ZINC EFFICIENCY AND DIVERSITY OF

MONTANA WHEAT AND BARLEY

by

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I dedicate this thesis to a judicious world where every living being can live freely and in balance with others.

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CHAPTER ONE – LITERATURE REVIEW

Introduction to Importance of Zn

The increase in human population in the recent decades has led to the accelerated use of farming practices and agricultural land to meet the food demand. Human population is predicted to reach 9 billion by 2050 which will further increase the demand for food and farmlands (United Nations, 2015). Intensive use of farmland causes reductions in nutrients from the soil and heavily influences grain nutritional value and yield capacity thereof gives rise to inadequate levels of dietary nutrient intake. Many cropping regions worldwide are deficient in Zn (Zn) causing reduced Zn concentration in cereals (Alloway, 2004). Therefore, regions where predominant diets are cereal based are at risk of facing major health complications lead by Zn deficiency.

Importance of Zn for Mammalians

Study of Zn and its enzymatic activity gained importance in the 20th century. In humans, Zn is the second most abundant trace element, found 2.3 g in an average adult body (Table, E., Table, 2001). In mammalian cells, homeostasis of Zn ions is controlled by cysteine rich proteins called metallothionein which chelates and sequesters excess Zn from the cell. Metallothionein depletes or accumulates in the cell depending on the amount of Zn in the cellular environment (McCall, 2001). Foremost discovery was the discovery of small protein domains that generally stabilized by metal ions, mostly Zn. These small protein domains are called “Zn fingers” and are involved in a broad range of

cellular activities including regulation of gene expression, apoptosis, DNA recognition and protein folding (Laity & Wright, 2001). Hence, it is important to understand and fully appreciate the significance of Zn in human nutrition. Assessment of Zn in humans matters especially in the developing world due significant deficiency of Zn in soils. According to WHO, Zn deficiency is causing an epidemic of undernutrition and more than two billion people worldwide are affected by this deficiency (WHO, 2015). Major problems attributed to Zn deficiency includes adolescent nutritional dwarfism, immunodeficiency and even psychiatric disorders (Gronli & Wynn, 2013)

Importance and Functions of Zn in Plants

Zn has functional roles in key cellular mechanisms, such as, photosynthesis, membrane integrity, protein synthesis, carbohydrate metabolism and fertility. Zn has a strong tendency to form tetrahedral complexes with nitrogen, oxygen and sulfur ligands and plays both catalytic and structural roles in enzymatic reactions. The enzyme, carbonic anhydrase (CA) which contains a Zn ion on its active site is responsible from the conversion of CO_2 to carbonic acid during photosynthesis (Alloway, 2004). Therefore, Zn deficiency reduces its activity. Although, CA is present both in C3 and C4 plants, it was suggested that C4 plants show more pronounced symptoms to Zn deficiency due to higher requirement of CA during photosynthesis. However, in case of extreme Zn deficiency, activity of CA is lost (Lindskog, 1997). In addition, chlorosis, a distinct deficiency symptom of Zn is partly associated with Zn and its deficiency is suggested to induce chlorophyll content reduction (Marschner, 2012). This reduction may be through oxidative damage

due to formation of free radicals, reactive oxygen species and peroxides which damages membrane integrity of cells. The reason behind can easily be correlated with reduced activity of Cu/Zn superoxide dismutase (SOD), an enzyme using Zn as a cofactor and responsible from scavenging free radicals. Other metal cofactors of SOD enzyme family include copper, iron and manganese. Furthermore, copper and Zn deficiency was shown to drastically reduce SOD activity and eventually resulted in declined shoot dry weight (Yu & Rengel, 1999). Increased disease risk of Zn deficiency is one of Zn's main aspects in plants. Along with Zn, manganese, phosphorus, boron and calcium have been suggested to localize around the root external surface and seem to directly play a role in cell integrity. Thus, deficiency of the above nutrient elements, especially Zn and phosphorous result in cellular leakage, favoring growth and infection by the surrounding pathogens (Graham & Hynes, 1992a). Furthermore, similar to CA and SOD, the activity of sucrose synthase is affected by Zn deficiency. In maize, sucrose synthase activity reduced by 20% under severe Zn deficiency which is directly associated with carbohydrate metabolism (Shrotri, 1980).

Challenges with Plant Zn Nutrition

Improving Zn content of plant edible parts by biofortification to benefit human nutrition is a strategy that is gaining global attention (Cakmak, 2008; Palmgren et al., 2008). As an agronomic approach, fertilizers are often used to compensate nutritional status of the soil and increase their availability to the plants. However, constraints of soil

characteristics such as pH, calcite content, organic matter possess an antagonistic effect on Zn bioavailability in soils. Furthermore, Zn soil fertilizers are applied on top soils, environmental conditions, for instance, drought causes drying of top soil preventing leaching of Zn to reach into the subsoil where root nutrient uptake takes place. Foliar Zn applications are apparently more practical approaches in overcoming yield losses and replenishing Zn concentration of seeds (Cakmak, 2008). Known Zn deficiency susceptible crop, rice, showed up to 10-fold increase in grain Zn concentration after foliar Zn application (Alloway, 2009; Boonchuay & Prom-U-Thai, 2013). Furthermore, foliar application of radiolabeled Zn (^{65}Zn) to wheat seedlings showed translocation of 40% of the total absorbed Zn translocation from the treated leaf to the roots under Zn deficient environment (Erenoglu et al., 2002). Although Zn foliar applications show immediate effect on seedling growth and increase Zn concentrations in cereal grains, repeated foliar applications are needed which is cost prohibitive especially for small-holder farmers. Strategies towards improving Zn cereal grain concentration should therefore be cost effective and sustainable. Zn and Fe are mainly deposited in higher concentrations in aleurone layer, embryo and lower in endosperm (Ozturk et al., 2006). Endosperm only accounts for 25% of total grain Zn (Lombi et al., 2011). During refinement process, large proportions of the aleurone layer and the embryo are removed, causes reduction of the nutritional value of cereal based food. Therefore, cereals with Zn enriched endosperm might be an effective solution.

Plant Zn Efficiency

As another option, genetic variation to Zn efficiency among cereal crops is a widely-used breeding approach to combat Zn deficiency and replenish Zn nutrition in cereal grains. Zn efficiency is a term to describe the ability of plants to maintain significant yields under low Zn is termed Zn efficiency (Graham et al., 1992b). Moreover, correlation between Zn efficiency and efficiency for other minerals have not been observed, suggesting independent genetically controlled utilization mechanisms for Zn efficiency are present. Previously, a 34 kDa polypeptide was found in the root cell plasma membrane of a Zn efficient wheat genotype induced under Zn deficiency stress (Rengel & Hawkesford, 1997). Moreover, as a coping mechanism to Zn deficiency, plants secrete root exudates for increasing element mobilization in the soil to ease the root nutrient absorption for the plant and the ability to retranslocate absorbed nutrients within the plant (Marschner & Cakmak, 1996). Although, Zn efficient genotypes are associated with enhanced yield, dry matter and Zn uptake under deficient conditions, root shoot Zn translocation and grain Zn filling processes are not necessarily improved (Hacisalihoglu, 2003; Holloway, 2010). Thus, understanding Zn homeostasis and genes that are associated with transport of Zn to plant edible parts are crucial for improving biofortification strategy of cereals.

Uptake and Translocation of Zn by Plants

Plants developed two strategies to take up nutrients from the soil. Strategy I plants used by all plants except graminaceous plants, include nongraminaceous species such as *Arabidopsis thaliana*, where they use protons and reductase enzymes released to rhizosphere to reduce soil pH as an element uptake strategy. Acidification of the rhizosphere causes pH to be dropped, increasing availability and facilitating easier uptake of elements by the roots. Strategy II plants include graminaceous plants, such as wheat and barley, in which non-protein amino acids called phytosiderophores, released as root exudate is the main tactic to uptake elements (Broadley, 2007; Marschner, 2012) Phytosiderophores form complexes with trace metal elements and the newly formed Zn-phytosiderephores complex has increased mobility. When the complex contacts with the root hairs, complex can easily be captured by the root hairs and diffused into the root epidermal cells (Alloway, 2009).

Acquisition of Zn from Soil

Zn is taken up in the form of Zn^{+2} via diffusion (Alloway, 2004). Unlike Fe, Zn does not need to be reduced before uptake. As Guerinot states, once taken up into the root epidermal cells, Zn is neither oxidized nor reduced; thus, the role of Zn in cells is based on its behavior as a divalent cation that has a strong tendency to form tetrahedral complexes with nitrogen, oxygen and sulfur ligands (Guerinot, 2000). After uptake, Zn moves towards the cortex via apoplastic and symplastic pathways in the root epidermal

cells. These tetrahedral complexes in the symplastic pathway are then recognized by protein transporters to be taken up and transported to the endodermal cells. Zn involved in the apoplastic pathway also travels through the cortex. However, once reached to Casparian strip, the entry of Zn is restricted; therefore, Zn in the apoplastic pathway is transported to the symplastic pathway by the transporter proteins residing in the plasma membranes of endodermal cells (Pinto & Ferreira, 2015).

Deposition of Zn in Seeds

Once passed the endodermis, Zn loading to the xylem takes place. Transport of Zn from root to shoot occurs via low molecular weight complexes, storage metalloproteins and free ions (Rengel, 2001). As mentioned above, tendency to form tetrahedral complexes mediates the translocation of Zn from xylem to leaves or loading to phloem. Furthermore, specialized cells also play a role in Zn transport entering and exiting xylem. These cells include transfer cells and vessel associated cells. Phloem Zn loading is the most important part in seed Zn deposition due to higher mobility of Zn in the phloem than xylem. The reason is associated with increased concentration of chelating solutes present in phloem sap. Although, phloem Zn loading mainly occurs via Zn transporter proteins within the xylem, Zn phloem loading may occur from leaves. Contrary to rice, wheat and barley deposit Zn in grains from phloem via transporter proteins (Pearson, 1995; Stomph, 2009).

Zn Transporters in Plants

There are several transporter families involved in Zn uptake and homeostasis in plants. Uptake of essential metals are dependent on concentration, metabolic energy for uptake, time and temperature. Although, ZRT-like and IRT-like proteins (ZIP family) of proteins considered to be the main transporters controlling plant Zn uptake from the soil, there are several other protein families involved. However, these transporter families differ in their localization, specificity and involvement in transportation. These families include, Metal Tolerance Proteins (MTP) or sometimes referred as Cation Diffusion Facilitator (CDF) family, P-Type ATPase or known as Heavy Metal ATPase (HMA) family and OPT (Oligopeptide Transporter) family (Colangelo & Guerinot, 2006; Eide, 2006).

