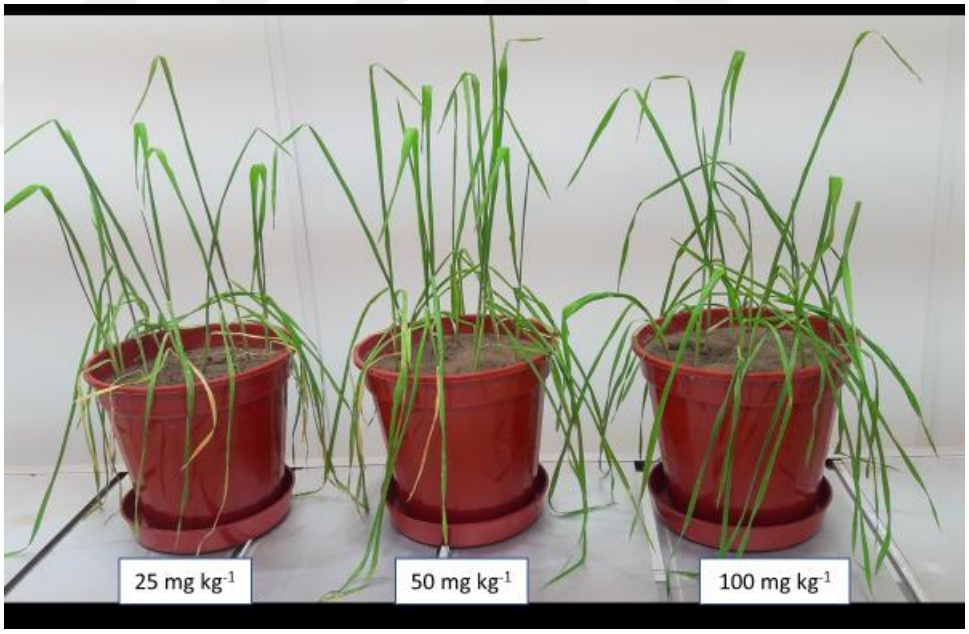

Fig 2.3: Wheat (*Triticum aestivum* L. cv. Tahirova) grown at low (50 mg N kg^{-1} soil), adequate (100 mg N kg^{-1} soil) or high (200 mg N kg^{-1} soil) N supply on a Zn-deficient soil and supplied with foliar Zn treatment of 0.25% $ZnSO_4.7H_2O$ was applied.

Biomass was increased significantly in maize with increasing the N supply in the soil. Foliar Zn application at low N supply had no effect on biomass (Table 2.3). Foliar

Zn application at high N supply increased the plant growth and hence the dry matter was increased significantly (Table 2.3, Fig 2.2). In wheat plants, however, this effect was not statistically significant. In maize, the shoot biomass of the high N plants without any foliar spray was 37% higher compared to that of the low N plants. Whereas, in wheat, shoot biomass of the high N plants with no foliar Zn treatment were 15% higher than those of low N plants (which is not statistically significant). This indicates that the low N treatment of 50 mg kg^{-1} of soil was already enough to support the growth of the wheat plants at the early vegetative stage. After foliar Zn application, biomass of high N maize plants was 73.8% higher than that of low N plants. However, in wheat the 20% increase in biomass production with foliar Zn was recorded (Table 2.3).

As expected, N concentration in younger leaves of maize and wheat plants were increased with increasing the soil N supply (Table 2.3). The medium N supply showed the intermediate values between low and high N supply. The differences were obvious and significant if the concentrations are compared among low and high N plants. The only exception was the reduced N concentrations in high soil N plants with foliar Zn spray probably due to increased biomass production and resulting dilution effect. Analysis of variance showed significant effect of plant species (maize and wheat), soil N supply and the foliar Zn supply on the dry matter production and N concentration. The interaction among all these variables had significant effect on dry matter production but not on N concentration of young leaves (Table 2.3).

Table 2.3: Effect of foliar Zn treatment of 0.25% $ZnSO_4.7H_2O$ on the dry matter production and leaf N concentration of Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) grown at low (50 mg N kg^{-1} soil), adequate (100 mg N kg^{-1} soil) or high (200 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil supplied with 0.5 mg Zn kg^{-1} soil.

Plant	Soil N (mg kg^{-1})	Foliar Application	Shoot Biomass (g $plant^{-1}$)	Leaf N (%)
Maize	50	None	0.89 ± 0.04 d	3.24 ± 0.25 fg
		$ZnSO_4$	0.88 ± 0.06 d	2.93 ± 0.33 g
	100	None	1.08 ± 0.05 c	3.87 ± 0.21 de
		$ZnSO_4$	1.15 ± 0.07 bc	3.39 ± 0.23 efg
	200	None	1.22 ± 0.06 b	4.53 ± 0.07 c
		$ZnSO_4$	1.53 ± 0.04 a	3.50 ± 0.12 ef
Wheat	50	None	0.26 ± 0.02 e	4.39 ± 0.27 cd
		$ZnSO_4$	0.25 ± 0.03 e	4.77 ± 0.18 bc
	100	None	0.25 ± 0.02 e	5.29 ± 0.36 ab
		$ZnSO_4$	0.27 ± 0.02 e	5.35 ± 0.19 a
	200	None	0.30 ± 0.05 e	5.53 ± 0.11 a
		$ZnSO_4$	0.30 ± 0.01 e	5.40 ± 0.23 a

*Dry matter HSD 0.05 (Crop, Soil N, Foliar Zn application, Soil N x Crop x foliar Zn application) = 0.03***, 0.03***, 0.03***, 0.11****

*N Conc. HSD 0.05 (Crop, Soil N, Foliar Zn application, Soil N x Crop x foliar Zn application) = 0.13***, 0.20***, 0.13***, N.S*

Newly grown young uncontaminated leaves were analyzed separately and Table 2.4 illustrate the effect of soil N on root and leaf Zn absorption in maize and wheat plants at vegetative growth stage grown at variable N levels supplied with low Zn supply in soil.

As expected, shoot Zn concentrations were increased with the application of foliar $ZnSO_4$ in both maize and wheat plants grown at low, medium and high N levels (Table 2.4). The increase in shoot Zn concentration as a result of foliar Zn application is significant in wheat but not in maize confirming the results presented in "Chapter 1".

Table 2.4: Effect of foliar Zn treatment of 0.25% $ZnSO_4.7H_2O$ on the Zn concentration (mg kg^{-1}) and Zn contents (µg $plant^{-1}$) of young new leaves of Maize (*Zea mays* L. cv. Shemal) and wheat (*Triticum aestivum* L. cv. Tahirova) grown at low (50 mg N kg^{-1} soil), adequate (100 mg N kg^{-1} soil) or high (200 mg N kg^{-1} soil) N supply on a Zn-deficient calcareous soil supplied with 0.5 mg Zn kg^{-1} soil.

Plant	Soil N (mg kg^{-1})	Foliar Application	Zn Concentration (mg kg^{-1})	Zn contents (µg $plant^{-1}$)
Maize	50	None	9.72 ± 1.66 cd	0.68 ± 0.15 ef
		$ZnSO_4$	11.4 ± 0.84 cd	1.28 ± 0.10 bc
	100	None	7.44 ± 0.31 d	0.65 ± 0.02 ef
		$ZnSO_4$	10.6 ± 0.56 cd	1.72 ± 0.05 b
	200	None	8.23 ± 0.80 d	0.67 ± 0.15 ef
		$ZnSO_4$	9.37 ± 0.59 d	2.38 ± 0.22 a
Wheat	50	None	10.3 ± 0.16 cd	0.48 ± 0.05 f
		$ZnSO_4$	31.4 ± 3.87 a	1.40 ± 0.05 bc
	100	None	11.6 ± 1.75 cd	0.50 ± 0.05 f
		$ZnSO_4$	27.5 ± 3.77 ab	1.42 ± 0.13 bc
	200	None	14.9 ± 1.70 c	0.91 ± 0.23 ef
		$ZnSO_4$	24.6 ± 1.34 b	1.53 ± 0.05 bc

*Zn Conc. HSD 0.05 (Crop, Soil N, Foliar Zn application, Soil N x Crop x foliar Zn application) = 1.18***, N.S, 1.18***, 4.99****

*Zn Contents. HSD 0.05 (Crop, Soil N, Foliar Zn application, Soil N x Crop x foliar Zn application) = 0.09***, 0.13***, 0.09***, 0.38****

Results showed that increasing N supply had a negative effect on shoot Zn concentrations in maize. In case of wheat, shoot Zn concentrations increased with increasing the soil N rate (Table 2.4). However, the shoot Zn contents were increased significantly with improving N status of the plants due to enhanced plant growth and more biomass production. In wheat plants, without foliar Zn application, the shoot Zn

concentrations increased with increasing N rates, however, the effect was not statistically significant (Table 2.4). With foliar spray, at low soil N plants, the Zn concentrations increased significantly compared to high N plants, however, contents were not increased much (Table 2.4).

2.3. Discussion

Nitrogen nutrition had a significant positive affect on Zn uptake from soil and foliar Zn application in both maize and wheat plants. Supplying adequate N in the soil, increased the shoot growth and biomass of the plants in both experiments reported in this study. In maize Zn deficiency symptoms were overcome by supplying adequate N rate, explaining that root uptake and utilization of Zn was improved with adequate N supply (Fig 2.1).

In experiment-I, at adequate Zn supply in soil, increasing the N nutrition increased the shoot Zn concentrations highly significantly in both maize and wheat plants (Table 2.1, 2.2). Although the effect was more significant in wheat compared to maize. At vegetative stage in both maize and wheat, increasing N supply was helpful in increasing the shoot biomass as well as the shoot Zn concentration when there is sufficient amount of Zn available in the soil (Table 2.2). The results found in current studies are with agreement to the many previous studies cited in literature (Cakmak *et al*., 2010b; Kutman *et al.*, 2010; Erenoglu *et al.,* 2011; Singh *et al.,* 2018). Kutman *et al*., 2010 showed that Zn concentration in wheat shoot and grains were increased due to increased supply of N either in soil or foliar providing the sufficient availability of Zn in soil. Erenoglu *et al.,* 2011 demonstrated the three-fold increase Zn root uptake by improving N nutrition by using radioactive isotope ^{65}Zn.

In experiment 2 where plants were grown on low soil Zn, biomass was increased with increasing N supply in the soil particularly in maize (Table 2.3). At high N application, foliar Zn application resulted in significant increase in shoot biomass (Table 2.3). Nitrogen nutrition was not effective in increasing the shoot Zn concentration, however, shoot Zn contents were increased significantly (Table 2.4). With increasing N supply in growth medium and applying foliar Zn, increased biomass of the plants resulted

in dilution of Zn in young leaves. In case of low soil N supply, foliar Zn treatment was highly effective in increasing the Zn concentration compared to high N supply, however, Zn contents were higher at high N supply (Table 2.4). Increased Zn contents explained the enhanced utilization of Zn in terms of biomass production. It is also well known that high N availability facilitates the remobilization and activity of Zn in plant tissues by increasing the level of Zn chelating compounds (Singh *et al.,* 2018). Similar results were reported by Kutma *et al.,* 2010 in durum wheat that under deficient Zn supply I growth medium N nutrition was not helpful in enhancing the shoot Zn concentration, however, shoot Z contents were greatly improved indicating the increased activity ad metabolism of Zn for biomass production.

There was a strong positive relation between Zn concentration and N was observed in both experiments in maize and wheat crops. It is hence suggested that Zn and N are closely linked to improve the Zn uptake and utilization when there is enough amount of these nutrients available in soil or in the form of foliar. These results are well supported with previous finding (Cakmak *et al*., 2010b; Kutman *et al.,* 2010; Erenoglu *et al.,* 2011; Habib *et al.,* 2012; Singh *et al.,* 2018).

Experiment 1 suggested that increased N nutrition enhance the Zn uptake from soil and helps in translocation to the young plant tissues in both wheat and maize but more efficiently in wheat. Root uptake is increased due to better plant growth caused by adequate N supply in the soil. Nitrogen also increased the transporter proteins responsible for uptake and translocation of Zn from root to young leaves (Waters et al., 2006; Haydon & Cobbett, 2007). Experiment II suggested that increasing soil N at deficient soil Zn does not increase the shoot Zn concentration but increase the shoot Zn contents. Nitrogen nutrition has a positive effect on the concentration of chelating compounds (Wirén et al., 1999; Haydon & Cobbett, 2007). High N supply has a positive effect on overall nitrogenous compounds including the Zn and other metal chelators and transporters (Kutman *et al.,*2010). Nitrogen nutrition is helpful to absorb the Zn from root as well as from leaf-applied foliar spray. Therefore, improving N applications, could be a very helpful tool for agronomic biofortification of Zn in cereal crops.

2.4. Conclusions

Micronutrient deficiencies particularly Zn and Fe also called as “hidden hunger” is a serious health problem increasing with an alarming rate in developing countries where population relay on cereal crops for staple food (Bouis, 2003). Agriculture strategies offer a workable and cost-effective solution to the problem by increasing the Zn contents in staple food like cereal crops through breeding or fertilization or combining both approaches (Cakmak, 2008). Improving nitrogen nutrition has proven very beneficial not only to increase the grain yield but also enhance the nutritional quality of the cereal grains by increasing the Zn and Fe concentrations (Kutman *et al.,* 2010). Improved N application increase the protein contents of the plants which involve in Zn uptake from root and leaf (in case of foliar applied Zn) and its transport toward sink. Maize and wheat absorb and accumulate more Zn in shoot in the presence of high soil N, provided the adequate availability of Zn in the soil. Under Zn deficient conditions, increased N helps to utilize the available Zn to increase biomass production. Giving a careful consideration to N nutrition as a part of agronomic biofortification of cereal crops may provide a better solution to food security and hidden hunger problems worldwide.