MTP/CDF family of transporters are responsible from transporting metals from the cytoplasm, either by efflux to the extracellular environment or sequestering in the intracellular organelles such as vacuoles, subsequently increasing tolerance to Zn deficiency as Zn is readily available in the vacuoles (Eide, 2006).

Similarly, HMA family also plays a role in Zn efflux from the cytoplasm into the extracellular environment and may transport Zn from root to shoot as found in *Arabidopsis thaliana* AtHMA2 transporter protein. Moreover, AtHMA2 may be activated at a lower extent by non-essential heavy metals, such as Cd^{2+}, Pb^{2+} and Ni^{2+} (Eren & Argüello, 2004). OPT family is responsible from peptide transport of metal ions complexed with phytosiderephores or nicotinamine. A member of the OPT family, YSL

(Yellow Stripe Like) protein is mainly associated with the transport of Fe, it may transport several other divalent cations including Zn, Ni, Cu and Mn (Palmgren et al., 2008). Furthermore, studies suggest YSL may participate in grain Zn deposition (Colangelo, 2006). Plants need to take up sufficient amount of Zn from the soil in order to maintain adequate Zn level in all plant parts and consequently transport Zn effectively and efficiently to edible plant parts, including the grain. ZIP family of transporters take part in Zn supply into leaves as well as Zn deposition into the grains. Therefore, understanding the ZIP family of transporters is crucial to genetically improve plant Zn status. Figure 1.1 summarizes the road map of Zn uptake in plants and the genes involved in transport based on literature review.

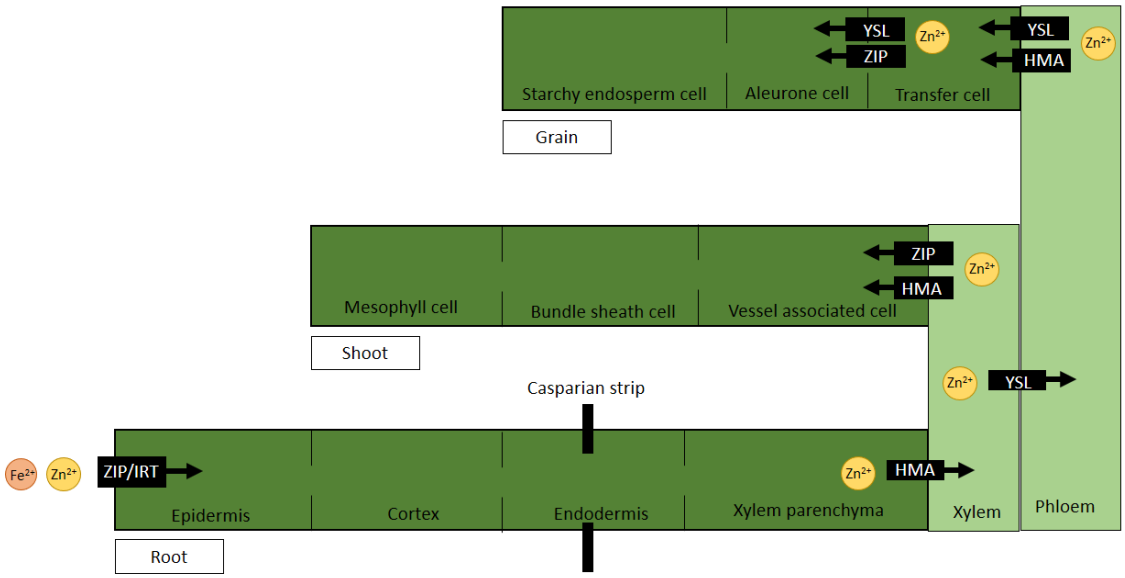


Figure 1.1. Summary of Zn trafficking from soil to grain (Palmgren et al., 2008; Waters & Sankaran, 2011). Figure was generated by the author based on literature review.

ZIP (ZRT-like and IRT-like Proteins) Family of Transporters

Many ZIP proteins have been identified in plants, yeast, bacteria, archaea, human and many other mammalians indicating that specific ZIP transporters may play different roles in metal transport and these proteins participate in a significant role in Zn homeostasis in organisms. Most ZIP family proteins are predicted to have eight transmembrane domains (TM) (alpha-helical segments) and similar membrane topologies with their N- and C- termini located on the extracellular region of the membrane (Figure 1.2). Guerinot states ZIP proteins range from 309 to 476 amino acids in length. Amino acid sequence of the cytoplasmic loop between TM 3 and TM 4 is highly variable among ZIP proteins. This region is predicted to be histidine rich and reside in the cytoplasm (Guerinot, 2000). Histidine rich structure is predicted to be involved in metal binding thereby functioning in Zn transport or its regulation. However, determination of the metal specificity of this structure has not yet been elucidated. For example, mutations in these residues of the ZRT1 protein in yeast did not inactivate the protein function but caused mislocalization (Gitan & Eide, 2003). On the contrary, deletion of this region in *Thlaspi japonicum* TjZNT1 protein increased the specificity for Zn^{+2} but did not affect the localization (Nishida, 2008). In addition, mutation of *Arabidopsis thaliana* root membrane protein IRT1 which is responsible from Fe uptake from the soil, eliminated IRT1 ability to transport Zn but not Fe, Cd and Mn (Rogers, 2000).

Moreover, other studies suggested the involvement of the extracellular N-terminal ends in metal ion selectivity. Mutation of the N-terminal region of *Thlaspi japonicum* TjZNT2 protein appeared to change its selectivity from Mn^{2+} to Zn^{2+} (Nishida, 2011).

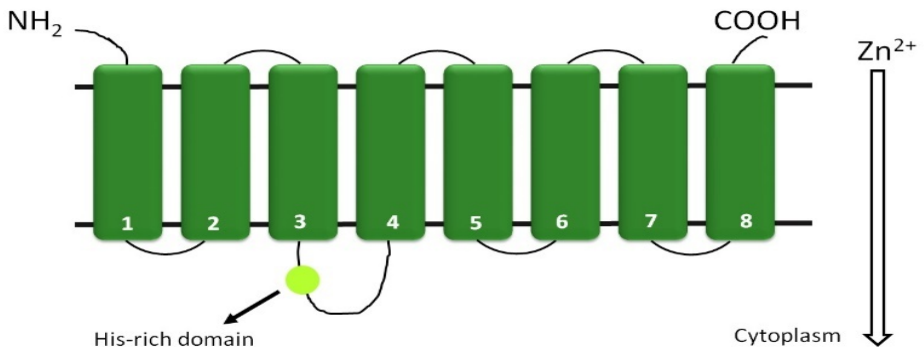


Figure 1.2. Predicted structure of ZIP family proteins (Guerinot, 2000). Figure was generated by the author based on literature review.

<u>ZIPs in Zn and Iron Transport</u>

ZIP proteins were first discovered in *Arabidopsis thaliana,* IRT1, which is a cation transporter expressed in the roots of iron deficient plants. IRT1 gene homologues, ZRT1 and ZRT2 found in *Saccharomyces cerevisiae* were discovered to encode a high affinity and a low affinity Zn transporter proteins, respectively, and ZRT1 activity increased in Zn limited cells (Zhao & Eide, 1996). However, when the cells were exposed to toxic levels of Zn, ZRT1 activity was lost. Although, yeast is a practical model organism to study Zn uptake, findings do not provide complete comprehension of the role of ZIP proteins in plant Zn homeostasis. Similar to IRT1, ZIP1, ZIP2 and ZIP3 genes of Arabidopsis were isolated via mutant yeast strain. Furthermore, expression of these Arabidopsis ZIP genes

could rescue mutant yeast strain in a Zn limited environment. Moreover, it was found that these ZIP genes were only specific to Zn (Guerinot, 2000). ZIP1, ZIP3 and ZIP4 were shown to be accumulated in response to Zn deficiency in plants. It was found that ZIP1 and ZIP3 are root specific while ZIP4 accumulated in both the shoots and the roots of Zn deficient plants (Grotz & Guerinot, 2006). In *Oryza sativa*, OsIRT1 and OsIRT2 genes has been isolated. OsIRT1 and OsIRT2 are expressed predominantly in roots, mainly epidermis and cortex, as well as in stems and these transporters are induced by Fe deficient conditions. Under Fe deficient conditions, both genes are highly expressed in the phloem of roots and stems (Ishimaru et al., 2006). Although, IRT2 protein in *Arabidopsis thaliana* could transport both Zn and Fe, OsIRT1 and OsIRT2 were specific to Fe in rice. The specificity of HvIRT1 protein in *Hordeum vulgare* was similar to that of Arabidopsis, where HvIRT1 was shown to restore growth of Zn, Fe and Mn uptake defective yeast (Pedas & Husted, 2009).

On the other hand, from wheat, only one ZIP protein from the tetraploid emmer wheat, TdZIP1, was discovered (Durmaz et al., 2009). Study demonstrated that ZIP1 transcript levels are elevated under Zn deficiency. Moreover, deletion of 20 amino acids from the last transmembrane domain of TdZIP1 caused mislocalization.

Research Questions

Zn is essential for all living organisms because of its functional, structural and regulatory roles in more than 300 enzymes found in eukaryotes (McCall, 2000). In plants and humans, trace metal deficiencies cause an altered expression or function of proteins at the metabolic level and may lead to physiological drawbacks in plants and even psychological problems in humans. Plants with improved Zn status may help to alleviate these issues globally. Therefore, it is crucial to understand genes involved in Zn homeostasis. Lack of information on the micronutrient status of Montana wheat and barley was the main reason of this study and most commonly cultivated Montana wheat and barley varieties were included in the study. The aim of this study was; (I) to identify ZIP1 and IRT2 genes in Montana wheat and barley cultivars, (II) to study the physiological response, effectiveness in Zn uptake capacity and Zn translocation to plant edible parts by subjecting these cultivars to Zn deficient and Zn adequate environments and (III) to comprehend the micronutrient diversity and Zn grain localization of local wheat and barley cultivars.