CHAPTER 3

CHARACTERIZATION OF BIOFORTIFIED HARVESTPLUS WHEAT GENOTYPES FOR ROOT UPTAKE, SHOOT TRANSLOCATION, FOLIAR ABSORPTION, RE-MOBILIZATION AND SEED DEPOSITION OF ZINC

3.1. Introduction

Chronic micronutrient deficiencies, particularly Zn and Fe, also known as "hidden hunger", is a major public health problem, affecting over two billion people worldwide particularly women and children in developing countries (Akhtar, 2013). More than 60% of the population living in developing countries is at the risk of low dietary Zn intake (Brown *et al.*, 2001). The process of increasing the density of mineral elements and vitamins in a crop by using the multiple strategies of agronomy, plant breeding, or transgenic techniques is called as biofortification (White and Broadely, 2011; Bouis and Saltzman, 2017). If the individuals are provided with biofortified staple food to consume on regular basis, considerable improvements can be generated in public health and nutrition (Bouis *et al.,* 2011b; Signorell *et al.,* 2015). Genetic as well as agronomic biofortification, are both considered as a reasonable solution to prevalent Zn deficiency problem in the human population of developing world (Pfeiffer and McClafferty, 2007).

Soil or foliar application of active Zn fertilizer to increase the Zn concentration of the edible part of food crops is agronomic biofortification. The effectiveness of Zn fertilizer depends on the application with accurate rate, the right time and appropriate plant stage. (Cakmak, 2008; Chattha *et al.*, 2017). However, genetic biofortification is the development of staple food crops, by using plant breeding techniques, with the capacity to accumulate higher level of micronutrients in edible plant portions, reduce the levels of anti-nutrients and elevate the levels of substances that help to increase the nutrient

absorption (Bouis, 2003; Welch and Graham, 2004; Bouis *et al.,* 2011). Genetic biofortification is thought to provide a sustainable and cost-effective solution to the global Zn deficiency problem by developing new crop cultivars with comparatively higher accumulating ability of Zn in grains (Welch and Graham, 2004; Cakmak, 2008; Bouis *et al.,* 2011; Meenakshi *et al.,*2010; Stein, 2010). It is done by exploring the natural available variation in the germplasm for the particular desired trait and utilize it for developing new genotypes by using plant breeding techniques (Cakmak, 2008; Velu *et al.,* 2014).

Wheat is a primary staple food in South Asia (Northern India and Pakistan) used to make traditional breads (*e.g.* chappati or roti) to be consumed almost in every meal and in every household (Baloch *et al.,* 2015). Despite the heavy consumption (>390 g per capita per day) of wheat, more than 26% of the population living in South Asia is diagnosed as Zn deficient. As described in previous chapter, cereals like wheat, maize and rice are inherently low in micronutrients, therefore, cereal-based foods do not provide enough Zn to meet the individual's daily Zn requirement. Human Zn deficiency in this region is also associated with noticeable Zn deficiency in soils as cereals are cultivated on severely Zn deficient soils (Asher, & Hynes, 1992; Welch & Graham, 2004; Cakmak, 2008; Cakmak *et al.,* 2010; Zou e*t al.,* 2012; Velu *et al.,* 2012; Cakmak, 2014; Cakmak and Kutman, 2017). Therefore, the South Asian agro-ecological zone including India and Pakistan was identified as potential target areas for adoption and commercialization of biofortified wheat. HarvestPlus program (www.harvestplus.org) along with collaboration with public and private partners took initiatives to develop the biofortified high-Zn wheat cultivars for the target areas (Bouis and Welch, 2010).

As a part of HarvestPlus program, "Biofortification Breeding Research", at International Maize and Wheat Improvement Center (CIMMYT), is taking lead role in development, evaluation, seed production and adoption of biofortified genotypes in partnership with numerous breeding programs at national level and private seed production groups in India and Pakistan (www.cimmyt.org). Initially breeding targets for the mentioned countries was to increase the Zn concentration by 10-12 mg kg^{-1} in wheat grain over the baseline (HarvestPlus Brief, 2006; Bouis and Welch, 2010; Bouis and Saltzman, 2017). The mean value for Zn concentration of popular varieties currently cultivated in the region is considered as baseline.

In order to develop a high-Zn cultivar, the first thing breeders need is the availability of sufficient genetic variation in germplasm for increased Zn concentration in the grain (Cakmak., 2008). To explore the genetic variation, plant breeders screen the available genetic resources like wheat accessions, promising lines, landraces in the primary gene pool and wild relatives and progenitors in secondary gene pool. If the germplasm screening results in useful genetic variation, the genes governing high Zn accumulation are transferred to the modern high yielding cultivars by crossing, back crossing or by using other plant breeding techniques (Cakmak *et al.,* 2000; Velu *et al.,* 2014; Velu and Singh, 2012).

The plant breeding targets for the previous 50 years were to increase the wheat production to overcome hunger and to feed the rapidly increasing world population. With the objective of genetic gain, semi dwarf, high yielding and disease resistant plants were selected over the years (Tilman *et al.,* 2002; Ortiz *et al.,* 2007; Trethowan *et al.,* 2007; Davis, 2009; Curtis & Halford, 2014; Velu and Singh, 2013). Unfortunately, nutritional measures like micronutrient and protein concentrations were largely disregarded. As a result, modern varieties of staple crops are commonly high-yielding and disease-resistant but not much nutritious (Cakmak, 2008). Therefore, improved cultivated wheats show a narrow genetic variation for Zn concentration (Oury *et al*., 2006) as compared to wild and primitive wheat species (Garvin *et al.,* 2006; Fan *et al.,* 2008; Davis, 2009; Shewry *et al.,* 2016).

Germplasm including more than 3000 bread and durum wheat accessions, tetraploid and diploid wild relatives and progenitors of wheat were collected from CIMMYT gene bank and were evaluated for Fe and Zn concentration (Monasterio and Graham, 2000, Ortiz-Monasterio *et al.,* 2007). Wild emmer (*Triticum turgidum* ssp. Dicoccoides), einkorn wheat (*Triticum monococcum*), wild goat grass (*Aegilops tauschii*) and wheat landraces showed substantial genetic variation for increased Zn in grain (Cakmak *et al.* 1999; Cakmak *et al.,* 2000; Monasterio and Graham, 2000; Cakmak *et al.,* 2004 a,b; Ortiz-Monasterio *et al.,* 2007; Peleg *et al.* 2008; ; Cakmak *et al.* 2010b). Among all the germplasm evaluated so far wild emmer wheat proved to have the highest Zn concentrations (14 to 190 mg Zn kg^{-1}) which is subsequently used by "Wheat wide crosses unit" of CIMMYT to develop the synthetic hexaploid wheat (*Triticum Turgidum* ssp. Dicoccon x *Aegilops tauschii*). Plant breeders at CIMMYT have transferred the genes

responsible for increased Zn from the reported high Zn sources like synthetics, diploid/tetraploid wild progenitors and landraces, to high yielding elite wheat backgrounds (Ortiz-Monasterio *et al.*, 2007)

With the several years of research efforts at CIMMYT, numerous high yielding, disease resistant Zn biofortified advance lines have been developed with significantly increased capacity of Zn accumulation in grains (HarvestPlus, 2010). These genotypes absorb greater quantities of Zn from soil and/or have capacity to remobilize greater quantities of Zn from shoots into grains (Distelfeld *et al.*, 2007). These CIMMYT-derived wheat lines were tested in target environments under HarvestPlus Yield Trial (HPYT) and resulted with a set of lines with 75-150% increased grain Zn compared to check cultivars. These candidate lines called "best bets" also claim to possess high yield potential, disease resistance, adoption and essential end use quality traits (Velu *et al.*, 2012; Velu and Singh, 2012; Velu and Singh, 2013).

With the collaboration of HarvestPlus and National Wheat breeding Program, Pakistani breeders developed and released the first high Zn wheat variety "Zincol-2016" by using traditional breeding techniques (http://www.harvestplus.org/node/1647). Zincol-2016 is nutritious, high yielding, well adapted to Pakistani environment and contains 37 mg kg^{-1} Zn in grain (+12 mg/kg) compared to the popular cultivated varieties in Pakistan (PARC, 2017; Baloch *et al.*, 2018). Similarly, in India several biofortified wheat varieties by using CIMMYT-derived lines were released which out yielded the conventionally growing varieties and at the same time had higher Zn concentration. "Zinc Shakti" is a new Indian Zn-biofortified variety with 40% higher Zn concentration released to the farmers in India (CIMMYT, 2017).

Although plant breeding and genetic biofortification are powerful approaches, they have some limitations which need special attentions (White and Broadley, 2011; Cakmak and Kutman, 2017). Definitely, the newly developed biofortified genotypes are highly dependent on plant-available soil Zn pool to absorb and accumulate more Zn in grains (Cakmak, 2008). It is already mentioned that most of the world's cereal cultivated soils including several South Asian countries are severely Zn deficient. Other factors like soil texture, high pH, low organic matter and reduced water supply also limit the Zn absorption by plant roots (Graham *et al.*, 1992; Marschner, 1993; Cakmak *et al.*, 1996; Alloway, 2009; Rengel, 2015). Under these adverse soil and climatic conditions with poor

bio-available Zn in rhizosphere, improved biofortified genotypes may not be able to express their full potential in terms of absorption of Zn from soil and accumulation in grain. To be able to achieve the targets of providing sufficient Zn to fulfil daily human Zn requirement, the biofortified lines should be able to extract and accumulate significant level of Zn in grain (up to 40-60 mg kg^{-1}) which seems impractical without Zn fertilizers (Cakmak, 2008; White and Broadley, 2011; Edward *et al.*, 2016; Cakmak and Kutman, 2017; Chen *et al.*, 2017). Hence, to support the long-term breeding efforts of genetic biofortification, soil and/or foliar Zn fertilizer applications are a complementary approach (Edward *et al.*, 2016).

Zinc efficiency (ZE) is an important agronomic character and is generally defined as the ratio of grain yield or straw dry-matter yield produced under Zn deficient conditions to that of under Zn fertilization (Cakmak *et al.*, 1996). Zinc efficient wheat genotypes are supposed to yield better on Zn-deficient soil (Singh *et al.*, 2005). However, grain Zn accumulation is influenced by several factors including genetic capacity of a genotype to extract Zn from soil, higher leaf absorption from foliar Zn fertilizers, translocation and remobilization at the time of grain filling (Waters *et al*. 2009; Kutman *et al*. 2012; Yilmaz *et al.*, 2017). Therefore, it is crucial to maintain a sufficient level of plant-available Zn in soil or instantly available large Zn pool in vegetative organs (foliar Zn) during seed-filling or both (Cakmak and Kutman, 2017).

Foliar Zn application is a proven tool to enhance grain Zn concentration. It is particularly useful when the soil conditions limit Zn availability for root absorption. Application of foliar Zn at the right plant development stage and rate can increase grain Zn concentration up to 83% in cereals like wheat (Zhou *et al.*, 2012; Phattarakul *et al.*, 2012; Cakmak and Kutman, 2017).

Flag leaves play an important role in synthesis and translocation of photo assimilates in the crop plants, therefore, directly affect the grain yield. Similarly, flag leaves are considered to be responsible for micronutrients (Fe and Zn) storage and remobilization to the grain at the time of grain filling. To investigate the contribution of flag leaf in mineral accumulation and remobilization to the grain, at early milk stage flag leaves were harvested and analyzed separately to know the elemental concentration and contents in flag leaf.

The objective of current study was to evaluate performance of the high-Zn Harvestplus-biofortified wheat genotypes (www.harvestplus.com) in comparison to a non-biofortified conventionally grown varieties as check under deficient and adequate soil Zn conditions. Ten genotypes developed by HarvestPlus Program in India and Pakistan were obtained along with two conventionally grown varieties. These biofortified lines were developed through long-term breeding activities under the HarvestPlus program in Pakistan and India, therefore, characterization of these genotypes for root uptake, shoot translocation, foliar absorption, re-mobilization and seed deposition of Zn can be helpful in adoption of these candidate lines as a cultivar.

There were three main questions addressed during these experiments

- How HP-biofortified genotypes differ from each other and from the check varieties in terms of soil Zn absorption and translocation capacity under deficient as well as adequate soil Zn conditions?
- How HP-biofortified genotypes differ from each other and from the check varieties in terms of Zn absorption and translocation capacity from foliar Zn sprays under deficient as well as adequate soil Zn conditions?
- Understand whether the higher grain Zn accumulation in the HP-biofortified genotypes is due to a higher root uptake from the growth medium or a greater root-to-shoot translocation capacity.

To answer the first two questions, the wheat genotypes were grown in green house with several soil and foliar Zn treatments (Experiment A) until maturity. To answer the third question, a time course depletion experiment (Experiment B) was conducted where genotypes were grown in nutrient solution medium.

3.2. Experiment A: Soil and/or foliar uptake and seed deposition of zinc in several HarvestPlus-Biofortified wheat genotypes grown in greenhouse conditions

3.2.1. Materials and Methods

3.2.1.1. Seed source

Seeds of 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally grown wheat cultivars (*Triticum aestivum* L.) were kindly provided by Dr. Hari Ram and Dr. Abdul Rashid from HarvestPlus Program in India and Pakistan respectively. Five genotypes obtained from Pakistan (NR-421, NR-435, NR-457, NR-488, NR-489) and five from India (HPBW-01, HPBW-02, HPPAU-05, HPPAU-07, HPPAU-10) are assumed to accumulate more Zn in the grain and agronomically superior than the traditional cultivars grown in India and Pakistan. The popular varieties from Pakistan (Faisalabad-2008) and India (HD-2967) were used as the experimental checks (Table 3.1).