CHAPTER TWO – MATERIALS AND METHODS

Plant Material

A total of 17 wheat and 4 barley cultivars were obtained from Montana State University (Table 2.1). Obtained seeds of each cultivar were grown in separate pots under greenhouse conditions (light/dark regime 16/8 h, temperature 25°C/20°C and photon flux density of 350 µmol m^{-2} s^{-1}) at the Plant Growth Center, MSU. Grains were randomly collected from different plants of the same cultivar in early 2017. Following preliminary Zn concentration data containing 45 wheat and 21 barley cultivars grown in Montana (preliminary data); 3 spring wheat, 4 durum wheat, 2 winter wheat and 2 barley cultivars were ranked in agreement with high or low Zn concentration among each crop and were included in hydroponics study (Table 2.2).

Crop	Cultivar	Uses
Barley	AC Metcalfe	Malting
Spring Wheat	Brennan	Baked goods
Winter Wheat	Carter	Baked goods
Durum Wheat	CDC Fortitude	Pasta
Spring Wheat	Chinese Spring	Cytogenetic studies
Spring Wheat	Corbin	Baked goods
Durum Wheat	Divide	Pasta
Spring Wheat	Fortuna	Baked goods
Durum Wheat	Havasu	Pasta
Barley	Haybet	Animal feed
Barley	Hector	Animal feed
Winter Wheat	Judee	Baked goods
Winter Wheat	Ledger	Baked goods
Spring Wheat	Mc Neal	Baked goods
Spring Wheat	Mott	Baked goods
Durum Wheat	Mountrail	Pasta
Winter Wheat	Norris	Baked goods
Durum Wheat	Silver	Pasta
Spring Wheat	Vida	Baked goods
Winter Wheat	Warhorse	Baked goods

Table 2.1. Cultivars included in grain element concentration study.

Crop	Cultivar	Grain Zn Concentration (mg kg^{-1})
Barley	AC Metcalfe	15 (Low)
Spring Wheat	Brennan	25 (Low)
Winter Wheat	Carter	17 (Low)
Spring Wheat	Chinese Spring	45 (High)
Durum Wheat	Divide	35 (High)
Spring Wheat	Fortuna	35 (High)
Durum Wheat	Havasu	49 (High)
Barley	Haybet	38 (High)
Winter Wheat	Ledger	22 (High)
Durum Wheat	Mountrail	24 (Low)
Durum Wheat	Silver	27 (Low)

Table 2.2. Zn concentrations of cultivars used in the hydroponics study.

DNA Isolation

Leaf DNA samples from wheat and barley cultivars included in the hydroponics study were collected. DNA was isolated using FastDNA® Kit and FastPrep® Instrument (MP Biomedicals, Santa Ana, CA).

Primer Design

Literature search on ZIP transporters in wheat obtained the mRNA complete coding sequence from National Center for Biotechnology Information (NCBI) webpage of *Triticum aestivum* ZIP transporter accession was taken based on the following accession; (ZIP 1 Accession number: AY864924.1). IRT transporter was obtained from *Oryza sativa* mRNA complete coding sequence (IRT 2 Accession number: AB126086.1) Refer to Appendix A for detailed information about the sequences. Genomic sequences of ZIP1

homolog and IRT2 ortholog mRNA complete coding sequences were obtained by BLAST searching in wheat genome assembly (IWGSC) (https://www.wheatgenome.org/). Exon-intron structures of the genomic sequences of the ZIP and IRT genes with their corresponding cDNA sequences were predicted using Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi). Primers were designed based on the assembled genomic DNA sequences of the ZIP and IRT genes including start and stop codon sites by using SnapGene version 3.1.4. The 18S rRNA (Gen-Bank accession no: X67238 was used as an internal control (Nicot, 2005). Primer pairs of ZIP1 that were unsuccessful in obtaining desired amplicons are listed in Table 2.4.

Name	Orientation	Molarity	Primer Sequence 5'- 3'	Amplicon Size (bp)
ZIP 1	Forward	20.2 nmol	GCC TTG AAG AAA CCA CCA TG	1240
	Reverse	23.1 nmol	TCA GTC CCA TAT CAT GAC GAC A	
IRT 2	Forward	14.9 nmol	TGC CAC AAC GTC CCC AAG	1794
	Reverse	34 nmol	TCA CGC CCA TTT GGC CAT G	

Table 2.3. Primer sequences of ZIP1, IRT2 and 18s rRNA used in PCR amplification study.

Table 2.3 Continued

Control	Forward	46 nmol	GGG CAT TCG TAT TTC ATA GTC AGA G	101
	Reverse	43.2 nmol	CGG TTC TTG ATT AAT GAA AAC ATC CT	

Name	Orientation	Primer Sequence 5'- 3'
ZIP 1	Forward	ATG GCG AAG ATG GCA AGG TCG
	Reverse	TCA GTC CCA TAT CAT GAC CAC
ZIP 1	Forward	GGC GAA GAT GGC AAG GTC GA
	Reverse	GTC CCA TAT CAT GAC CAC GGC C

Table 2.4. Sequences of ZIP1 primer pairs used in PCR amplification that were unsuccessful in obtaining desired amplicons.

PCR Analysis

Polymerase chain reactions (PCR) using ZIP 1 and IRT 2 primers were carried out in a total volume of 25 µl. The reactions were performed with concentrations of the reagents as follows: 5X OneTaq GC Buffer, 0.5 µL OneTaq High GC Enhancer, 0.5 µL of 10 mM dNTP mix, 1 µL of 10 µM forward and 1 µL of 10 µM reverse primers, 1 µL DNA template, and 0.2 µL of 5 mM OneTaq DNA Polymerase. Amplifications were performed in BioRad C1000 Touch Thermal Cycler. After amplification, PCR products were run on 1%

agarose gel in 5X TBE buffer at 140 V for about 40 minutes. The bands were stained with GelRed and visualized under UV light. NEB™ 2-Log DNA Ladder, EZ Load™ 100 bp Molecular Ruler and EZ Load™ 1 kb Molecular Ruler were used to determine the size of DNA bands. PCR experiments were repeated for three times.

PCR Conditions

Cycles	Temperature (°C)	Duration
1	94 °C	00:30
35	94 °C	00:30
	57 °C	00:45
	68 °C	3:00
1	68 °C	5:00
	4 °C	∞

Table 2.5. PCR conditions used in the amplification of ZIP1, IRT2 and 18S rRNA.

Gel Extraction

The agarose gel fragments, both amplified with ZIP1 and IRT2 primers with expected molecular weights, were excised with a clean scalpel. The bands of interest were purified using Zymoclean™ Gel DNA Recovery Kit following the manufacturer's protocol. DNA was eluted in ≥ 6 µL of Elution Buffer and quantified by measuring the absorbance

of samples at 260 nm using a NanoDrop spectrophotometer. The samples were stored at 4°C.

Sequencing

Sequencing of extracted gel samples were obtained by GenScript (GenScript Corp., NJ, USA) following the company instructions.

Dithizone Staining

To localize Zn in wheat and barley grains, 1,5-diphenyl thiocarbazone (Merck) solution was prepared by dissolving in 500 mg L^{-1} in analysis-grade pure methanol. Individual dry grains from each cultivar were imbibed in sterile distilled water for 1 hour at room temperature and excised longitudinally along the crease with a scalpel. Seeds were stained in DTZ solution for thirty minutes at room temperature. Samples were rinsed thoroughly with sterile distilled water and blotted dry with Kimwipes. For DTZ staining of germinating seeds, ten individual seeds from each cultivar were surface sterilized with 75% ethanol for one minute and 30% sodium hypochlorite for twenty minutes and rinsed thoroughly with sterile distilled water for six times. Seeds were later placed on sterile filter paper (Whatman No:1) moistened with sterile distilled water and germinated in Petri dishes for 36 hours at 25°C in incubator. After germination, seeds were stained as previously. Seed flour samples of each cultivar were obtained by grinding seeds for five minutes in a clean coffee grinder for five minutes (Mr. Coffee, Ohio). 0.2 grams of powder sample from each cultivar were placed in 24 well plates and stained 200 uL of DTZ solution

for thirty minutes. Stained cereal grains and flour samples were immediately visualized by AmScope SM-1BSX-64S stereomicroscope and photographed by a high-resolution camera. Quantification of dithizone staining is achieved via extraction of red color from the flour samples (1 µL) by methanol followed by centrifuging at 5000 g for ten minutes. All extracts were then measured spectrophotometrically at 512 nm on a Genesys™ 30 Spectrophotometer (Fisher Scientific, Fairlawn, NJ). Zn specificity of dithizone was determined by incubating seeds of AC Metcalfe, a low Zn concentration (15 mg kg^{-1}) with 0.01 M, 0.05 M and 0.1 M of Cu, Cd, Fe, Mn and Zn sulfate salts for 20 hours. After incubation, seeds were washed thoroughly with sterile milli-Q water and dried at 50°C until the seeds were completely dry. Ground seeds were stained with DTZ as previously described.

Hydroponics Experiment

Detection of Zn deficiency tolerance (ability of plants to maintain significant yields under low Zn) among wheat and barley cultivars were performed by surface sterilization of seeds as mentioned previously and grown on sterile filter paper (Whatman No:1) for seven days at 25°C in incubator. After seven days, two seedlings from each cultivar were transplanted into a hydroponics sponge in a continuously aerated in 10 L of hydroponics bucket system (Pathonor brand, https://www.amazon.com/dp/B01N3UVHVC?psc=1). There were 11 open space in each bucket, therefore, seedlings from each cultivar (11

cultivars) were evenly distributed. Experiment was designed in a complete randomized design with a total of three replications. Experiment was run twice.

Two seedlings were thinned to a single seedling after 4 days. The composition of the nutrient solution was as follows: 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 100 µM Fe-EDTA, 1.0 µM H_3BO_3, 1.0 µM $MnSO_4$, 0.2 µM $CuSO_4$ and 0.02 µM $(NH_4)_6Mo_7O_{24}$. Solutions were prepared using deionized water. There were two treatment groups (Zn deficient and Zn sufficient). Zn sufficient solution contained adequate Zn (1 µM $ZnSO_4$) and the deficient solution was absent in Zn. Plants were grown in a growth chamber under controlled conditions (light/dark regime 16/8 h, temperature 24°C/22°C and photon flux density of 100 µmol m^{-2} s^{-1}) at the Plant Growth Center, MSU. Nutrient solutions were renewed every 4 days. Before renewal of the solution, previous solution was completely drained, inside of the bucket was brushed and cleaned with deionized water. After 21 days of growth in the growth chamber, shoots and roots were separated and rinsed with deionized water thoroughly to remove adsorbed elements on the tissue surface. Shoot includes leaf blade, sheath and stem. Roots and shoots were dried at 35°C until dry weights were constant and weighed for determination of dry matter production. Zn efficiency of the cultivars were determined as the ratio of dry weight at deficient Zn to dry weight at adequate Zn per plant and the root-to-shoot Zn translocation index was calculated as the ratio of total shoot Zn content to total Zn content (root and shoot) per plant (Rengel & Graham, 1996).

Cultivar Chinese Spring was included as a control in the hydroponics experiment due to possessing both ZIP1 and IRT2 genes. Study included nine wheat (Fortuna, Brennan, Chinese Spring, Ledger, Carter, Divide, Mountrail and Silver) and two barley (AC Metcalfe and Haybet) cultivars subjected to two treatments (-Zn and +Zn). Durum wheat cultivar Havasu, failed to germinate sufficiently and was eventually eliminated from the study.

Total Mineral Analysis

The dried shoot and root samples from the hydroponics experiment were ground. Approximately 0.5 g ground samples were digested using a 6 mL nitric acid / 12 mL hydrogen peroxide digestion and the concentrations of Al, B, Ca, Cd, Cu, Fe, K, Mg, Mn, P, S and Zn were determined using a Perkin Elmer 5400 inductively coupled plasma optical emission spectrometry (ICP-OES) analyzer (Zarcinas, 1987). Additionally, an amount of 2 g of whole grain samples from each cultivar were ground and analyzed for elements. The whole grain analysis was performed on 22 cultivars with three replicates randomly collected from different plants of the same cultivar. Data provided courtesy of AGVISE Laboratories, Northwood, ND. Content of the elements were calculated as follows: Content = Dry Weight x Concentration.

Data Analysis

Statistical analysis was performed using the SPSS statistical software (version 1.0.0.642). Data from two experiments were combined and significant differences between means were determined using the least significant difference (LSD) mean separation procedure at the 5% level. Correlation coefficients were obtained using Pearson correlation analysis.

CHAPTER THREE – RESULTS

Gene Models of ZIP1 and IRT2

Exon-intron structures of the genomic sequences of the ZIP1 and IRT2 genes with their corresponding cDNA sequences were constructed using Splign and NCBI Blast, respectively. Figure 3.1 and Figure 3.2 illustrate the structures of ZIP1 and IRT2 and their alignment to the *Triticum aestivum* mRNA and *Oryza sativa* mRNA, respectively. Span section indicates the lower and the higher alignment bounds of the complementary DNA (either ZIP1 cDNA or IRT2 cDNA in this case) to the common wheat genomic DNA. Therefore, ZIP1 gene was found to be 1240 bp in length whereas IRT2 was 1794 bp. Both genes possessed two exons (colored area) and one intron. Overall (ZIP1 91.48%) and identity (IRT2 89%) indicates the percent amount of cDNA sequence overlapped with the sequence of genomic DNA.

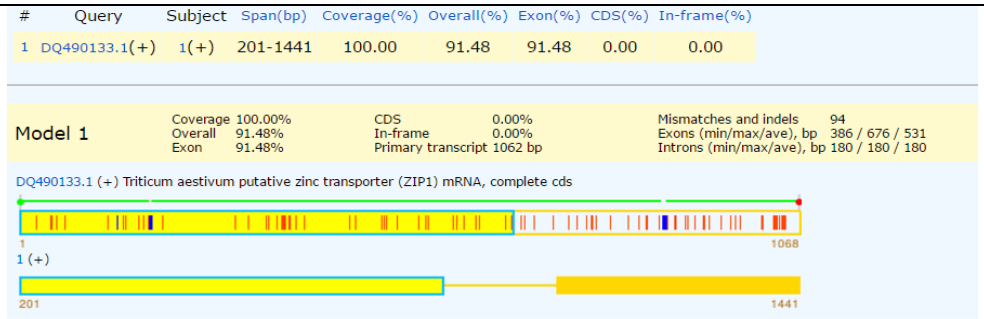
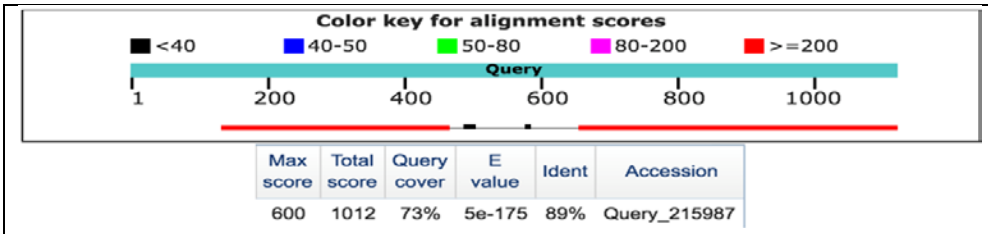


Figure 3.1. Exon-intron structures of the genomic sequence of the ZIP1 constructed using Splign.



Max score	Total score	Query cover	E value	Ident	Accession
600	1012	73%	5e-175	89%	Query_215987

Figure 3.2. Exon-intron structures of the genomic sequence of the IRT2.

PCR Amplification and Gel Extraction using ZIP1 and IRT2 Primers

Results obtained from PCR amplification using ZIP1 primer were successful in some of the cultivars. Amplified fragments from different samples were about 1240 kb, as expected. Spring wheats; Fortuna, Chinese Spring and Brennan, winter wheats; Ledger and Carter, durum wheats; Havasu, Divide, Mountrail confirmed the existence of ZIP1 gene. Chinese Spring was used as a control plant in this study. However, ZIP1 gene could not be amplified under these conditions in durum wheat Silver and barley cultivars AC Metcalfe and Haybet. In addition, IRT2 gene was successfully amplified in Fortuna, Brennan, Ledger and Chinese Spring. The fragments around 1200 kb for ZIP1 and 1794 kb for IRT2 were extracted from gels to be purified and sequenced for conformation. 18S rRNA primer was used as an internal control. Expected bands (101 bp) were successfully obtained from each cultivar. Table 3.1 summarizes the PCR results.

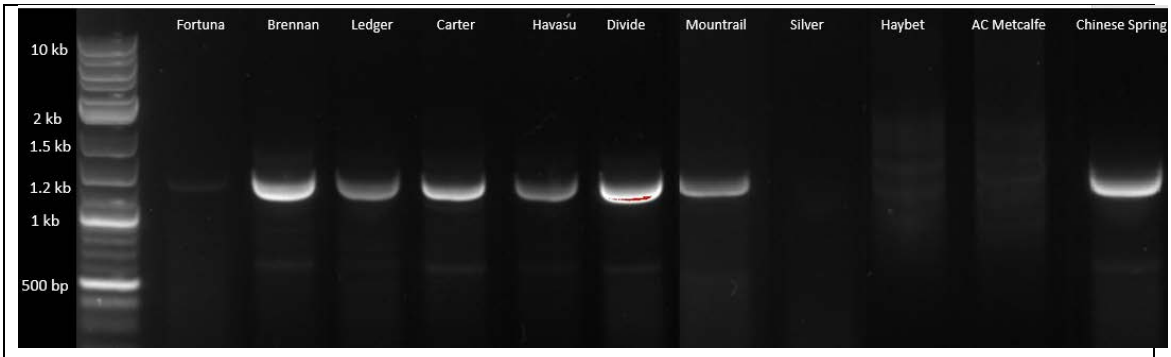


Figure 3.3: PCR gel picture using ZIP 1 primer. Expected bands (1240 bp) were obtained from the leaf DNA samples of Fortuna, Brennan, Ledger, Carter, Havasu, Divide, Mountrail, Silver, Haybet, Tioga and Chinese Spring were used as a template. 10 kb marker was used in order to detect molecular weights.

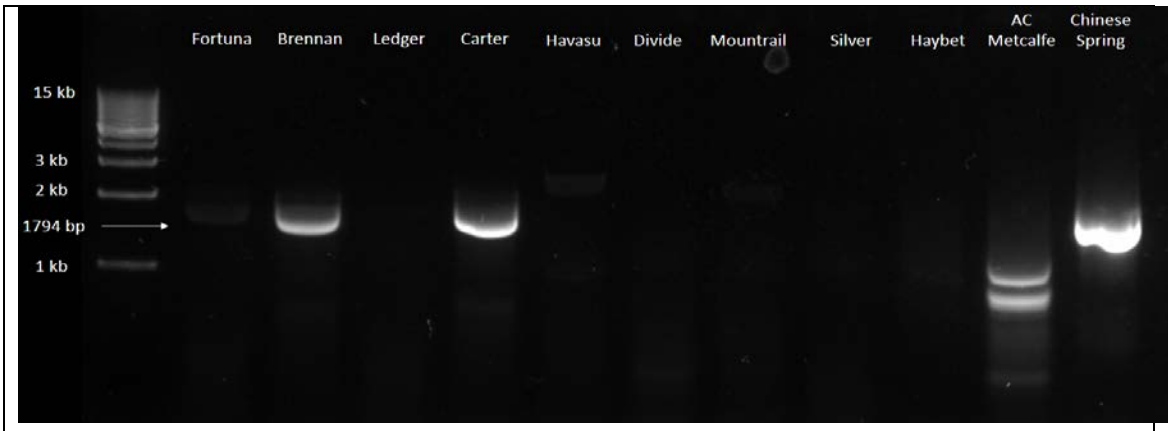


Figure 3.4: PCR gel picture using IRT 2 primer. Expected bands (1794 bp) were obtained from the leaf DNA samples of Fortuna, Brennan, Ledger, Carter, Havasu, Divide, Mountrail, Silver, Haybet and Chinese Spring were used as a template. 15 kb marker was used in order to detect molecular weights.