Table 3.1: List of the biofortified genotypes obtained from HarvestPlus Biofortification Program

Pakistan	India
Faisalabad 2008*	HD-2967*
NR-421	HPBW-01
NR-435	HPBW-02
NR-457	HPPAU-05
NR-488	HPPAU-07
NR-489	HPPAU-10

**Locally cultivated popular cultivars used as experimental checks.*

3.2.1.2. Growth Conditions

First part of the experiment was carried out to investigate the differences among the HP-biofortified lines in term of their capacity of Zn uptake from soil supplied with low or adequate Zn. The experiment had a factorial design with four independent (pot) replicates with total of 96 pots. The soil was prepared as described in "General Materials and Methods". Initial N rate was 250 mg N kg^{-1} soil, additional N was supplied at tillering, booting and early milk stages each at a rate of 50 mg N kg^{-1} soil as $Ca(NO_3)_2.4H_2O$. Low and adequate Zn levels were established by adding 0.5 mg kg^{-1} Zn (low Zn supply) and 5 $mg.kg^{-1}$ Zn (adequate Zn supply) to the soil respectively in the form of $ZnSO_4.7H_2O$. Initially 14 seeds were sown in each pot and the resulting seedlings were thinned to 10 per pot at the three-leaf stage. Plants were grown in a computer-controlled greenhouse (details described in General Materials and Methods"). Pots were irrigated with DI-water every day and randomized every two weeks for uniform exposure to possible climatic variations.

3.2.1.3. Foliar Fertilizer application

In the second part of the experiment, Zn uptake capacity of HP-biofortified lines from foliar sprays was tested in second set of plants grown in identical condition (both set of plants were grown simultaneously as described above). At booting (*i.e.* before anthesis) and early milk stages (*i.e.* after anthesis) plants were sprayed with a solution of 0.4% $ZnSO_4.7H_2O$ and 0.02% Tween-20. The soil surface was covered with paper towel before foliar sprays to avoid soil contamination.

3.2.1.4. Harvesting and analysis

Plants from the first set of the experiment were sampled for shoot mineral analysis at the early booting and early milk stages (three plants from each pot were harvested at each stage). The same number of plants were also harvested from second duplicate set in order to keep the uniform plant density in all pots. At the early milk stage shoot sampling,

flag leaves from each plant were sampled separately in order to investigate the elemental concentration in flag leaves contributing to grain yield and grain mineral accumulation. Shoots and flag leaves sampled separately were washed with DI water, dried, weighed and analyzed for mineral concentrations as described in "General Materials and Methods". At full maturity, spikes and straw were harvested separately. Straw samples were weighed to determine straw biomass. Spikes were threshed with a laboratory thresher; grains were washed with DI-water and oven-dried before mineral analysis. Zinc concentration and contents in grains were measured and calculated as described in "General Materials and Methods" section.

3.2.2. Results

3.2.2.1 Biomass, Zn concentration and contents at booting stage

Mean values of biomass production shoot Zn concentration and contents at early booting stage of 12 genotypes grown at low and adequate soil Zn is presented in Table 3.2. Analysis of variance showed that varied soil Zn treatment (i.e. low or adequate) had no significant effect on shoot biomass production of wheat genotypes at the booting stage. However, there was a significant ($P<0.001$) genotypic variation in shoot biomass of the genotypes used in this study.

At adequate soil Zn, HPPAU-05 produced the maximum (1.25 g plant^{-1}) and the Indian Check cultivar (HD-2967) produced minimum (0.86 g plant^{-1}) shoot biomass, whereas at low Zn, NR-421 produced the lowest shoot biomass (0.92 g plant^{-1}) (Table 3.2). The ANOVA indicated that soil Zn and genotype interaction was statistically insignificant (Table 3.2).

Table 3.2: Shoot biomass, Zn concentration and content at booting stage in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil).

Genotypes	Shoot Biomass (g $plant^{-1}$)		Zn concentration (mg kg^{-1})		Zn content (µg $plant^{-1}$)	
	Low Zn	Adequate Zn	Low Zn	Adequate Zn	Low Zn	Adequate Zn
Faisalabad 2008	**1.04 abc**	**1.09 abc**	**13.8 c**	**38.4 b**	**14.4 c**	**42.1 ab**
NR-421	0.92 c	0.99 abc	17.2 c	49.2 a	16.0 c	48.8 ab
NR-435	0.97 abc	1.11 abc	14.6 c	44.7 ab	14.1 c	49.6 ab
NR-457	0.95 bc	0.98 abc	15.2 c	48.8 a	14.4 c	47.8 ab
NR-488	0.95 bc	0.99 abc	14.4 c	47.1 ab	13.7 c	46.5 ab
NR-489	0.94 c	1.01 abc	14.3 c	40.3 ab	13.4 c	40.6 ab
HD-2967	**0.95 bc**	**0.86 c**	**13.3 c**	**42.5 ab**	**12.7 c**	**36.4 b**
HPBW-01	0.99 abc	1.11 abc	14.4 c	49.0 a	14.2 c	53.3 a
HPBW-02	1.01 abc	1.06 abc	13.7 c	49.5 a	13.8 c	52.6 a
HPPAU-05	1.23 abc	1.25 a	12.1 c	42.5 ab	14.9 c	53.1 a
HPPAU-07	1.12 abc	0.95 bc	13.3 c	43.2 ab	14.8 c	40.8 ab
HPPAU-10	1.03 abc	1.00 abc	13.4 c	46.8 ab	13.8 c	46.6 ab
Mean	**1.01 A**	**1.03 A**	**14.1 B**	**45.2 A**	**14.2 B**	**46.5 A**

*Shoot biomass HSD 0.05 (Soil Zn, Genotype, Soil Zn X Genotype) = NS, 0.19***, N.S*

*Zn concentration HSD 0.05 (Soil Zn, Genotype, Soil Zn X Genotype) = 1.49***, 6.21***, N.S*

*Zn content HSD 0.05 (Soil Zn, Genotype, Soil Zn X Genotype) = 2.09***, 8.67***, 13.7**

As expected, there was a significant *($p<0.001$)* effect of soil Zn fertilization on shoot Zn concentration of all the genotypes (Table 3.2). The mean shoot Zn concentration of wheat genotypes at low Zn supply was 14.1 mg kg^{-1} with maximum concentration in NR-421 (17.2 mg kg^{-1}) and minimum in HPPAU-05 (12.1 mg kg^{-1}). The mean Zn concentration at adequate soil Zn was 45.2 mg kg^{-1} with highest Zn concentration in HPBW-02 (49.5 mg kg^{-1}) and lowest in the check variety, Faisalabad 2008 (38.4 mg kg^{-1}). Analysis of variance showed a significant *($p<0.001$*) genotypic variation for shoot Zn concentration at booting stage. Almost all biofortified genotypes when grown at adequate soil Zn, exhibited more shoot Zn concentration as compared to the check varieties Faisalabad-2008 (38.4 mg kg^{-1}) and HD-2967 (42.5 mg kg^{-1}) indicating an enhanced capacity of root uptake and/or shoot translocation of Zn. Among the biofortified lines, maximum shoot Zn concentration was recorded in HPBW-02 (49.5 mg kg^{-1}) and

minimum in NR-489 (40.3 mg kg^{-1}) under adequate Zn supply. Soil Zn and genotype interaction on shoot Zn concentration was not significant (Table 3.2).

At the booting stage, mean shoot Zn content at low Zn was 14.2 µg $plant^{-1}$ and increased significantly (*p<0.001*) to 46.5 µg $plant^{-1}$ with adequate Zn application. At low soil Zn, the Indian check cultivar (HD-2967) produced lowest (12.7 µg $plant^{-1}$) Zn contents and Pakistani genotype (NR-421) produced highest (16.0 µg $plant^{-1}$). At adequate soil Zn maximum shoot Zn contents were found in genotype HPBW-01 (53.3 µg $plant^{-1}$) and minimum in Indian check cultivar HD-2967 (36.4 µg $plant^{-1}$). There was a significant genotypic variation (*p<0.001*) observed among the 12 genotypes tested. Contrary to shoot biomass and Zn concentration, soil Zn supply significantly interacted with shoot Zn content of genotypes (*p<0.05*) (Table 3.2).

3.2.2.2. Biomass, Zn concentration and contents at early milk stage

Table 3.3 presents the mean values of the dry matter produced in whole plant shoot (except flag leaf) g $plant^{-1}$ and the flag leaves separately harvested at the early milk stage. At early milk stage, ANOVA indicated that soil Zn had a statistically significant (*p<0.01*) effect on whole plant biomass production. The mean value of all the genotypes grown at low soil Zn was recorded as 3.43 mg kg^{-1} whereas the mean for the genotypes grown at adequate soil Zn was 3.67 mg kg^{-1}. Soil Zn had no significant effect on dry weight of individual flag leaves. No significant genotypic variation or soil Zn x genotype interaction was observed for dry matter produced by whole plant; however, genotypes differed significantly in terms of flag leaf biomass. Maximum flag leaf dry matter (0.19 mg $plant^{-1}$) was produced by HPBW-01 and minimum (0.10 mg $plant^{-1}$) by HPPAU-07 (Table 3.3).

Soil Zn application had a significant effect *(p<0.001)* on shoot Zn concentration of all the genotypes (Table 3.4). The mean shoot Zn concentration grown at low soil Zn (8.03 mg kg^{-1}) was significantly increased adequate soil Zn (31.7 mg kg^{-1}). Flag leaf Zn concentration was also significantly affected by soil Zn with mean value (13.3 mg kg^{-1}) under low Zn and (32.5 mg kg^{-1}) at adequate soil Zn conditions (Table 2.4). There was a significant genotypic variation *(p<0.001)* for Zn concentration in plant shoot (without flag leaf). At low soil Zn supply, minimum Zn concentration was found in HPPAU-07

(6.29 mg kg^{-1}) while maximum in NR-488 (9.14 mg kg^{-1}). Under adequate soil Zn supply, lowest Zn concentration was measured in Faisalabad-2008 (25.7 mg kg^{-1}) while highest in NR-488 (36.2 mg kg^{-1}). However, for flag leaf Zn concentration, genotypic variation was not statistically significant (Table 3.4). Analysis of variance (ANOVA) revealed a significant interaction between soil Zn and genotypes for Zn concentration of whole plant shoot ($p<0.05$) as well as for flag leaf ($p<0.01$) (Table 3.4).

As expected, soil Zn had a significant effect *(P<0.001)* on Zn contents in plant shoot (without flag leaf), in flag leaf and hence, overall Zn contents of plants (Table 3.5). The mean value of Zn contents of shoot (without flag leaf) was 27.5 µg $plant^{-1}$ at low soil Zn and 116 µg $plant^{-1}$ at adequate soil Zn supply. Similarly, flag leaf Zn contents increased from 1.89 µg $plant^{-1}$ at low Zn to 4.43 µg $plant^{-1}$ at adequate Zn supply. Overall Zn contents were also increased significantly ($p<0.001$) with the mean value of 29.4 µg $plant^{-1}$ at low Zn and 121 µg $plant^{-1}$ at adequate Zn supply. At low Zn supply in soil, minimum total shoot Zn contents were found in HPPAU-07 (24.1 µg $plant^{-1}$) and maximum in HPPAU-10 (33.0 µg $plant^{-1}$). At adequate soil Zn, all biofortified genotypes produced more Zn contents compared to check variety Faisalabad-2008. Faisalabad-2008 produced lowest (87.6 µg $plant^{-1}$) Zn contents whereas, HPBW-01 produced highest (140 µg $plant^{-1}$). Analysis of variance showed that genotypic variation for Zn content was also significant *(P<0.01)* for plant shoot, flag leaf and total plant contents. However, interaction between soil Zn and genotypes had a significant *(P<0.05)* effect on Zn contents in plant shoot and whole plant but not in flag leaf (Table 3.5).

Table 3.3: Shoot biomass plant shoot (without flag leaf), in flag leaf and total shoot biomass at early milk stage in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil).

	Shoot Biomass (g $plant^{-1}$)					
Genotypes	**Shoot (without flag leaf)**		**Flag leaf**		**Total**	
	Low Zn	**Adequate Zn**	**Low Zn**	**Adequate Zn**	**Low Zn**	**Adequate Zn**
Faisalabad 2008	**3.50 a**	**3.19 a**	**0.14 ab**	**0.11 b**	**3.65 a**	**3.30 a**
NR-421	3.25 a	3.54 a	0.13 ab	0.16 ab	3.38 a	3.69 a
NR-435	3.32 a	3.54 a	0.14 ab	0.11 b	3.45 a	3.65 a
NR-457	3.40 a	3.60 a	0.14 ab	0.13 ab	3.54 a	3.73 a
NR-488	3.10 a	3.51 a	0.12 ab	0.14 ab	3.22 a	3.65 a
NR-489	3.26 a	3.29 a	0.19 a	0.15 ab	3.44 a	3.44 a
HD-2967	**3.23 a**	**3.37 a**	**0.11 ab**	**0.13 ab**	**3.34 a**	**3.50 a**
HPBW-01	3.09 a	3.78 a	0.19 a	0.17 ab	3.28 a	3.95 a
HPBW-02	3.08 a	3.48 a	0.13 ab	0.16 ab	3.21 a	3.64 a
HPPAU-05	3.57 a	3.96 a	0.11 b	0.12 ab	3.67 a	4.08 a
HPPAU-07	3.40 a	3.60 a	0.15 ab	0.10 b	3.56 a	3.71 a
HPPAU-10	3.27 a	3.58 a	0.15 ab	0.16 ab	3.43 a	3.74 a
Mean	**3.29 B**	**3.54 A**	**0.14 A**	**0.14 A**	**3.43 B**	**3.67 A**

*Shoot (without flag leaf) biomass HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 0.16**, N.S, N.S*

*Flag leaf dry matter HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = N.S, 0.05**, N.S*

*Total shoot biomass HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 0.08***, N.S, N.S*

Table 3.4 Shoot Zn concentration (without flag leaf) and in flag leaf separately at early milk stage in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil).

Zn Concentration (mg kg^{-1})				
Genotypes	**Shoot without flag leaf**		**Flag leaf**	
	Low Zn	**Adequate Zn**	**Low Zn**	**Adequate Zn**
Faisalabad 2008	**7.12 d**	**25.7 c**	**13.3 c**	**31.2 ab**
NR-421	9.09 d	31.2 abc	16.2 c	33.0 ab
NR-435	7.58 d	29.0 bc	12.4 c	31.3 ab
NR-457	7.89 d	29.4 abc	14.1 c	30.3 b
NR-488	9.14 d	36.2 a	13.7 c	32.9 ab
NR-489	7.85 d	33.7 ab	15.1 c	30.8 ab
HD-2967	**8.27 d**	**31.8 abc**	**14.1 c**	**36.4 a**
HPBW-01	8.05 d	33.9 ab	12.1 c	32.7 ab
HPBW-02	8.18 d	35.0 ab	12.7 c	31.9 ab
HPPAU-05	7.79 d	32.1 ab	11.8 c	31.2 ab
HPPAU-07	6.29 d	28.5 bc	12.2 c	35.8 ab
HPPAU-10	9.12 d	33.4 ab	12.5 c	33.0 ab
Mean	**8.03 B**	**31.7 A**	**13.3 B**	**32.5 A**

*Shoot (without flag leaf) Zn Concentration HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 1.01***, 4.21***, 6.66**

*Flag leaf Zn Concentration HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 0.89***, N.S, 5.84***

Table 3.5: Zinc contents in shoot (without flag leaf), flag leaf and total at early milk stage in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil).

Zn Contents (µg $plant^{-1}$)						
Genotypes	**Shoot without flag leaf**		**Flag leaf**		**Total**	
	Low Zn	**Adequate Zn**	**Low Zn**	**Adequate Zn**	**Low Zn**	**Adequate Zn**
Faisalabad 2008	**26.0 c**	**84 b**	**1.93 d-g**	**3.39 b-f**	**27.9 c**	**87.6 b**
NR-421	30.8 c	115 ab	2.13 d-g	5.15 ab	32.9 c	120 ab
NR-435	26.1 c	106 ab	1.69 fg	3.40 b-f	27.8 c	109 ab
NR-457	28.0 c	110 ab	1.99 d-g	3.92 a-d	30.0 c	114 ab
NR-488	29.5 c	132 a	1.70 fg	4.56 a-c	31.2 c	137 a
NR-489	27.0 c	113 ab	2.80 c-g	4.72 a-c	29.8 c	118 ab
HD-2967	**27.5 c**	**111 ab**	**1.53 fg**	**4.82 ab**	**29.1 c**	**116 ab**
HPBW-01	26.6 c	135 a	2.26 d-g	5.39 a	28.8 c	140 a
HPBW-02	26.5 c	128 a	1.65 fg	5.07 ab	28.1 c	133 a
HPPAU-05	28.4 c	132 a	1.25 g	3.84 a-d	29.6 c	136 a
HPPAU-07	22.3 c	106 ab	1.84 f-g	3.71 a-e	24.1 c	109 ab
HPPAU-10	31.1 c	125 a	1.92 d-g	5.22 ab	33.0 c	130 a
Mean	**27.5 B**	**116 A**	**1.89 B**	**4.43 A**	**29.4 B**	**121 A**

*Plant (without flag leaf) Zn contents HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 5.50***, 22.8**, 36.1**

*Flag leaf Zn contents HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 0.30***, 1.26**, N. S*

*Total Zn contents HSD 0.05 (Soil Zn, Genotypes, Soil Zn X Genotypes) = 5.67***, 6.98**, 9.86**

3.2.3.3. Yield, Zn concentration, and other parameters at physiological maturity

Grain Yield

Soil and foliar Zn fertilization had significant positive effect on grain yield plant^{-1} (Table 3.6). The mean values of all genotypes grown at low soil Zn without foliar spray was 2.6 g plant^{-1} which increased significantly to 2.96 g plant^{-1} with foliar spray. At adequate soil Zn, mean value without foliar was recorded as 3.07 g plant^{-1} and with foliar as Zn 3.17 g plant^{-1}. Grain yield (g plant^{-1}) was increased in all biofortified genotypes with adequate soil Zn application (the effect was statistically non-significant) with the exception of one Pakistani genotype (NR-457). Soil Zn application had no effect on grain yield of check cultivar Faisalabad-2008 (Table 3.6). Foliar Zn application had a significant effect ($p<0.01$) in increasing yield plant^{-1} average of all genotypes including check cultivars. Analysis of variance indicates a significant ($p<0.05$) genotypic variation in grain yield plant^{-1} for soil Zn application (Table 3.10). Highest grain yield (3.69 g plant^{-1}) was observed in HPPAU-07 (Soil + Foliar Zn application) whereas lowest yield (2.18 g plant^{-1}) was recorded in NR-488 grown at low soil Zn with no foliar treatment (Table 3.6). Interaction of soil Zn with other sources of variation had no significant effect on grain yield (Table 3.10).

Grain Zn Concentration

As expected, soil Zn application, foliar Zn application and soil + foliar Zn application had a significant ($p<0.001$) positive effect on grain Zn concentrations (Table 3.10). The mean value of grain Zn concentration of all the genotypes grown at low soil Zn was 15.7 mg kg^{-1} which significantly ($p<0.001$) increased to 46.8 mg kg^{-1} (198%) at adequate soil Zn, to 51.5 mg kg^{-1} (228%) with foliar Zn and to 70.4 mg kg^{-1} (348%) with soil + foliar Zn application (Table 3.6).

There was a significant ($p<0.001$) genotypic variation for grain Zn concentration with soil or foliar or both (soil + foliar) Zn application (Table 3.10). With low Zn supply in soil, without foliar application, the Indian check cultivar HD-2967 was found with the highest grain Zn concentration (19.2 mg kg^{-1}), HPPAU-07 with lowest grain Zn (12.0 mg kg^{-1}) whereas the Pakistani check variety Faisalabad-2008 had 14.5 mg kg^{-1}. At adequate soil Zn, maximum grain Zn concentration was observed in HPBW-01 (57.2 mg kg^{-1}) and minimum in NR-457 (39.2 mg kg^{-1}). With foliar Zn application (0.04% $ZnSO_4.7H_2O$) at booting and early milk stage, there was a significant increase in grain Zn concentration

of all the genotypes including the check cultivars (Table 3.6). Generally, all the biofortified genotypes responded to foliar Zn and soil + foliar Zn application better than check varieties. Pakistani check (Faisalabad-2008) had lowest grain Zn concentration (57.2 mg kg^{-1}) among all genotypes with soil + foliar Zn application. HPBW-01 showed maximum grain Zn concentration i.e., 70.7 mg kg^{-1} with foliar Zn application and 93.7 mg kg^{-1} with soil + foliar Zn application (Table 3.6). The interaction between soil Zn and genotypes had significant effect ($p<0.05$) on grain Zn concentration. The interaction between foliar Zn application genotypes also had significant ($p<0.001$) effect on grain Zn concentration (Table 3.10).

Grain Zn Contents

Grain Zn contents of all the genotypes tested were significantly ($p<0.001$) affected by soil, foliar and soil + foliar Zn application (Table 3.10). At low soil Zn with no foliar application, the mean value for Zn contents 40.5 µg $plant^{-1}$ with minimum in HPPAU-07 (28.7 µg $plant^{-1}$) and maximum in NR-457 (50.8 µg $plant^{-1}$). Pakistani check cultivar (Faisalabad-2008) gave higher Zn contents than three Pakistani biofortified genotypes, while Indian check cultivar (HD-2967) was better than all Indian biofortified genotypes when grown on low Zn soil skipping the foliar application (Table 3.7). However, with adequate soil Zn application, all the Pakistani HP-biofortified genotypes except NR-457 were able to absorb more Zn from soil and showed the higher grain Zn contents than the check variety (Faisalabad-2008). Similarly, all Indian HP-biofortified genotypes showed higher Zn contents than the check cultivar (HD-2967) except HPPAU-07. The mean value for grain Zn contents grown at adequate soil Zn without foliar was 142 µg $plant^{-1}$ with lowest in NR-457 (115 µg $plant^{-1}$) and highest in HPBW-01 (177 µg $plant^{-1}$). Generally, there was 3.5 folds increase in grain Zn contents with the application of soil Zn compared to the low Zn application (Table 3.7).

Foliar Zn application on low soil Zn plants showed a significant increase (3.7 folds) in grain Zn contents with an average of 150 µg $plant^{-1}$. Maximum contents were found in HPBW-02 (208 µg $plant^{-1}$) and minimum in the check cultivar HD-2967 (109 µg $plant^{-1}$). With soil + foliar Zn application, grain Zn contents increased up to 5.4 folds compared to control with a mean value of 220 µg $plant^{-1}$. Genotype HPBW-02 showed the maximum grain Zn contents (291 µg $plant^{-1}$) whereas NR-489 produced minimum (189 µg $plant^{-1}$). Check variety Faisalabad-2008 had the lowest Zn contents (185 µg $plant^{-}$

^{1}) among all genotypes (Table 3.7) and the India check cultivar had lowest Zn contents (205 µg plant^{-1}) among all the Indian HP-biofortified genotypes.

Harvest plus biofortified genotype "HPBW-01" performed best in terms of Zn contents when grown on adequate soil Zn, whereas, HPBW-02 showed highest Zn contents with foliar application or soil + foliar application. At low soil Zn conditions, without foliar application, NR-457 showed highest Zn contents (Table 3.7).

Interaction of soil and foliar Zn application had a significant ($p<0.001$) effect on grain Zn contents, however, interaction of soil Zn with other variables had non-significant effect (Table 3.10). Analysis of variance revealed the significant genotypic variation ($p<0.001$) for grain Zn contents. Moreover, interaction of genotypes with foliar Zn also had a significant ($p<0.001$) affect (Table 3.10).

Other yield components

Straw yield of the genotypes was significantly affected by soil Zn and foliar Zn application ($p<0.001$). Mean value for the straw yield of all genotypes grown at low Zn soil without foliar application was 5.3 g plant^{-1} which increased significantly to 5.82 g plant^{-1} with foliar Zn. At adequate soil Zn mean value was observed 5.98 g plant^{-1} which increased to 6.29 g plant^{-1}, however, this affect was not statistically significant (Table 3.7). Maximum straw yield (7.02 g plant^{-1}) was produced by adequate soil Zn genotype HPPAU-07 whereas minimum (4.29 g plant^{-1}) was observed in low soil Zn NR-489. A significant genotypic variation was also revealed by the ANOVA, however, interaction of any of the variables had no significant effect on straw yield (Table 3.10).

Soil Zn and soil + foliar Zn application had a significant positive effect on mean values of number of spikes per plant (Table 3.8). Mean at low Zn soil without foliar application was recorded 2.01 spikes plant $^{-1}$ which increased significantly to 2.26 spikes plant $^{-1}$ at adequate soil Zn. With soil + foliar application, the mean increased significantly to 2.41 spikes plant $^{-1}$ (Table 3.8). Analysis of variance indicated significant ($p<0.001$) genotypic variation as well (Table 3.10). Soil Zn, foliar Zn and genotypes interacted significantly ($p<0.01$) to increase the number of spikes plant^{-1} (Table 3.10).

Soil Zn application reduced the thousand grain weight (TGW) significantly ($p<0.05$) (Table 3.10), however, no significant effect was observed on mean values

(Table 3.8). Foliar Zn treatment also had no significant effect. Analysis of variance indicates that there was a significant ($p<0.001$) genotypic variation for TGW but, no significant interaction among variables were observed (Table 3.8, Table 3.10)

Grain yield spike^{-1} and number of grain spike^{-1} were increased with soil, foliar and soil + foliar Zn application (Table 3.9), however, this effect was statistically insignificant. A significant ($p<0.001$) genotypic variation was observed for both of these traits. None of the variable interaction showed significant effect on grain yield spike^{-1} or number of grain spike^{-1} (Table 3.10).

Table 3.6: Grain yield and grain Zn concentration in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil) with and without foliar spray (0.4% $ZnSO_4.7H_2O$ + 0.02% Tween-20) at booting and early milk stage

Genotypes	Grain Yield (g $plant^{-1}$)				Zn Concentration (mg kg^{-1})			
	Low Zn		Adequate Zn		Low Zn		Adequate Zn	
	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar
Faisalabad 2008	**2.85 ab**	**3.33 ab**	**2.85 ab**	**3.25 ab**	**14.5 k**	**44.0 f-j**	**45.4 f-j**	**57.2 c-j**
NR-421	2.85 ab	3.09 ab	3.22 ab	3.53 ab	15.9 k	45.5 f-j	44.3 f-j	58.2 c-j
NR-435	2.61 ab	3.18 ab	3.32 ab	3.26 ab	14.4 k	45.7 f-j	43.5 g-j	62.8 c-f
NR-457	3.22 ab	3.33 ab	2.95 ab	3.10 ab	15.8 k	44.2 f-j	39.2 j	60.7 c-h
NR-488	2.18 b	2.53 ab	2.84 ab	2.75 ab	17.4 k	66.4 b-e	55.2 c-j	83.2 ab
NR-489	2.54 ab	2.57 ab	3.22 ab	2.87 ab	15.2 k	53.5 c-j	43.7 f-j	65.9 b-e
HD-2967	**2.47 ab**	**2.54 ab**	**3.12 ab**	**3.15 ab**	**19.2 k**	**42.8 h-j**	**42.8 h-j**	**65.4 b-e**
HPBW-01	2.45 ab	2.52 ab	3.18 ab	2.74 ab	16.9 k	70.7 b-d	57.2 C-j	93.7 a
HPBW-02	2.63 ab	3.39 ab	2.77 ab	3.53 ab	15.6 k	62.4 c-g	51.6 d-j	82.5 ab
HPPAU-05	2.49 ab	2.94 ab	2.93 ab	2.99 ab	13.3 k	48.5 e-j	48.5 e-j	83.1 ab
HPPAU-07	2.42 ab	3.27 ab	3.20 ab	3.69 a	12.0 k	44.1 f-j	40.7 ij	59.2 c-i
HPPAU-10	2.54 ab	2.81 ab	3.26 ab	3.20 ab	18.1 k	50.6 e-j	49.0 e-j	72.0 bc
Mean	**2.60 B**	**2.96 A**	**3.07 A**	**3.17 A**	**15.7 D**	**51.5 B**	**46.8 C**	**70.4 A**

Table 3.7: Straw yield and grain Zn contents in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil) with and without foliar spray (0.4% $ZnSO_4$.7H2O + 0.02% Tween-20) at booting and early milk stage.