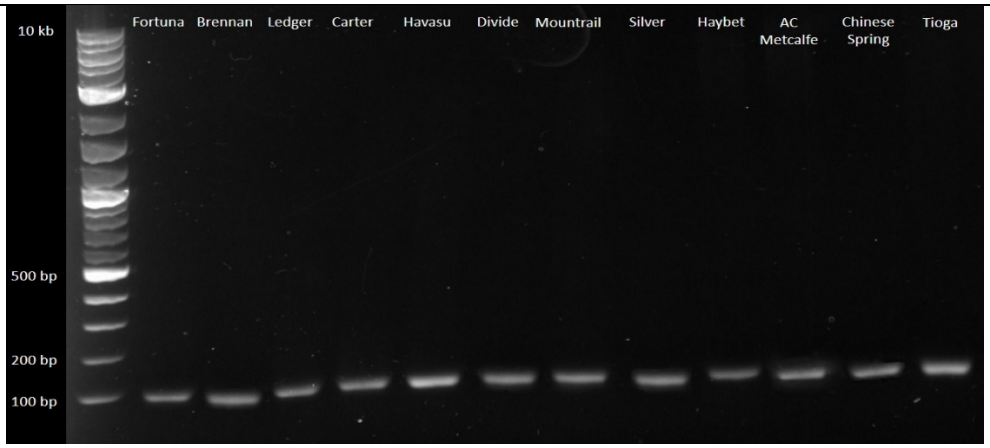


Figure 3.5: PCR gel picture using 18S rRNA primer. 10 kb marker was used in order to detect molecular weights.

Cultivar	ZIP1	IRT2	Control (18S rRNA)
Fortuna	+	+	+
Brennan	+	+	+
Ledger	+	-	+
Carter	+	+	+
Havasu	+	-	+
Divide	+	-	+
Mountrail	+	-	+
Silver	-	-	+
Tioga	+	+	+
Haybet	-	-	+
AC Metcalfe	-	-	+
Chinese Spring	+	+	+

Table 3.1 Summary of PCR results.

Sequence Analysis

ZIP1 gene sequenced from Chinese Spring matched 98% with the sequence from *T. aestivum* whereas IRT2 sequence from Chinese Spring matched 99%. Please refer to the Appendix C for details.

Dithizone Staining of Wheat and Barley Grains

Wheat and barley grains were excised along the crease and stained with diphenyl thiocarbazone (DTZ) to study the deposition of Zn in grain, when bound to Zn, DTZ forms a pink/red colored complex and is highly selective for this metal. DTZ staining of matured seeds showed that Zn was found to be present mostly in the embryo and aleurone/sub-aleurone layer in wheat as well as in barley seeds. Pink/ red color formation intensified as the Zn concentration increased (Figure 3.6). However, color intensity was not significant when the Zn concentration was lower than 25 mg kg^{-1}.

Dithizone Staining of Whole Grain Flour

Dithizone staining of whole grain flour did not show significant red color formation as most of the grain Zn levels were less than 33 mg kg^{-1} (Figure 3.7). Moreover, during DTZ staining of flour samples, sample matrix color may interfere with the stain. For instance, McNeal showed a brownish color when ground (Figure 3.7). Therefore, stain color was not visible even though the Zn concentration was higher than 25 mg kg^{-1}. Spectrophotometric quantification of flour samples indicated there was a significant correlation between seed Zn concentration and the spectral absorbance of the dithizone stained flour extracts, suggesting DTZ staining of grain flour did correlate with increasing Zn concentration, however, color formation was not visible to the naked eye (Figure 3.8). Zn specificity of dithizone staining was performed on low Zn

concentration (15 mg kg^{-1}) AC Metcalfe seeds. Clearly, only increasing Cd levels did interfere with the dithizone and formed an orange/red color. However, red color was not as distinct as Zn, therefore, much higher Cd levels may interfere with Zn and reduce the accuracy of the DTZ staining method (Figure 3.9).

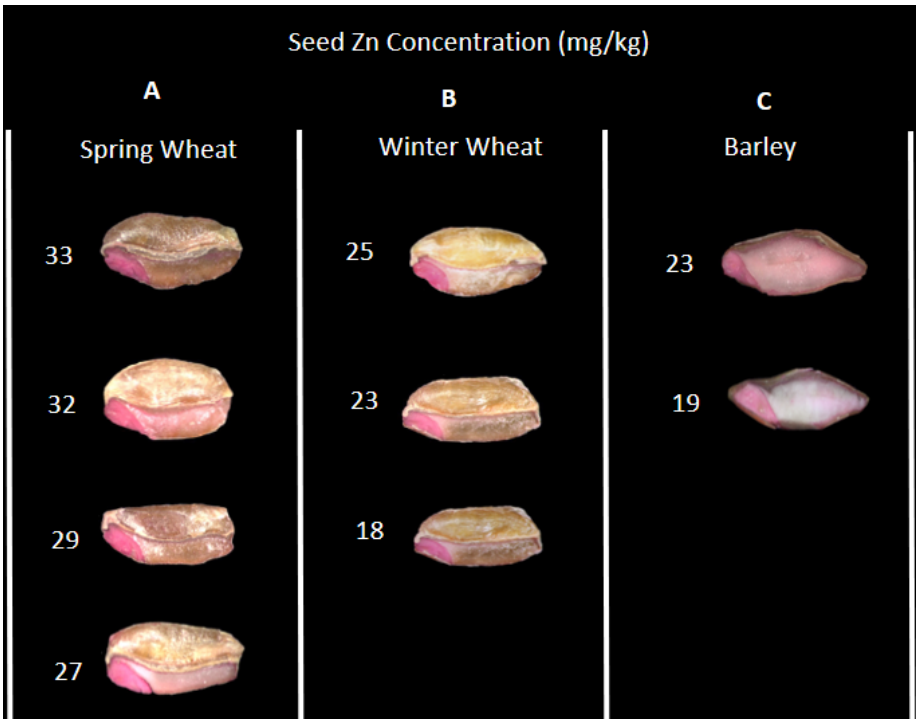


Figure 3.6. Localization of Zn in dry seeds of **(A)** from top to bottom; *T. aestivum* L. cv McNeal, *T. aestivum* L. cv Mott, *T. aestivum* L. cv Vida and *T. aestivum* L. cv Corbin. **(B)** from top to bottom; *T. aestivum* L. cv Norris, *T. aestivum* L. cv Judee and *T. aestivum* L. cv Warhorse **(C)** from top to bottom; *H. vulgare* L. cv Hector and *H. vulgare* L. cv CDC Fortitiude shown here after 30 minutes of DTZ staining at room temperature. Concentrations represent seed Zn levels.

Figure 3.7. DTZ staining of whole grain flour of wheat and barley cultivars. Cultivars are; Mc Neal, Mott, Vida, Corbin, Norris, Hector, Judee, CDC Fortitude and Warhorse respectively. Numbers below represent Zn concentration of seeds.

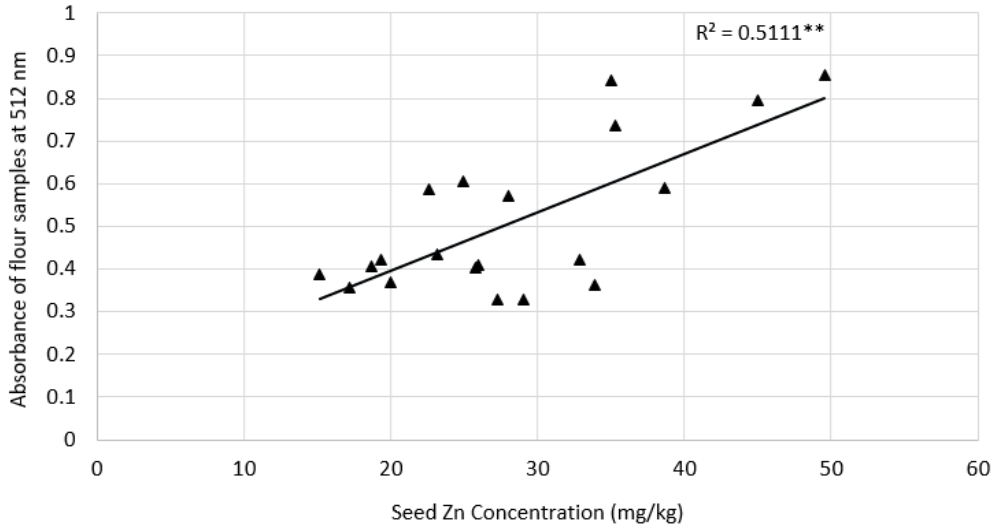


Figure 3.8. Relationship between Zn concentration and spectral absorbance of DTZ stained whole grain flour samples of Montana wheat and barley cultivars. Each triangle represents the average of spectral absorbance and average seed Zn concentration of three independent replications. (**, $p < 0.01$)

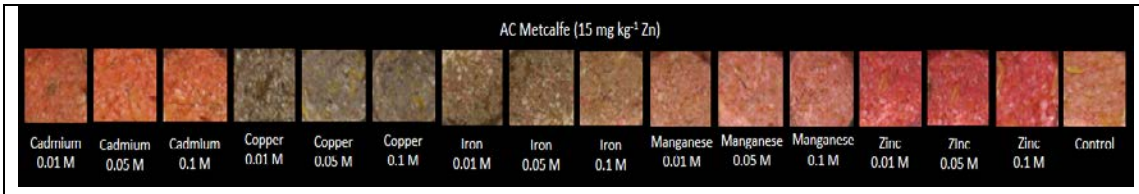


Figure 3.9. Zn specificity of dithizone staining. Seeds of AC Metcalfe were incubated in three concentrations (0.01 M, 0.05 M, 0.1 M) of Cd, Cu, Fe, Mn and Zn solutions in order to demonstrate the interference of other metals in red color formation by DTZ staining.

Micronutrient Diversity of Montana Wheat and Barley Grains

Seeds from each cultivar was grown under greenhouse conditions and harvested for analysis. Average Zn concentration of wheat and barley grains ranged from 15 to 49 mg kg^{-1} whereas Fe concentrations ranged from 34 to 51 mg kg^{-1} (Table 3.2). The highest grain Zn concentrations were detected in spring wheat with an average of 33 mg kg^{-1} and lowest in winter wheat (20 mg kg^{-1}). There was a significant difference in grain Zn concentration between the means of winter wheat and durum wheat cultivars ($p < 0.05$) (Figure 3.10). On average, spring wheat grain Zn concentrations were 39% higher than that of winter wheat grains (Figure 3.11). Moreover, mean of durum wheat Cd levels were significantly higher than the mean of other crops. Grain Fe and P concentrations were highest in Chinese Spring (Figure 3.12). Grain Zn, grain Fe and grain P concentrations across all cultivars significantly correlated (Figure 3.13), however, no correlation was detected for grain Cd concentrations and rest of the elements ($r = 0.36$, $p = 0.12$). Sulfur,

constituent of proteins involved in Fe and Zn binding, correlated significantly with both of these elements (Figure 3.14).