Genotypes	Straw Yield (g $plant^{-1}$)				Zn Contents (µg $seed^{-1}$)			
	Low Zn		Adequate Zn		Low Zn		Adequate Zn	
	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar
Faisalabad 2008	**5.51 a-d**	**6.38 a-c**	**6.00 a-d**	**6.15 a-d**	**41.2 i-k**	**146 e-h**	**128 gh**	**185 b-g**
NR-421	5.61 a-d	6.30 a-c	6.19 a-d	6.68 ab	45.1 i-k	140 f-h	142 f-h	205 b-f
NR-435	5.31 a-d	6.21 a-c	6.22 a-d	6.60 a-c	36.4 jk	145 e-h	142 f-h	205 b-f
NR-457	6.22 a-d	6.25 a-c	5.82 a-d	6.03 a-d	50.8 i-k	145 e-h	115 g-i	189 b-g
NR-488	4.69 cd	5.32 a-d	5.75 a-d	6.29 a-c	37.9 jk	164 d-h	157 d-h	229 a-d
NR-489	5.03 b-d	4.29 d	5.91 a-d	5.78 a-d	38.7 jk	139 f-h	141 f-h	189 b-g
HD-2967	**5.56 a-d**	**5.32 a-d**	**5.87 a-d**	**6.56 a-c**	**47.3 i-k**	**109 h-j**	**134 f-h**	**205 b-f**
HPBW-01	5.03 b-d	5.16 a-d	6.47 a-c	5.72 a-d	40.8 i-k	176 c-h	177 c-h	257 ab
HPBW-02	5.17 a-d	6.13 a-d	5.19 a-d	6.63 ab	40.9 i-k	208 b-f	143 f-h	291 a
HPPAU-05	5.18 a-d	5.87 a-d	5.66 a-d	5.77 a-d	33.3 k	140 f-h	141 f-h	245 a-c
HPPAU-07	5.06 b-d	6.45 a-c	6.58 a-c	7.02 a	28.7 k	142 f-h	129 gh	218 a-e
HPPAU-10	5.28 a-d	6.09 a-d	6.06 a-d	6.29 a-c	45.1 i-k	141 f-h	159 d-h	223 a-d
Mean	**5.30 C**	**5.82 B**	**5.98 AB**	**6.29 A**	**40.5 C**	**150 B**	**142 B**	**220 A**

Table 3.8: Number of spikes per plant and TGW grain weight in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil) with and without foliar spray (0.4% ZnSO4.7H2O + 0.02% Tween-20) at booting and early milk stage.

Genotypes	No. of spikes $plant^{-1}$				TGW (g)			
	Low Zn		Adequate Zn		Low Zn		Adequate Zn	
	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar
Faisalabad 2008	**2.06 b-d**	**2.31 b-d**	**2.44 bc**	**2.06 b-d**	**43.5 a-i**	**43.6 a-i**	**44.9 a-i**	**43.8 a-i**
NR-421	2.06 b-d	2.19 b-d	2.56 b	2.50 bc	42.7 a-j	41.7 a-k	39.1 g-k	40.3 d-k
NR-435	1.94 b-d	2.25 b-d	2.50 bc	2.56 b	45.0 a-i	45.3 a-i	43.8 a-i	43.3 a-i
NR-457	2.31 b-d	2.44 bc	2.25 bcd	2.00 b-d	44.2 a-i	42.9 a-j	41.4 c-k	42.0 b-k
NR-488	1.31 d	1.50 cd	1.81 bcd	1.63 b-d	46.5 a-e	43.9 a-i	43.2 a-i	46.3 a-f
NR-489	2.13 b-d	2.01 b-d	1.94 bcd	2.31 b-d	41.9 a-k	43.1 a-i	42.1 b-k	40.4 e-k
HD-2967	**2.13 b-d**	**2.25 b-d**	**2.19 bcd**	**3.69 a**	**43.5 a-i**	**42.3 b-k**	**40.8 e-j**	**42.0 b-k**
HPBW-01	1.81 b-d	1.69 b-d	1.81 bcd	2.06 b-d	45.5 a-i	46.9 a-d	44.5 a-i	43.9 a-i
HPBW-02	1.94 b-d	1.94 b-d	1.69 bcd	2.25 b-d	47.4 a-c	46.4 a-e	45.0 a-i	44.7 a-i
HPPAU-05	1.94 b-d	2.19 b-d	2.25 bcd	2.44 bc	44.2 a-i	48.3 ab	48.4 ab	49.1 a
HPPAU-07	2.00 b-d	2.31 b-d	2.67 ab	2.50 bc	38.7 h-k	35.8 k	36.5 jk	38.4 i-k
HPPAU-10	2.13 b-d	2.56 bc	2.69 ab	2.56 b	38.5 h-k	40.0 e-k	40.2 d-k	39.6 f-k
Mean	**2.01 C**	**2.15 BC**	**2.26 AB**	**2.41 A**	**43.5 A**	**43.3 A**	**42.5 A**	**42.8 A**

Table 3.9: Grain yield spike^{-1} and no. of grains spike^{-1} in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil) with and without foliar spray (0.4% $ZnSO_4.7H_2O$ + 0.02% Tween-20) at booting and early milk stage

Genotypes	Grain yield (g spike^{-1})				No. of grains spike^{-1}			
	Low Zn		Adequate Zn		Low Zn		Adequate Zn	
	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar	- Foliar	+ Foliar
Faisalabad 2008	**1.38 ab**	**1.45 ab**	**1.20 ab**	**1.58 ab**	**31.8 ab**	**33.5 ab**	**26.7 ab**	**36.1 ab**
NR-421	1.40 ab	1.42 ab	1.26 ab	1.43 ab	32.8 ab	34.1 ab	32.3 ab	35.7 ab
NR-435	1.32 ab	1.42 ab	1.32 ab	1.27 ab	29.3 ab	31.3 ab	30.3 ab	29.6 ab
NR-457	1.41 ab	1.37 ab	1.31 ab	1.56 ab	31.8 ab	31.9 ab	31.7 ab	37.2 ab
NR-488	1.70 ab	1.73 a	1.59 a	1.79 a	36.4 ab	39.3 a	36.9 ab	38.1 a
NR-489	1.21 ab	1.27 ab	1.68 a	1.26 ab	28.9 ab	29.5 ab	39.8 a	31.2 ab
HD-2967	**1.16 ab**	**1.12 ab**	**1.44 ab**	**0.93 b**	**26.8 ab**	**26.8 ab**	**35.5 ab**	**22.3 b**
HPBW-01	1.34 ab	1.48 ab	1.74 a	1.41 ab	29.7 ab	31.7 ab	39.4 a	32.4 ab
HPBW-02	1.38 ab	1.74 a	1.68 a	1.57 ab	29.3 ab	37.6 ab	37.5 ab	35.2 ab
HPPAU-05	1.29 ab	1.37 ab	1.30 ab	1.22 ab	29.2 ab	28.4 ab	27.2 ab	25.1 ab
HPPAU-07	1.24 ab	1.41 ab	1.22 ab	1.49 ab	32.1 ab	39.3 a	33.7 ab	38.7 a
HPPAU-10	1.20 ab	1.19 ab	1.23 ab	1.25 ab	31.2 ab	29.9 ab	30.9 ab	32.4 ab
Mean	**1.34 A**	**1.41 A**	**1.41 A**	**1.40 A**	**30.8 A**	**32.8 A**	**33.5 A**	**32.8 A**

Table 3.10: Analysis of variance (ANOVA) of the effects of genotypes, foliar and soil applications of Zn on the grain yield, grain Zn concentration, straw dry weight, grain Zn contents, no. of spikes per plant, TGW, grain yield per spike and number of grains per spike in 10 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and two conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008 and HD-2967) grown with low (0.5 mg kg^{-1}) or adequate (5 mg kg^{-1}) Zn supply in Zn-deficient soil (DTPA-Zn: 0.13 mg kg^{-1} soil) with and without foliar spray (0.4% $ZnSO_4.7H_2O$) at booting and early milk stage.

Source of Variation	DF	Grain yield		Grain Zn Conc.		Straw Yield		Grain Zn Content	
		MS	F.Pr	MS	F.Pr	MS	F.Pr	MS	F.Pr
Soil Zn (A)	1	5.56241	<0.001	29750.5	<0.001	15.76	<0.001	357075	<0.001
Foliar Zn (B)	1	2.47067	0.0015	42364.1	<0.001	8.24	<0.001	419497	<0.001
Genotypes (C)	11	0.61287	0.0048	741.6	<0.001	1.52	<0.001	3722	<0.001
A X B	1	0.78541	0.0701	1800.8	<0.001	0.48	0.3178	11781	<0.001
A X C	11	0.29163	0.2684	92.4	0.0349	0.82	0.0715	1049	0.1501
B X C	11	0.33075	0.1779	243.3	<0.001	0.86	0.0569	2324	<0.001
A X B X C	11	0.0569	0.994	42	0.5446	0.39	0.6262	436	0.8188
Exp. Error	144	0.23591		46.8		0.47		715	

Source of Variation	DF	No. of spikes $plant^{-1}$		1000 grain wt		Grain yield $spike^{-1}$		No. of grains $spike^{-1}$	
		MS	F.Pr	MS	F.Pr	MS	F.Pr	MS	F.Pr
Soil Zn (A)	1	3.07547	<0.001	26.107	0.0366	0.0675	0.2726	92.269	0.0886
Foliar Zn (B)	1	1.06505	0.0076	0.5	0.7707	0.05333	0.3293	21.267	0.4118
Genotypes (C)	11	1.28161	<0.001	131.16	<0.001	0.34756	<0.001	147.673	<0.001
A X B	1	0.00047	0.9547	2.168	0.5442	0.09188	0.2009	82.819	0.1065
A X C	11	0.26354	0.0561	8.989	0.1259	0.02841	0.894	27.101	0.5775
B X C	11	0.22176	0.1271	2.674	0.9271	0.09515	0.0767	57.382	0.0543
A X B X C	11	0.36308	0.0066	8.884	0.1322	0.0921	0.0896	44.273	0.174
Exp. Error	144	0.14505		5.866		0.05566		31.387	

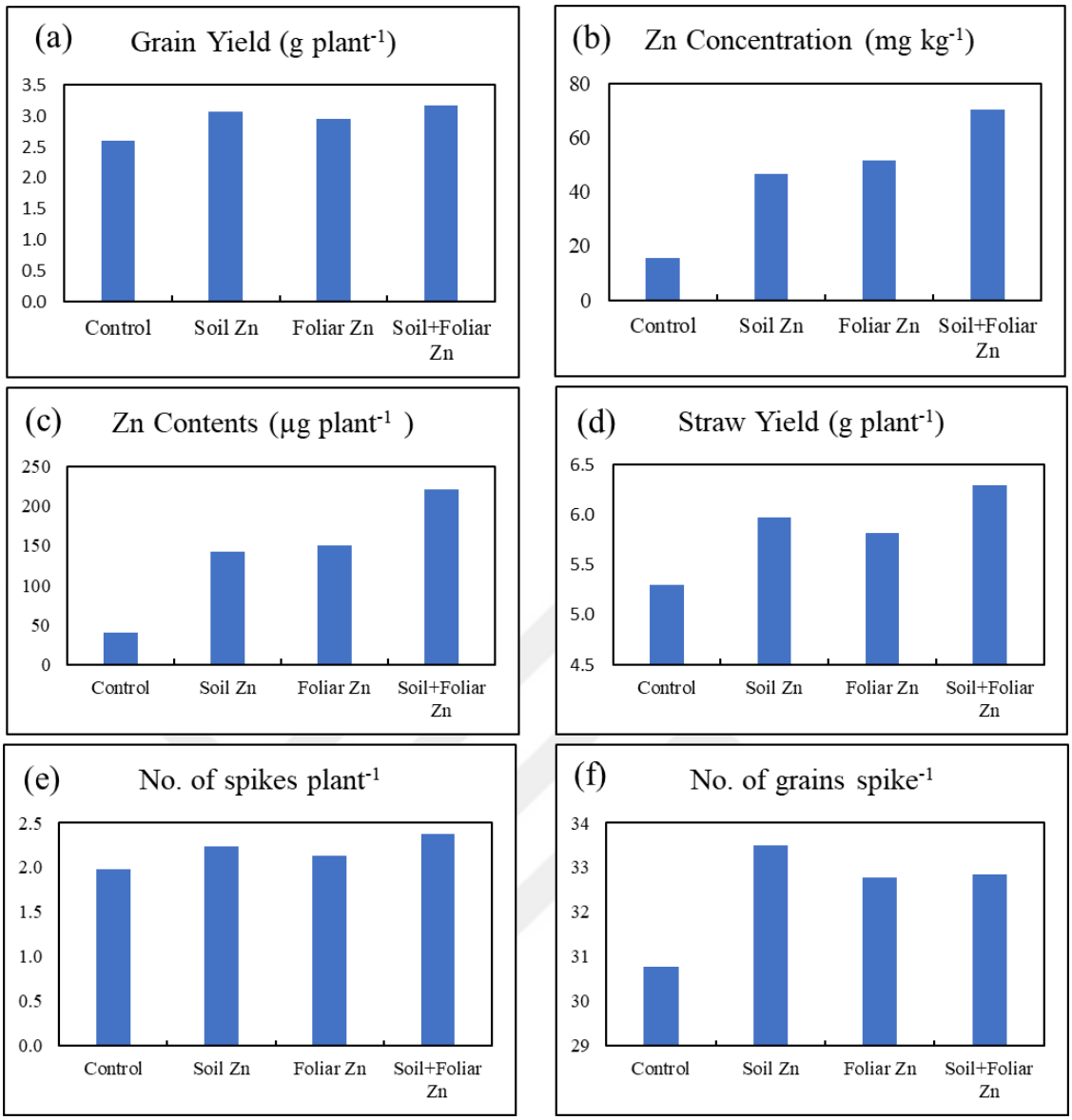

Fig 3.1: Effect of low soil Zn (control), adequate soil Zn (Soil Zn), foliar spray (Foliar Zn) and adequate soil Zn with foliar spray (soil + foliar) on mean values of (a) grain yield, (b) grain Zn concentration, (c) grain Zn content, (d) straw yield, (e) number of spike plant^{-1}, (f) number of grains plant^{-1} in 11 CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and one conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008).

Grain yield (g plant^{-1}), on average, was increased with soil Zn and soil + foliar Zn, however, foliar Zn sprays alone also increased the grain yield significantly (Fig 3.1 a). Grain Zn concentration (mg kg^{-1}) was increased significantly with all the Zn treatments applied, however, soil + foliar Zn application were very effective in increasing the grain Zn concentration up to considerable high levels (Fig 3.1 b). Mean Zn contents in grains were increased at a higher rate with soil + foliar Zn application as compared to soil or foliar Zn application alone (Fig 3.1 c). Similarly, straw yield and number of spikes per

plants (Fig 3.1 d, e) were more influenced with the soil and soil + foliar Zn application, although foliar Zn application alone also increased these traits significantly as compared to control (low soil Zn treatment). The combination of soil and foliar Zn application had a positive effect on grain yield and other yield components except the number of grains per spike (Fig 3.1). It seems number of grains per spike were reduced with foliar Zn sprays, as soil Zn application alone increased the number of grains per spike significantly (Fig 3.1 f).

Correlation matrix show a positive significant correlation between grain yield (g plant^{-1}) and grain Zn concentration mg kg^{-1} (Table 3.11). Grain yield (g plant^{-1}) had a positive correlation with all the agronomic and micro nutrients trait except the TGW (g). Grain Zn concentration (mg kg^{-1}) was increased with straw yield as well and it also has a strong positive correlation with the grain Zn contents. (Table 3.11)

Table 3.11: Correlation matrix for different agronomic and micronutrient traits

Characters	Zn conentration (mg kg^{-1})	Zn content (µg plant^{-1})	No. spikes plant^{-1}	Straw yield (g plant^{-1})	Grain yield spike^{-1} (g)	TKW (g)	No. grain spike^{-1}
Grain yield (g plant^{-1})	0.18*	0.51***	0.46***	0.82***	0.35***	-0.26	0.46***
Zn conentration (mg kg^{-1})		0.92***	0.14	0.26***	0.10	0.14	0.04
Zn Content (µg plant^{-1})			0.28***	0.52***	0.21**	0.04	0.19**
No. spikes plant^{-1}				0.53***	-0.59	-0.30	-0.46
Straw yield (g plant^{-1})					0.15*	-0.23	0.24***
Grain yield spike^{-1}						0.18**	0.89***
TKW (g)							-0.27

Significant at *=$p<0.05$, ** = $p<0.01$, *** = $p<0.001$

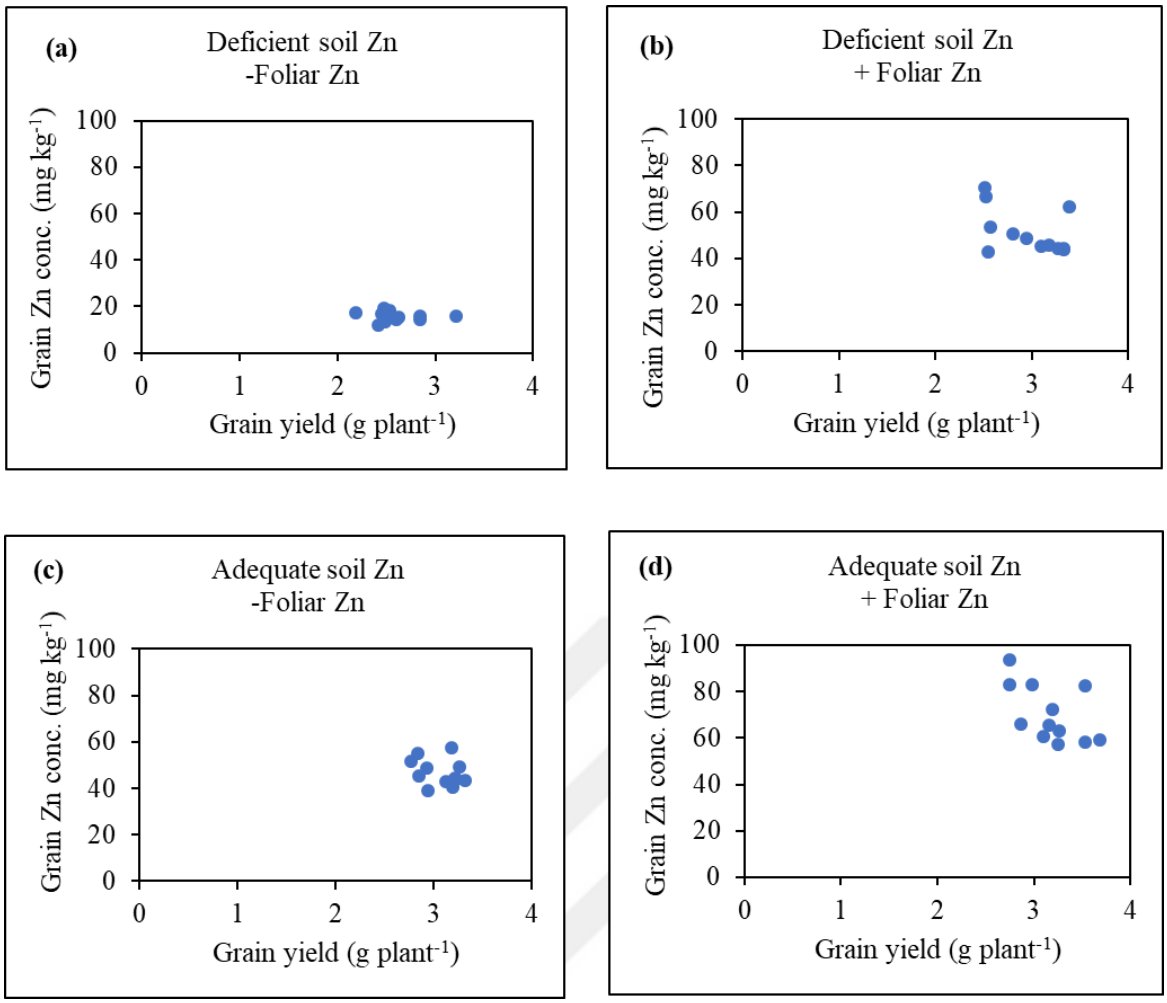

Fig 3.2: Relationship between grain yield (g plant^{-1}) and grain Zn concentration (mg kg^{-1}) of 12 genotypes grown at (a) low Zn soil (b) low soil Zn with foliar spray (c) adequate Zn soil (d) adequate soil Zn with foliar spray

Scatterplot between grain yield (g plant^{-1}) and grain Zn Concentration (mg kg^{-1}) of the biofortified genotypes tested under different soil and foliar Zn applications is given in Fig 3.2. Under low Zn soil without foliar Zn fertilization (Fig 3.2, a) HP-biofortified genotypes and the local checks were not able to deliver the grain Zn above 20 mg kg^{-1} and grain yield was also below 3 g plant^{-1} except one genotype. With foliar Zn spray (Fig 3.2, b) grain Zn concentration is increased significantly with an increase in grain yield also. Adequate soil Zn treatment (Fig 3.2, c) increased the yield significantly, however, soil + foliar Zn application (Fig 3.2, d) were found effective for both grain yield and grain Zn concentration.

3.3. Experiment-B: Studying the root uptake and root-to-shoot translocation of Zn in HP-Biofortified Pakistani wheat cultivars by time-course depletion experiment

A short-term nutrient solution culture experiment was conducted by using HP biofortified Pakistani genotypes. The objective of this experiment was to assess the differences in the root Zn uptake capacity of the biofortified genotypes. Because of non-availability of seeds of Indian genotypes, this experiment was only conducted for Pakistani lines.

3.3.1. Materials and Methods

The following HP-biofortified genotypes from Pakistan were used in the experiment: Faisalabad 2008 (check variety), NR-421, NR-435, NR-457, NR-488 and NR-489.

Seeds were germinated in perlite moistened with a saturated $CaSO_4$ solution for 5 days at room temperature. Twenty-five seedlings per pot were transferred to 3 L pots containing a standard nutrient solution (details are described in "General Material and Methods" section). Plants were supplied with either low (10^{-8} M) or adequate Zn (10^{-6} M) in the form of $ZnSO_4.7H_2O$. Nutrient solutions were continuously aerated and refreshed every 3–4 days. When the plants were 18 days old, they were transferred to five times diluted nutrient solution containing 2 x 10^{-6} M Zn as $ZnSO_4.7H_2O$.

For calculation of the root Zn uptake from the nutrient solution, 10 mL of solution was sampled at two-time points (i.e. 0 h and 4.5 h) from all pots and analysed for Zn by ICP-OES. Throughout the experiment, the volume of solution in the pots was kept constant by adding DI water to compensate the evaporative loss. At the end of the uptake period, root and shoot of the plants were harvested separately. To remove the non-absorbed Zn on the root surface and non-chelated apoplastic Zn in root tissue, roots were

rinsed in DI water, incubated in 1 mM $CaCl_2$ and 1 mM Na EDTA for 3 min each and finally rinsed again with DI water. Root and shoot samples were dried and analysed by ICP-OES according to the procedure mentioned in "General Material and Methods" section.

3.3.2. Results

Shoot, root dry weight, total biomass and root to shoot ratio of the 18 days old plants are given in the Table 3.12. Results shows that applying adequate Zn in the nutrient medium solution increased the shoot dry weight but reduced the root weight. Mean value for shoot biomass at low Zn was 81.7 mg plants^{-1} which increased significantly to 93.6 mg plants^{-1} with adequate Zn supply (Table 3.12). At low Zn supply mean value for root dry weight was significantly higher 37.9 mg plants^{-1} compared to the adequate Zn plants 35.1 mg plants^{-1}. However, overall biomass production as well as shoot:root ratio was increased significantly at adequate Zn supply. Analysis of variance revealed the significant ($p<0.001$) genotypic variation for shoot, root, total biomass and shoot:root ratio (Table 3.12). Genotype NR-489 and check variety Faisalabad-2008 produced significantly higher biomass as compared to other genotypes under both low and adequate Zn supply (Table 3.12).

Zinc efficiency of root, shoot and biomass were calculated as percentage of biomass production at low Zn to the biomass production at adequate Zn. There were no significant differences among genotypes for shoot and total biomass efficiency (Table 3.13), however, there was a significant variation ($P<0.05$) among the genotypes for root Zn efficiency (Table 3.13). NR-489 being most efficient (125.8%) compared to the check variety Faisalabad-2008 (95.6 %) (Table 3.13).

Cumulative uptake and uptake rate of additional Zn ($2x10^{-6}$ M supplied as $ZnSO_4{\cdot}7H_2O$) by 25 plants with time course (h^{-1}) is given in the Table 3.14. Cumulative uptake (µmol Zn 25 plants^{-1}) was significantly ($p<0.01$) higher in low Zn supply plants compared to that of adequate Zn (Table 3.14). There was a significant ($p<0.001$) genotypic variation for cumulative uptake µmol Zn 25 plants^{-1}. Uptake rate (µmol Zn 25 h^{-1}plants^{-25}) was also significantly ($p<0.01$) higher in low Zn supplied plants compared

to that of adequate Zn. Genotypes differ significantly (*p<0.001*) for uptake rate (µmol Zn 25 h^{-1}plants^{-25}) with low Zn NR-489 being most efficient with highest uptake rate.

Table 3.12. Biomass production of 18-days-old five CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and one conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008) grown with low (10^{-8} M) or adequate (10^{-6} M) Zn supply in greenhouse. Additional Zn ($2x10^{-6}$ M) was supplied as $ZnSO_4.7H_2O$ 5 hours before the harvesting.

		Dry matter production (mg plant^{-1})			
Zn supply in nutrient solution	**Genotypes**	**Shoot**	**Root**	**Biomass**	**shoot:root ratio**
Low Zn	Faisalabad-2008	103.0 ± 11.3 abc	40.2 ± 3.5 ab	143 ± 13 ab	2.57 ± 0.27 bc
	NR-421	77.5 ± 4.2 ef	36.4 ± 2.9 abc	114 ± 6 de	2.14 ± 0.18 cd
	NR-435	77.2 ± 3.5 ef	36.3 ± 1.4 abc	113 ± 3 de	2.13 ± 0.14 cd
	NR-457	74.4 ± 7.8 ef	36.3 ± 6.9 abc	111 ± 9 de	2.13 ± 0.57 cd
	NR-488	61.0 ± 3.8 f	35.0 ± 2.9 bc	96 ± 7 e	1.75 ± 0.06 d
	NR-489	96.8 ± 7.0 bcd	43.4 ± 3.9 a	140 ± 8 abc	2.24 ± 0.27 cd
	Mean	81.7 B	37.9 A	119.6 B	2.2 B
Adequate Zn	Faisalabad-2008	119.9 ± 8.2 a	42.1 ± 3.3 ab	162 ± 11 a	2.85 ± 0.08 ab
	NR-421	87.1 ± 6.8 cde	36.6 ± 3.0 abc	124 ± 10 bcd	2.38 ± 0.04 bc
	NR-435	81.7 ± 6.2 cde	31.3 ± 2.2 c	113 ± 8 de	2.61 ± 0.04 bc
	NR-457	86.1 ± 7.9 cde	34.2 ± 2.0 bc	120 ± 10 cd	2.52 ± 0.11 bc
	NR-488	72.8 ± 3.9 ef	31.5 ± 2.5 c	104 ± 6 de	2.31 ± 0.10 bcd
	NR-489	113.9 ± 10.6 ab	34.6 ± 2.3 bc	148 ± 11 a	3.30 ± 0.34 a
	Mean	93.6 A	35.1 B	128.6 A	2.7 A

*Shoot dry weight HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 4.22***, 10.9***, N.S*

*Root dry weight HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 1.95**, 5.01***, 8.22**

*Total biomass HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 5.24**, 13.5***, N.S*

*Root:Shoot ratio HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 0.14***, 0.35***, 0.58***

Cumulative Zn uptake per gram of root dry weight were significantly (*p<0.01*) high in low Zn plants with maximum observed in low Zn NR-421. A significant (*p<0.001*) genotypic variation was found for cumulative uptake and uptake rate. The uptake rates (µmol Zn h^{-1} g^{-1} dw) were significantly (*p<0.05*) high in plants grown at low

Zn compared to that of adequate Zn. The genotypic (*p<0.001*) variation was also significant. NR-421 can be considered as most efficient genotype with highest uptake rate at low Zn supply (Table 3.14).