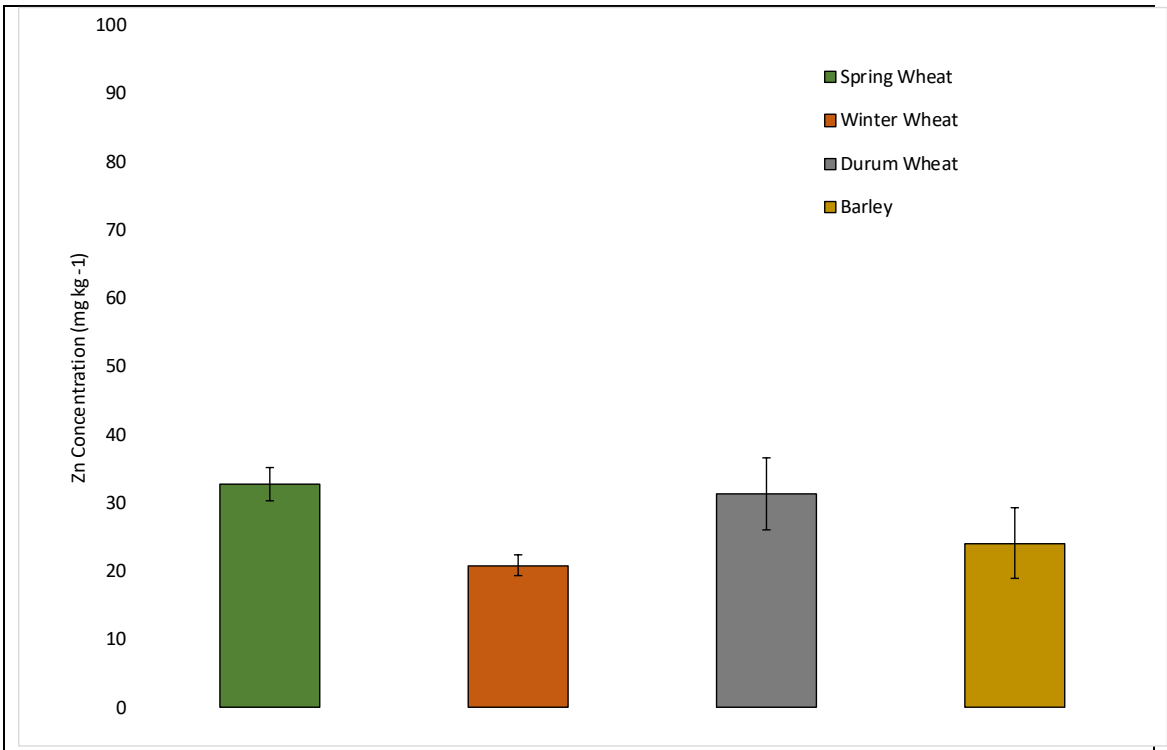


Figure 3.10. Average seed Zn concentrations of wheat and barley crops. Vertical bars represent ± standard error of three independent replications. Letters denote significant difference at the 0.05 level between means of crop varieties.

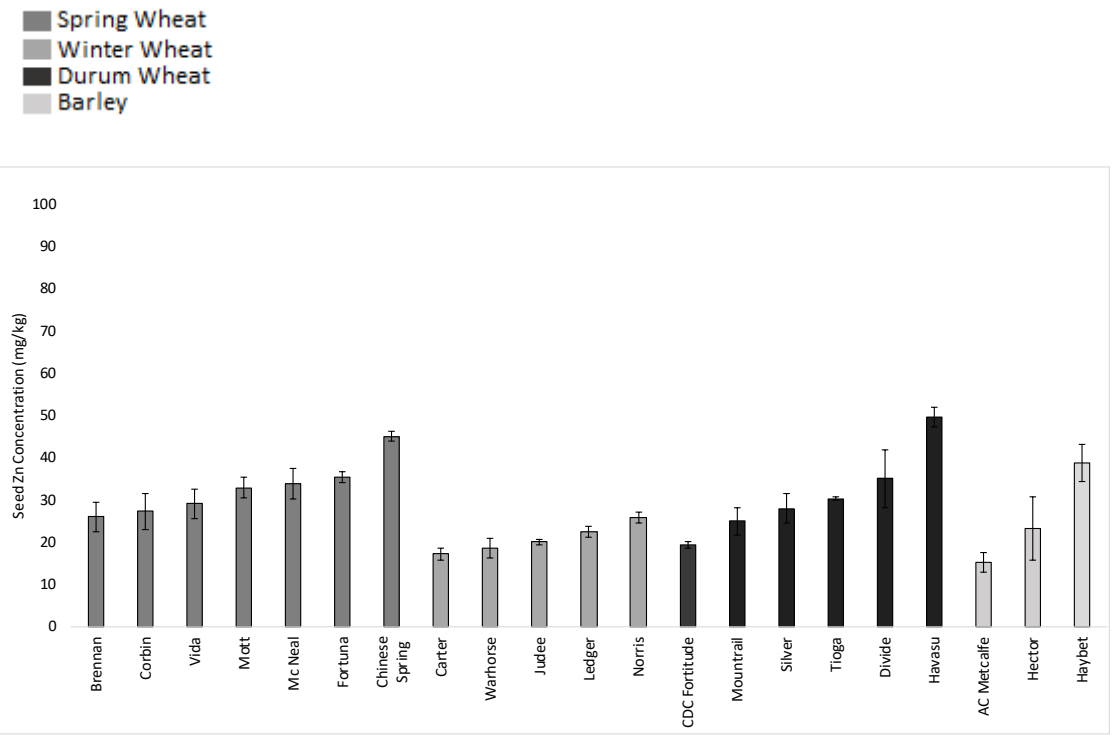


Figure 3.11. Seed Zn concentrations of Montana wheat and barley cultivars in increasing order. Vertical bars represent means ± standard error of three independent replications.

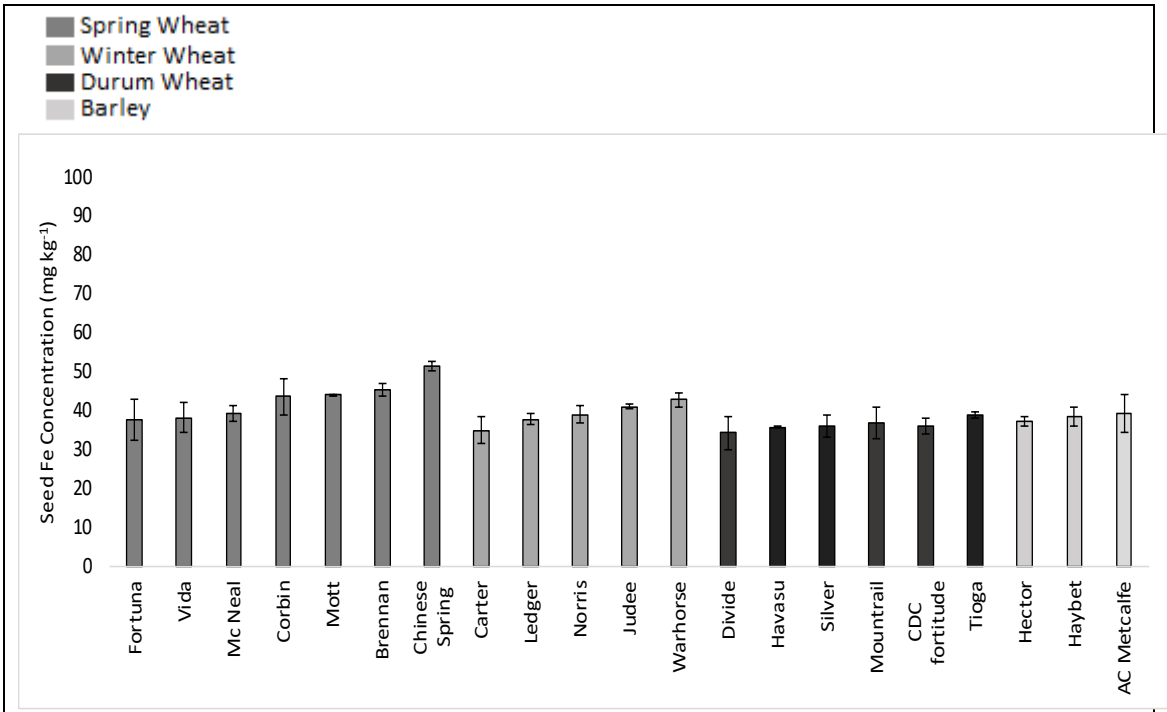


Figure 3.12. Seed Fe concentrations of Montana wheat and barley cultivars in increasing order. Vertical bars represent means ± standard error of three independent replications.