Table 3.13. Zn efficiency of root shoot and biomass (root+shoot) of 18-days-old plants of HP biofortified wheat genotypes grown in nutrient solution with low (10^{-8} M) or adequate Zn (10^{-6} M) supply in greenhouse. Additional Zn ($2x10^{-6}$ M) was supplied as $ZnSO_4.7H_2O$ 5 hours before the harvesting.

	Zn efficiency (%)		
Genotypes	**Root**	**Shoot**	**Biomass**
Faisalabad-2008	95.6 ± 3.4 b	86.2 ± 10.6 a	89 ± 8 a
NR-421	99.4 ± 3.6 b	89.2 ± 6.1 a	92 ± 4 a
NR-435	116.1 ± 8.7 ab	95.2 ± 12.5 a	101 ± 11 a
NR-457	106.1 ± 19.4 ab	86.7 ± 9.7 a	92 ± 3 a
NR-488	111.7 ± 14.2 ab	84.0 ± 5.6 a	92 ± 8 a
NR-489	125.8 ± 11.2 a	85.6 ± 10.1 a	95 ± 11 a

*Root Zn efficiency HSD 0.05 (Genotype) = 26.0**

Shoot Zn efficiency HSD 0.05 (Genotype) = N.S

Total biomass Zn efficiency HSD 0.05 (Genotype) = N.S.

Zinc concentration in root and shoot of 18 days old plants supplied with additional Zn ($2x10^{-6}$ M supplied as $ZnSO_4 \cdot 7H_2O$) in nutrient solution 5 hours before harvesting is provided in Table 3.15. Shoot and root Zn concentrations were affected significantly (*p<0.01*) with the Zn supply in nutrient solution, however, there was a genotypic variation (*p<0.05*) only for shoot Zn concentration. Under low Zn supply mean value for shoot Zn concentration was 35.4 mg kg^{-1} which increased significantly to 43.4. mg kg^{-1} at adequate Zn conditions (Table 3.15). Interestingly root Zn concentration in low Zn supply plants was significantly higher than that of adequate Zn plants. Mean values of all genotypes were 119 mg kg^{-1} and 102 mg kg^{-1} at low and adequate Zn conditions respectively. However, root Zn concentration of the check variety Faisalabad-2008 was lowest among all genotypes at low Zn conditions (Table 3.15). These results suggest that there was no difference in Zn absorption and root to shoot translocation among biofortified genotypes and check cultivar in a given period of time.

Table 3.14. Cumulative uptake and uptake rate of Zn ($2x10^{-6}$ M additional Zn supplied as $ZnSO_4.7H_2O$) to 18-days-old plants of HP biofortified wheat genotypes grown in nutrient solution with low (10^{-8} M) or adequate Zn (10^{-6} M) supply in greenhouse. Additional Zn ($2x10^{-6}$ M) was supplied as $ZnSO_4.7H_2O$ 5 hours before the harvesting.

Zn supply in nutrient solution	Genotypes	Cumulative uptake (µmol Zn 25 plants^{-1})	Uptake rate (µmol Zn h^{-1} 25 plants^{-1})	Cumulative uptake (µmol Zn g^{-1} root dw)	Uptake rate (µmol Zn h^{-1} g^{-1} root dw)
Low Zn	Faisalabad-2008	2.67 ± 0.20 abc	0.59 ± 0.04 abc	2.68 ± 0.42 abc	0.60 ± 0.09 abcd
	NR-421	3.06 ± 0.47 a	0.68 ± 0.10 a	3.36 ± 0.49 a	0.75 ± 0.11 a
	NR-435	2.84 ± 0.16 ab	0.63 ± 0.04 ab	3.14 ± 0.23 ab	0.70 ± 0.05 a
	NR-457	2.47 ± 0.42 abcd	0.55 ± 0.09 abcd	2.36 ± 0.38 abcde	0.72 ± 0.19 ab
	NR-488	2.46 ± 0.13 abcd	0.55 ± 0.03 abcd	2.82 ± 0.12 abc	0.63 ± 0.03 abc
	NR-489	3.16 ± 0.55 a	0.70 ± 0.12 a	2.90 ± 0.29 abc	0.64 ± 0.06 abc
Adequate Zn	Faisalabad-2008	2.28 ± 0.48 abcde	0.51 ± 0.11 abcde	2.16 ± 0.38 bcde	0.48 ± 0.09 abcde
	NR-421	1.76 ± 0.49 cdef	0.39 ± 0.11 cdef	1.95 ± 0.67 cde	0.43 ± 0.15 bcde
	NR-435	1.99 ± 0.72 cdef	0.44 ± 0.16 bcde	2.56 ± 0.95 abcd	0.57 ± 0.21 abcd
	NR-457	1.56 ± 0.44 def	0.35 ± 0.10 def	1.81 ± 0.43 cde	0.40 ± 0.09 cde
	NR-488	1.00 ± 0.20 f	0.22 ± 0.05 f	1.27 ± 0.18 e	0.28 ± 0.04 e
	NR-489	1.24 ± 0.36 ef	0.28 ± 0.08 ef	1.43 ± 0.43 de	0.32 ± 0.09 de

*Cumulative uptake (µmol Zn 25 plants^{-1}) HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 0.63**, 0.25***, 1.04**

*Uptake rate (µmol Zn h^{-1} 25 plants^{-1}) HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 0.14 **, 0.05***, 0.23**

*Cumulative uptake (µmol Zn g^{-1} root dw) HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 0.70**, 0.27***, N.S*

Uptake rate (µmol Zn h^{-1} g^{-1} root dw) HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 1.15, 0.17***, N.S*

Table 3.15. Shoot and root Zn concentration of 18-days-old five CIMMYT-based biofortified wheat genotypes (*Triticum aestivum* L.) and one conventionally-grown wheat cultivar (*Triticum aestivum* L., cv. Faisalabad-2008) grown with low ((10^{-8} M) or adequate (10^{-6} M) Zn supply in greenhouse. Additional Zn ($2x10^{-6}$ M) was supplied as $ZnSO_4.7H_2O$ 5 hours before the harvesting.

		Zn concentration (mg kg^{-1})	
Zn Supply	**Genotype**	**Shoot**	**Root**
Low	Faisalabad-2008	36.6 ± 4.6 ab	89.4 ± 7 b
	NR-421	39.1 ± 0.8 ab	126 ± 26 ab
	NR-435	42.6 ± 1.6 ab	126 ± 17 ab
	NR-457	33.9 ± 15.7 ab	117 ± 26 ab
	NR-488	33.8 ± 15.5 ab	120 ± 17 ab
	NR-489	26.3 ± 10.2 b	137 ± 17 a
	Mean	35.4 B	119 A
Adequate	Faisalabad-2008	35.4 ± 16.5 ab	107 ± 6 ab
	NR-421	46.5 ± 2.6 ab	103 ± 19 ab
	NR-435	51.6 ± 5.8 a	107 ± 31 ab
	NR-457	46.9 ± 1.4 ab	103 ± 17 ab
	NR-488	44.7 ± 2.7 ab	91.7 ± 9 ab
	NR-489	35.0 ± 2.1 ab	100 ± 14 ab
	Mean	43.4 A	102 B

*Shoot Zn concentration HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 5.13 **, 13.2 *, N.S*

*Root Zn concentration HSD 0.05 (Zn supply in nutrient solution, Genotypes, Zn supply in nutrient solution X Genotypes) = 10.9 **, N.S, N.S*

3.4. Discussion

This study demonstrated the effectiveness of Zn fertilizer in the form of soil, foliar and soil + foliar applications for improving growth, yield and nutrients uptake by Harvest Plus- Biofortified wheat genotypes. Adding $ZnSO_4$ fertilizer to the Zn deficient soil increased the grain yield, straw biomass, Zn concentration and contents in shoot and grains, number of spikes per plant and number of grains per spike in wheat biofortified lines (Table 3.6, 3.7, 3.8, 3.9). Grain yield increases caused by soil Zn application alone and in combination with soil + foliar Zn were recorded as 18% and 22%, respectively (Fig 3.1). Numerous studies reported the application of Zn fertilizer to soil helped to correct the Zn deficiency symptoms and to increase the crop yield (Yilmaz *et al.,* 1997; Cakmak, 2008b; Abid *et al.,* 2013; Zhao *et al.,* 2014). Razvi *et al*. (2005) reported significantly higher grain yield, straw yield, harvest index and dry matter production at harvest by the application of Zn, over the rest of the treatments. Likewise, Khan *et al.* (2007) reported an increase in the number of tillers, spike m^{-2}, spike length, plant height and 1000 grain weight of wheat significantly, in an experiment on wheat and rice using two levels of zinc (5 & 10 kg ha^{-1}) over control.

In present study, interestingly 13.8% increases in grain yield were recorded with foliar Zn application alone (Table 3.6, Fig 3.1). These results are contradictory with many previous studies where foliar Zn spraying did not affect yield traits of wheat. Numerous previous studies reported that grain yield was less dependent on foliar Zn supply (Cakmak *et al.,* 2010 a,b; Wang *et al.,* 2012; Zhang *et al.,* 2012b; Zou *et al.,* 2012; Zhao *et al.,* 2014, Xia *et al.,* 2018). One study reported that foliar Zn spraying increased grain yield under drought conditions (Karim *et al.,* 2012). Increases in wheat grain yield with foliar Zn alone was reported in only one location in Pakistan out of seven countries probably due to calcareous nature of Pakistani soil which reduces the Zn availability to roots (Ram *et al.,*2016).

A significantly positive correlation was observed between the grain yield and grain Zn concentration in present study (Table 3.11). Graham *et al.,* (1999), Welch and

Graham (2004) and Velu *et al.,* (2012) also reported that there was no negative association of grain Zn with grain yield in wheat. Few reports in literature revealed slightly negative correlation between Zn and grain yield in wheat (Zhao *et al.,* 2009; Gomez Becerra *et al.,* 2010, Gomez-Coronado *et al.* 2016). Some previous studies have shown that wheat grain Zn concentrations are negatively correlated with grain yields because of the "dilution" effect (Zhao *et al.,* 2009; Velu *et al.,* 2014). However, agronomic management approaches (e.g., fertilization) have not been given careful consideration in these studies (Chen *et al.,* 2017).

In this study, all the three treatments; adequate soil Zn, foliar Zn and soil + foliar Zn, were found effective in significantly increasing the grain Zn concentrations in HP-biofortified (Fig 3.1). Among different wheat genotypes tested, grain Zn concentrations varied with different soil and/or foliar treatment. On average (including checks), Zn spraying increased grain Zn concentrations by 36 mg kg^{-1} (more than 2 folds) compared with low Zn application. Increases caused by adequate soil Zn application alone were only 31 mg kg^{-1} (2 folds) whereas soil + foliar Zn application increased 54.6 mg kg^{-1} (3.5 folds) (Fig 3.2).

Many studies in literature reported that foliar Zn supply alone significantly increased grain Zn accumulation in wheat and rice crops (Jiang *et al*. 2007; Cakmak *et al.,* 2010a; Wang *et al.,* 2012; Phattarakul *et al.* 2012; Xue *et al.* 2012; Zhang *et al.,* 2012b, Mabesa *et al.* 2013; Ram *et al.,* 2017; Xia *et al.,* 2018). In current studies, on average of all genotypes, foliar fertilizer application increased the Zn concentration up to 36 mg kg^{-1}. Experiments conducted in seven countries (China, India, Kazakhstan, Mexico, Pakistan, Turkey and Zambia) covering 23 sites over 3 years by using 10 different wheat cultivars showed that 83.5% increase (more than 10 mg·kg^{-1}) in grain Zn was achieved by foliar Zn spraying alone (Zou *et al.,* 2012). There was a positive correlation between Zn concentration in grain with foliar Zn rates (Zhang *et al.,* 2012b), which shows that Zn translocation to grain is not the limiting factor in wheat. Several studies reported that Zn concentrations in wheat grains were largely influenced by genotype, environment and genotype x environment interaction (Gomez-Becerra *et al.,* 2010; Joshi *et al.,* 2010; Murphy *et al.,* 2011).

Although it is worth pointing out that foliar Zn spraying represents an effective way to grain Zn biofortification of wheat, however, plant development stage at the time

of foliar Zn application is also important in term of grain Zn accumulation. Considerable increases in grain Zn concentration usually happen when Zn is sprayed to plants before and/or right after anthesis (i.e., just prior to heading and at early milk stages, respectively) (Ram *et al.,* 2016). In present study HP-biofortified genotypes were sprayed with Zn fertilizer at booting stage and early milk stage which gave the highly significant increases in grain Zn concentration. Other studies also reported that magnitude of increase in grain Zn concentration with foliar Zn application depends largely on the growth stage of crop plants at which foliar Zn application is applied (Cakmak *et al.* 2010a; Phattarakul *et al.* 2012; Mabesa *et al.* 2013; Boonchuay *et al.* 2013; Stomph *et al.* 2014).