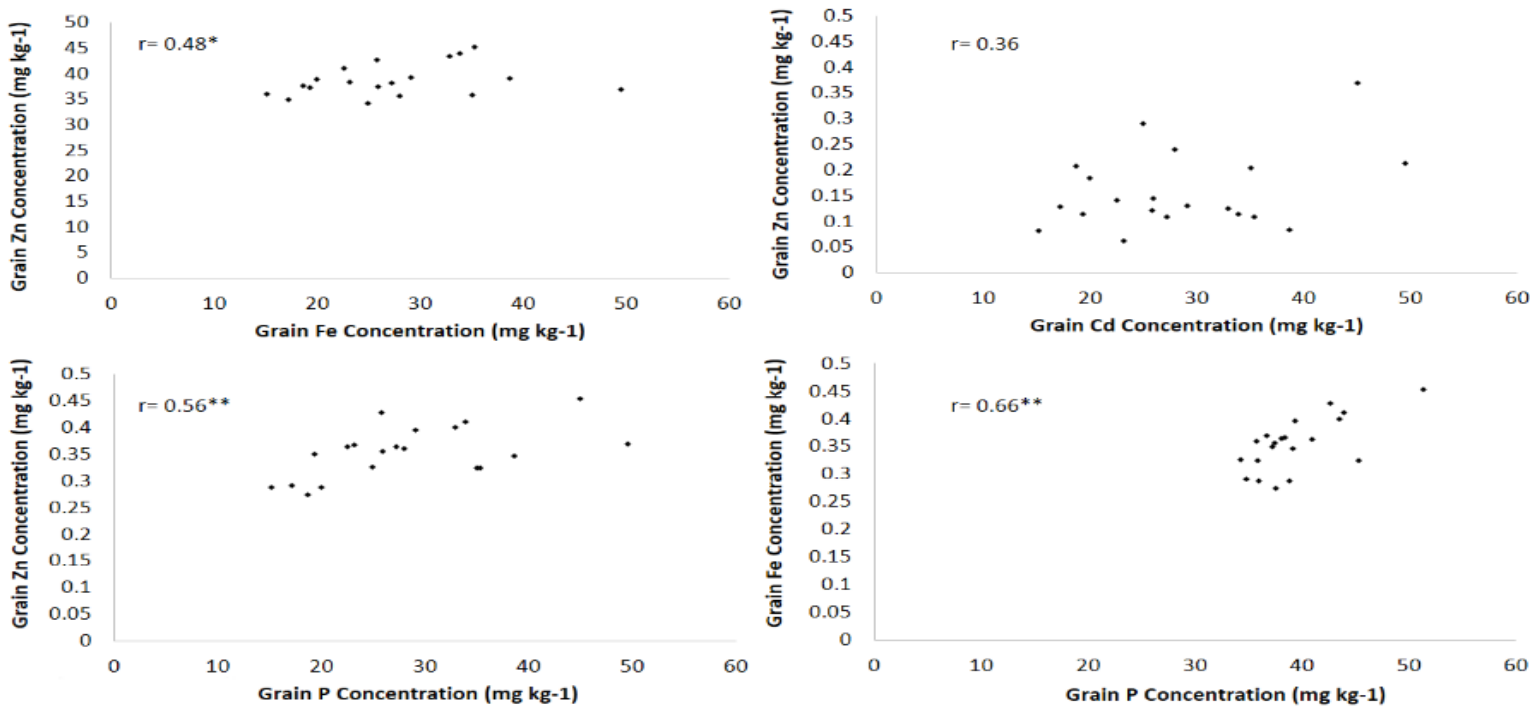


Figure 3.13. Relationships between grain element concentrations. * and ** indicates significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively.

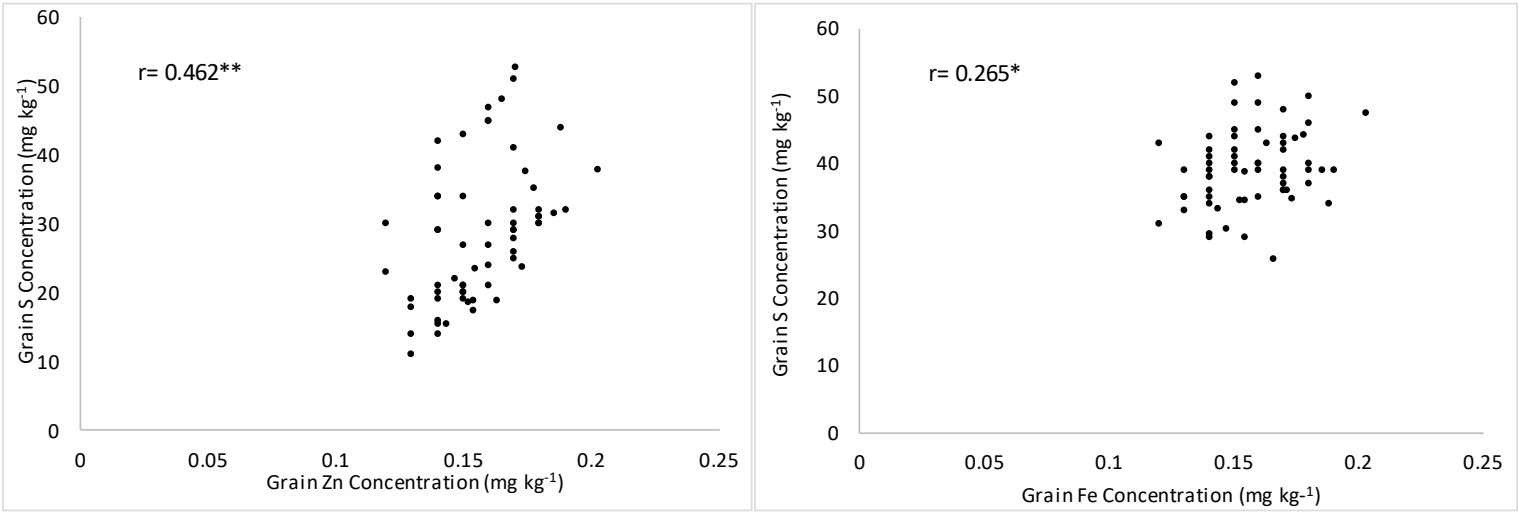


Figure 3.14. Relationships between grain S and grain Zn and Fe element concentrations. * and ** indicates significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Cultivar	Seed Zn Concentration (mg kg-1)	Seed Fe Concentration (mg kg-1)	Seed Cd Concentration (mg kg-1)	Seed P Concentration (mg kg-1)
Spring Wheat				
Brennan	25.94 ± 3.6 [1]	37.49 ± 2.0 [1]	0.14 ± 0.03 [1]	0.36 ± 0.01 [1]
Corbin	27.25 ± 4.3 [1]	38.08 ± 1.5 [1]	0.11 ± 0.02 [1]	0.36 ± 0.03 [1]
Vida	29.09 ± 3.5 [1]	39.32 ± 2.6 [1]	0.13 ± 0.04 [1]	0.40 ± 0.03 [2]
Mott	32.89 ± 2.5 [1]	43.48 ± 2.8 [1]	0.13 ± 0.02 [1]	0.40 ± 0.02 [2]
Mc Neal	33.87 ± 3.6 [1]	43.89 ± 0.4 [2]	0.11 ± 0.04 [1]	0.41 ± 0.01 [2]
Fortuna	35.33 ± 1.3 [2]	45.3 ± 3.3 [2]	0.11 ± 0.03 [1]	0.41 ± 0.001 [2]
Chinese Spring	45 ± 1.2 [3]	51.33 ± 0.1 [3]	0.37 ± 0.02 [1]	0.45 ± 0.01 [3]
Mean	32.77 a	42.7 e	0.16 d	0.39 g
Winter Wheat				
Carter	17.18 ± 1.4 [1]	34.83 ± 4.7 [1]	0.13 ± 0.04 [1]	0.29 ± 0.02 [2]
Warhorse	18.67 ± 2.3 [1]	37.61 ± 5.0 [1]	0.21 ± 0.02 [2]	0.27 ± 0.01 [1]
Judee	20 ± 0.6 [1]	38.84 ± 1.9 [2]	0.18 ± 0.01 [2]	0.29 ± 0.003 [2]
Ledger	22.56 ± 1.3 [1]	41 ± 4.2 [2]	0.14 ± 0.03 [1]	0.36 ± 0.01 [3]
Norris	25.82 ± 1.2 [2]	42.67 ± 2.1 [2]	0.12 ± 0.02 [1]	0.43 ± 0.02 [4]
Mean	20.84 b	38.99 ef	0.16 d	0.33 h
Durum Wheat				
CDC Fortitude	19.33 ± 0.9 [1]	37.25 ± 5.4 [1]	0.11 ± 0.02 [1]	0.35 ± 0.01 [1]
Mountrail	24.93 ± 3.3 [2]	34.27 ± 3.9 [1]	0.29 ± 0.07 [2]	0.33 ± 0.02 [1]
Silver	27.97 ± 3.5 [2]	35.71 ± 1.1 [1]	0.24 ± 0.08 [3]	0.36 ± 0.03 [2]
Tioga	30.33 ± 0.33 [2]	38.67 ± 0.88 [2]	0.41 ± 0.07 [4]	0.34 ± 0.00 [1]
Divide	35.04 ± 6.8 [2]	35.81 ± 1.2 [1]	0.2 ± 0.07 [3]	0.32 ± 0.02 [1]
Havasu	49.56 ± 2.3 [3]	36.72 ± 1.4 [1]	0.21 ± 0.02 [3]	0.37 ± 0.01 [2]
Mean	31.36 a	36.40 f	0.24 c	0.34 h
Barley				
AC Metcalfe (**Malting**)	15.15 ± 2.3 [1]	36 ± 3.9 [1]	0.08 ± 0.03 [1]	0.29 ± 0.02 [1]
Hector (**Feed**)	23.18 ± 7.5 [1]	38.37 ± 0.6 [1]	0.06 ± 0.02 [2]	0.37 ± 0.01 [2]
Haybet (**Feed**)	38.67 ± 4.4 [2]	39.12 ± 2.2 [1]	0.08 ± 0.03 [1]	0.35 ± 0.04 [2]
Mean	25.33 a	37.83 f	0.07 d	0.33 h

Table 3.2. Seed Zn, iron, cadmium and phosphorus concentrations of Montana wheat and barley cultivars. ± values represent standard error of three independent replications per grain. Letters denote significant difference at the 0.05 level between means of crop varieties within columns. Superscripted numbers denote significant difference at the 0.05 level between cultivars within each crop species.

Zn Efficiency and Plant Growth

Hydroponics experiment was performed to detect Zn efficiency and Zn mobilization to shoots. Plants were supplied with adequate Zn (1 µM $ZnSO_4$) and deficient Zn (no Zn) levels for 21 days. Deficiency symptoms were visible around 14th day. Deficiency symptoms were observed as stunted growth and interveinal chlorosis and necrosis which are the typical symptoms appear under Zn deficiency (Figure 3.15). Mean shoot heights of spring wheat, durum wheat, winter wheat and barley cultivars

were not significantly different when subjected to Zn deficiency. However, significant difference between treatment groups was observed and adequate Zn supply increased mean shoot heights of barley and durum wheat cultivars more than other crops (Figure 3.16). Moreover, when considered as percent increase, mean shoot length of durum wheat cultivars increased 110% followed by 58% increase with spring wheat, 42% increase with barley and 32% increase with winter wheat cultivars. Zn efficiency was determined by considering tolerance index and leaf deficiency symptoms. According to Zn efficiency, there was no significant difference among crops, but when taken individually, cultivars Carter, Fortuna and Brennan were recorded as Zn deficiency tolerant; whereas Chinese Spring, Ledger and all durum wheat and barley cultivars were recorded as susceptible. Although, Zn efficiency of Brennan was less than any of the durum wheat cultivars, leaf symptoms of chlorosis and necrosis was not as pronounced as the susceptible cultivars (Figure 3.17). Therefore, Brennan was determined to be tolerant to Zn deficiency. There was a significant difference in shoot and root dry weight between -Zn and +Zn treated plants. Furthermore, leaf numbers of cultivars were significantly increased when subjected to adequate Zn, however, there was no significant difference for leaf number among subjects (Table 3.4). Despite being involved in the hydroponics study, durum wheat cultivar Havasu failed to germinate sufficiently to be involved in the study.