Present study presents the significantly positive effect of soil Zn application on the grain Zn accumulation (Table 3.6). These results are in accordance with some studies which showed the effectiveness of soil Zn fertilization on grain Zn accumulation. Maqsood *et al.* 2009 reported 51.7 to 69.9% increase in grain Zn concentration by soil application of Zn (6 mg Zn kg^{-1}) in several wheat genotypes in comparison with local check. Similarly, soil Zn fertilization at the rate of 9 mg kg^{-1} enhanced the grain yield and grain Zn concentration by 29% and 95% respectively (Hussain *et al.* 2012). However, in some studies, soil application had no effect on significantly increasing the grain Zn (Phattarakul *et al.* 2009; Zou *et al.,* 2012; Rehman *et al.,* 2018). In agreement to the literature, soil + foliar Zn fertilization was very effective in the present study in increasing grain yield and grain Zn concentration as well. Grain Zn concentration was improved up to 80% with soil + foliar application of Zn (Bharti *et al.* 2013). Many other studies also reported the significantly increases in grain Zn concentration and grain yield by the combined application of soil + foliar Zn (Cakmak *et al.* 2010b; Zhou *et al.,* 2012; Phattarakul *et al.,* 2012; Rehman *et al.,* 2018).

Genotypes differ from each other in grain yield, grain Zn concentration and even response of soil and foliar Zn application. In current studies of testing different HP-biofortified genotypes with soil and foliar Zn resulted in significantly different response and significantly higher genotypic variation (Table 3.10). Indian check cultivar "HD-2967" showed the highest grain Zn concentration under low soil Zn condition without foliar fertilization among all the HP biofortified lines. HP-biofortified genotype "HPBW-01" performed best among all the genotypes in response to soil, foliar and soil + foliar fertilizer applications. Therefore, "HPBW-01" can be considered as the best genotype in

terms of Zn absorption from soil and/or foliar fertilizer applications and efficiently translocating it in grain for deposition. With soil Zn application alone, grain Zn concentration in "HPBW-01" increased to 57 mg kg^{-1}, with foliar Zn application alone increased to 70 mg kg^{-1} and with soil + foliar fertilizer further increased to 93.7 mg kg^{-1}. It means cultivation of HPBW-01 on adequate soil Zn or with soil fertilizer alone on deficient soil Zn can provide the recommended Zn supply. Previous studies in literature also showed wide variation for grain Zn concentration among wheat genotypes (Oury *et al.,* 2006; Zhao *et al.,* 2009; Masood *et al.,* 2009; Velu *et al.,* 2011; Badakhshan *et al.,* 2013; GomezCoronado *et al*. 2016; Khokhar *et al.,* 2018). Results presented in this study confirm that cultivars obviously differ in grain yields, yield components, grain Zn concentrations, response to fertilizer applications and other nutritional traits.

There are different biofortification targets of Zn level in wheat grains to meet the Zn requirement by human body, set by different group of scientists and organization. HarvestPlus program (http://www.harvestplus.org; Hao *et al*., 2015) set the target concentration of Zn in wheat grain as 38 mg kg^{-1}. Other studies reported that the target value for wheat grain Zn concentration was set to be 38–50 mg kg^{-1} for biofortification (Allen *et al.,* 2006; Ortiz-Monasterio *et al.,* 2007; Pfeiffer and McClafferty, 2007; Tang *et al*., 2008; Wang *et al.,* 2012; Hao *et al.,* 2015). Results in current studies show that the target value was completely achieved in all biofortified lines as well as in check cultivars with soil, foliar and soil + foliar application of Zn fertilizer. It seems that biofortified genotypes are not able to achieve these targets without the application of Zn fertilizers under deficient soil Zn conditions. Maximum grain Zn concentrations achieved was 19.2 mg kg^{-1} by the Indian check variety "HD-2967" under Zn deficient conditions in this study without soil or foliar Zn. Therefore, the results presented in current research confirmed that foliar Zn spraying alone or with soil Zn is necessary to enhance both grain yield and grain Zn concentrations.

The variation in grain Zn concentration of HP biofortified genotypes cannot be explained by the differences in root Zn uptake at seedling stage. Although the results in current study (experiment B) confirmed that HP-biofortified genotypes are efficient in absorbing and translocating the absorbed Zn to shoot compared to check variety in a given time period. But it is difficult to correlate the higher root uptake and shoot concentration with the grain Zn accumulation. Because there are several key factors affecting grain Zn

accumulations other than root absorption at vegetative stage. Higher absorption by the plant roots during a limited time (4.5 h in this study) cannot justify the high grain Zn accumulation. Previously similar experiments result clearly reported that root Zn uptake capacity of genotypes and shoot accumulation of Zn during early growth stage has no relation to the grain Zn accumulation (Yilmaz *et al.*, 2017)

The uptake experiment in present study indicates a very efficient system of root absorption and translocation of Zn deficient biofortified genotypes as compared to the local check variety. Although genotypic variation has a primary influence in grain Zn concentrations, other environmental factors like soil water content and precipitation has also important role in grain Zn accumulation (Gomez-Coronado *et al.*, 2016). A large magnitude of variation is reported in different fields or pot experimental studies. Cultivating different wheat cultivars are always considered as a major source of variation for the grain yield and grain Zn accumulation (Masood *et al.*, 2009, GomezCoronado *et al*. 2016; Khokhar *et al.*, 2018). The variation found in grain Zn increases caused by soil, foliar, or soil + foliar Zn application were also wide in different experiments. Therefore, it is complex to develop a most effective Zn biofortification strategy for the cereal crops like wheat. All factors including cultivars, soil and other environmental conditions, and artificial managements (e.g., fertilization and foliar application times) should be considered and managed in a proper way. In areas of the world where cereals are grown on Zn deficient soils, Gomez-Coronado *et al.* (2016, 2017) suggested that selecting the efficient cultivars for Zn absorption and grain accumulation capacity combined with appropriate soil and foliar Zn applications could be a best strategy for the Zn biofortification.

3.5. Conclusion

The current research confirms that HP-biofortified high Zn wheat genotypes have capacity to absorb, utilize and translocate Zn from soil and/or foliar Zn fertilizers more efficiently as compared to the conventionally grown check cultivars. These genotypes were developed by long-term breeding activities at CIMMYT as a part of HarvestPlus

biofortification program and released in the target countries (India and Pakistan). Plant breeders have transferred the genes responsible for increased Zn from the reported high Zn sources like synthetics, diploid/tetraploid wild progenitors and landraces, to high yielding elite wheat backgrounds. However, a fertilizer strategy along with high Zn wheat genotype cultivation is necessary to be able to achieve the grain Zn concentrations according to the preset target levels specially in Zn deficient soils. Although these lines are good absorber and have more capacity to extract Zn from soil, but these genotypes cannot represent their full genetic potential in terms of grain Zn accumulation in Zn deficient calcareous soils. Under Zn deficient conditions, HP-biofortified lines grown without any additional Zn fertilizer do not provide the sufficient grain Zn to fulfil daily Zn requirement by human body. In current study the strategy of foliar Zn spraying along with soil Zn application was found very effective to biofortify wheat with Zn as well as to increase the grain yield. There was a significant genotypic variation among the genotypes, however, all the genotypes were able to achieve the grain Zn targets with the soil and/or foliar Zn application.

In south Asian countries like India and Pakistan where soils are calcareous and Zn deficient, the strategy of growing genetically biofortified wheat cultivars with an added application of Zn in soil and foliar form is the best approach to improve yield and grain Zn accumulation. Under such a scenario, the targets for biofortification will be rapidly achieved by combining agronomic and genetic strategies and hence to overcome the malnutrition problem.

C. GENERAL DISCUSSION AND CONCLUSION

Pregnant and lactating women require more Zn which is not fulfilled with cereal based diet as cereals are genetically low in Zn and other micronutrients (Cakmak *et al.*, 2008; Bouis, 2003).

Cereals (Wheat, maize and rice) are the most important source of the world's total food. According to Food and Agriculture Organization (FAO, 2015) more than 51% daily caloric requirement is provided by the combination of wheat, maize and rice. Rice alone is a major part of the diet for more than half the world's population. In many parts of the world especially in Latin America, Africa, Southern Europe and some Asian countries, maize is consumed as food grain. Wheat is a primary staple food in South Asia (Northern India and Pakistan) used to make traditional breads called chappati or roti which is important part of almost every meal and in every house (Baloch *et al.*, 2015). The average wheat consumption in South Asia is around 400g per person per day. More than 26% of the population living in region consuming wheat as staple food is diagnosed as Zn deficient. Cereals like wheat, maize and rice are inherently low in micronutrients, therefore, cereal-based foods do not provide enough Zn to meet the individual's daily Zn requirement.

Agriculture strategies offer a practical and cost-effective solution to the problem by increasing the Zn concentration in staple food like cereal crops through breeding (genetic biofortification) or fertilization (agronomic biofortification) or combining both approaches. Agronomic biofortification provides an instant solution to the problem by applying Zn fertilizer to the soil and/or to plant as a foliar spray. Knowledge about root and leaf absorption of Zn, its re-translocation and grain deposition in wheat and maize is crucial to improve the nutritional value of cereal crops and to efficiently address malnutrition problem. Previous studies including the results from International HarvestZinc project (www.harvestzinc.org) reported that wheat responded to foliar Zn fertilization very positively and significantly in terms of increases in grain Zn as

compared to maize, however, reasons remained unknown. Studies reported in first chapter were aimed elucidating the physiological reasons behind the different responses of wheat and maize to foliar Zn fertilizers. A series of experiments were conducted on wheat and maize under controlled environmental conditions in soil and nutrient solution with varied supply of Zn. Modern and sensitive Zn tracing and visualizing techniques were used like stable isotope of ^{70}Zn and Zn fluorescence dye "Zinpyre". Maize plants showed significantly less uptake of foliar applied Zn compared to wheat plants regardless the age of plants and/or the volume of fertilizer solution.

Human Zn deficiency in this region is also associated with noticeable Zn deficiency in soils as cereals are cultivated on severely Zn deficient soils (Asher, & Hynes, 1992; Welch & Graham, 2004; Cakmak, 2008; Cakmak *et al.,* 2010; Zou e*t al.,* 2012; Velu *et al.,* 2012; Cakmak, 2014; Cakmak and Kutman, 2017). Therefore, the South Asian agro-ecological zone including India and Pakistan was identified as potential target areas for adoption and commercialization of biofortified wheat. HarvestPlus program (www.harvestplus.org) along with collaboration with public and private partners took initiatives to develop the biofortified high-Zn wheat cultivars for the target areas (Bouis and Welch, 2010).

The Harvest plus wheat genotypes used in this study were developed through long term breeding activities at CIMMYT as a part of genetic biofortification program. The objective of HarvestPlus Biofortification Program is to increase the Zn concentrations in the edible portions of staple food crops through plant breeding. There is very promising progress in this program and numerous Zn biofortified cereal genotypes were developed and released in many target areas of developing countries. Although genetic biofortification is a cost-effective and widely accepted strategy, however, achieving sufficiently high Zn concentrations in grains to be able to effect human nutrition is directly related to the available Zn in soils to the plant roots (Cakmak 2008). Therefore, in regions of the world where low Zn solubility in soils is a problem, high Zn genotypes may also depend on application of Zn-containing fertilizers, e.g., agronomic biofortification (White and Broadley 2005; Cakmak 2008; Alloway 2009). Soils in south Asian countries like India and Pakistan are calcareous and Zn deficient. Insufficient precipitations and lack of proper irrigation resulting in low moisture is another limitation reducing the Zn availability to plant roots. Under these adverse soil and environmental

conditions, cultivating the genetically improved high Zn cultivars may not be able to achieve the set targets of increased Zn in grain (+12 mg kg^{-1}) without the synergistic fertilizer strategy. Several studies reported that agronomic biofortification provides a fast and effective solution to increase Zn concentration in several grain crops, mainly in wheat and rice (Cakmak *et al.* 2010b; Phattarakul *et al.* 2012; Zou *et al*. 2012).

Because in plant systems, there is a similar mechanism of absorption of heavy metals and accumulation in grains, if genetically biofortified wheat cultivars are grown on heavy metal contaminated soils (e.g. Cadmium and Lead) they can accumulate higher amount of these toxic metals in grains. This is another important concern regarding the use of genetically biofortified wheat genotypes. However, studies have shown that adding Zn fertilizers in heavy metal contaminated soil can reduce the uptake and accumulation of heavy metals. As these metals compete with Zn for uptake, if there is more available Zn pool in the rhizosphere, plants can uptake more Zn instead of Cd or Pb (Qaswar *et al.,* 2017; Ishfaq *et al.,* 2018).

Here, based on the current results, we propose a strategy to improve the grain Zn nutritional quality while ensuring high yields and protecting the soil and environment at the same time. At least three factors should be managed for a nutritious and profitable wheat production by farmers: (i) adoption/cultivation of genetically improved biofortified wheat cultivars with high yield, high grain Zn concentration and bioavailability, low anti nutrients like phytate and high resistance to biotic and abiotic stresses; (ii) maintaining the adequate Zn and N levels in the soil by adding soil Zn and N fertilizers, respectively. Improving soil N not only increases the grain yield but also the nutritional value of cereal crops. Soil Zn applications increase the biologically available Zn pool to the plant roots even in high-pH and low-organic matter soils, while at the same time reducing heavy metal accumulation (e.g. Cd and Pb) in grains; (iii) enhancing the Zn pool in plant foliage by foliar Zn applications at the time of booting and early milk stages. Foliar Zn application is proved to be an efficient and economical way to increase the leaf Zn uptake and its subsequent re-translocation to grains. Foliar Zn sprays can be applied in combination with soil Zn application depending upon the deficiency condition and set targets.

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