Cultivar	Leaf Number -Zn	Leaf Number +Zn
Spring Wheat		
Fortuna	17.25 ± 3.75	29.33 ± 3.82
Brennan	15.00 ± 2.33	26.50 ± 3.50
Chinese Spring	10.75 ± 5.25	29.17 ± 8.17
Winter Wheat		
Ledger	16.50 ± 2.83	30.67 ± 0.35
Carter	19.50 ± 1.50	28.84 ± 2.84
Durum Wheat		
Divide	21.50 ± 2.12	24.00 ± 3.12
Mountrail	13.67 ± 1.15	35.33 ± 2.22
Silver	16.00 ± 2.23	30.33 ± 2.74
Barley		
Haybet	13.33 ± 1.28	34.33 ± 3.35
AC Metcalfe	12.00 ± 0.17	35.50 ± 8.50

Table 3.4. Leaf number of each cultivar after subjected to -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent the standard error of two independent replicates.

Figure 3.15. Zn deficiency symptoms observed on the leaves of durum wheat cultivar Silver.

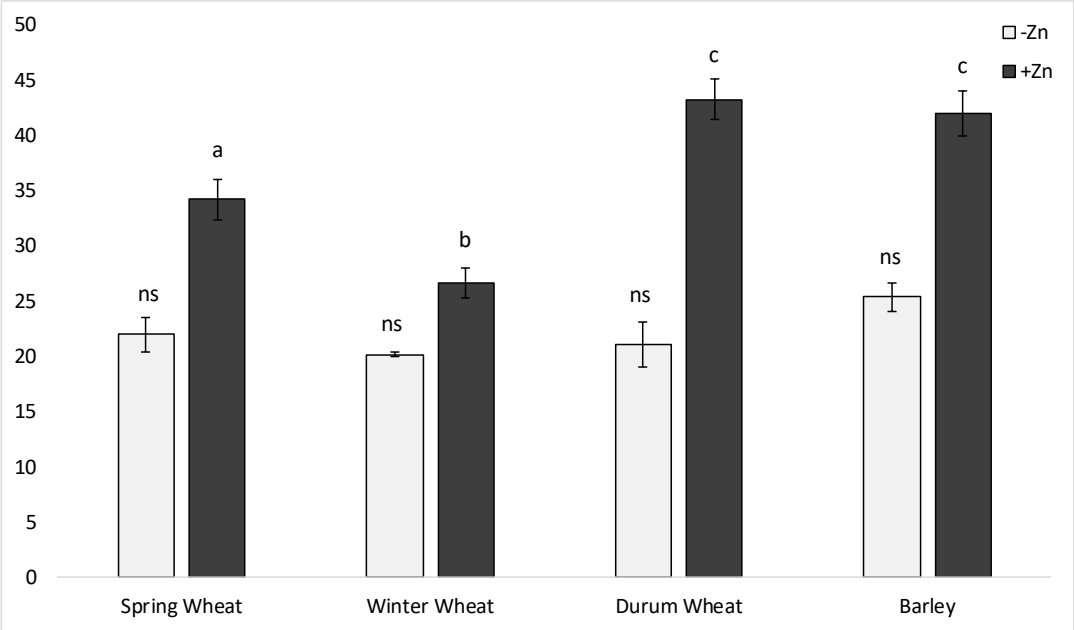


Figure 3.16. Mean shoot height of wheat and barley crops. Vertical bars represent means ± standard error of two independent replications. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant).

Figure 3.17. Physiological response of spring wheat cultivar Brennan when subjected to Zn deficient and Zn sufficient environments.

Cultivar	Shoot Dry Weight (g plant^{-1}) -Zn		Shoot Dry Weight (g plant^{-1}) +Zn		Zinc Efficiency (%)		Leaf Symptoms
Spring Wheat							
Fortuna	0.82 ± 0.06	1	1.35 ± 0.31	1	65	1	2
Brennan	0.58 ± 0.05	2	1.66 ± 0.22	1	35	2	2
Chinese Spring	0.39 ± 0.12	3	1.84 ± 0.56	1	21	3	5
Mean	0.6 ± 0.13		1.62 ± 0.14		40		
	ns		bc		ns		
Winter Wheat							
Ledger	0.57 ± 0.15	1	1.04 ± 0.02	1	39	1	3
Carter	0.71 ± .10	2	1.36 ± 0.14	2	71	2	1
Mean	0.64 ± 0.07		1.2 ± 0.16		55		
	ns		bd		ns		
Durum Wheat							
Divide	0.7 ± 0.14	1	1.68 ± 0.21	1	42	1	4
Mountrail	0.6 ± 0.12	1	1.77 ± 0.13	2	34	1	5
Silver	0.66 ± 0.08	1	1.61 ± 1.11	3	41	1	4
Mean	0.65 ± 0.03		1.69 ± 0.05		39		
	ns		b		ns		
Barley							
Haybet	0.93 ± 0.08	1	2.28 ± 0.09	1	41	1	3
AC Metcalfe	0.59 ± 0.17	2	2.01 ± 0.53	1	29	1	4
Mean	0.76 ± 0.17		2.14 ± 0.13		35		
	ns		a		ns		

Table 3.3. Shoot dry weight, Zn tolerance index of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent the standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant). Superscripted numbers denote significant difference at the 0.05 level between cultivars within each crop species.

Element Analysis

Element analysis was performed using plant shoots included in hydroponics study by ICP/OES. Generally, Zn supply significantly increased shoot Zn concentration and content (shoot dry weight x shoot Zn concentration) in all crop varieties. Table 3.5 represents the shoot concentrations of Zn and contents of Zn and Fe of all cultivars. According to Table 3.5, spring wheat cultivars were relatively higher than rest of the

crop varieties in shoot Zn concentration and content. Although significant increase among treatments were detected, no significant difference was observed between neither of the crop varieties for shoot Zn concentration or content. Moreover, there was no significant correlation between Zn efficiency and/or shoot Zn concentration and shoot Zn content with or without Zn supplementation; suggesting an increase in Zn deficiency tolerance was not reflected by the increase in Zn concentration or Zn content. Highest shoot Zn concentration increase was observed in Haybet, followed by Carter. Percent increase in shoot Zn content was not correlated with percent increase in shoot Zn concentration. Therefore, Zn deficiency tolerant cultivars cannot be differentiated from the susceptible ones by increased Zn concentrations or contents. As Table 3.5 illustrates, deficiency susceptible cultivars harbor higher shoot Fe concentrations in Zn deficient conditions than tolerant ones while doing the opposite during Zn sufficient conditions, suggesting ZIP genes in these cultivars may have specificity for Fe as well. However, shoot Fe contents of barley cultivars were not affected by the Zn supply, therefore Fe specificity of ZIP genes may not be the only parameter involved in Fe uptake difference.

Root to Shoot Zn, Iron, Phosphorus and Cadmium Translocation Index

Root to shoot Zn translocation index was calculated as the ratio of total shoot Zn content to total Zn content (root and shoot) per plant. As expected, all cultivars translocated more Zn to the shoot when supplied with Zn (Table 3.6). Zn deficiency

tolerant cultivar Carter showed the highest Zn translocation index (45%) than any other cultivar under deficiency. However, no relation was found in regards to Zn efficiency and Zn translocation. Deficiency susceptible durum wheat cultivars translocated less Zn to the shoots when supplied with Zn. On the contrary, durum wheat and barley cultivars translocated more Fe to the shoots under Zn deficiency. Interestingly, grain Fe concentration and Fe translocation index under Zn deficiency was significantly correlated (p=0.019), suggesting grains with lower Fe levels translocate more Fe to edible parts under Zn deficiency. However, the relation is dependent on the grain Zn levels. Cultivars with higher grain Zn concentrations grown under Zn sufficient condition translocated significantly lower Fe to the shoots (p= -873). Moreover, grain Zn, Fe, P and Cd concentrations were found to inversely affect Zn translocation under deficiency. On the other hand, cultivars with lower grain Zn concentrations demonstrated greater ability in translocating Zn to the shoots when Zn supplied. P translocation index was adversely affected under Zn sufficient conditions (Figure 3.18). Whereas, Zn levels did not show any effect on Cd translocation (Figure 3.19).

Cultivar	Shoot Zn Concentration (mg kg^{-1}) -Zn		Shoot Zn Concentration (mg kg^{-1}) +Zn		Shoot Zn Content (µg plant^{-1}) -Zn		Shoot Zn Content (µg plant^{-1}) +Zn		Shoot Fe Content (µg kg^{-1}) -Zn		Shoot Fe Content (µg kg^{-1}) +Zn	
Spring Wheat												
Fortuna	13.00 ± 1.00	1	34.33 ± 1.72	1	10.34 ± 0.22	1	46.50 ± 3.14	1	94.63 ± 27.69	1	97.20 ± 17.21	1
Brennan	17.33 ± 9.00	1	23.17 ± 0.83	2	8.01 ± 2.89	1	39.37 ± 5.13	1	70.05 ± 7.65	2	112.00 ± 23.95	2
Chinese Spring	36.50 ± 28.00	1	38.00 ± 4.00	1	10.05 ± 5.52	1	47.56 ± 8.66	2	41.57 ± 11.20	3	98.78 ± 35.25	3
Mean	22.28 ± 7.22		31.83 ± 4.46		9.47 ± 0.73		44.48 ± 2.57		68.75 ± 15.33		102.66 ± 4.69	
	ns		ns		a		ns		c		f	
Winter Wheat												
Ledger	15.67 ± 8.67	1	36.00 ± 4.33	1	7.20 ± 1.97	1	45.99 ± 0.97	1	67.77 ± 28.05	1	59.20 ± 6.08	1
Carter	8.17 ± 2.84	1	28.50 ± 3.83	2	6.03 ± 2.77	1	31.72 ± 2.62	2	88.21 ± 1.39	2	85.87 ± 27.43	2
Mean	11.92 ± 3.75		32.25 ± 3.75		6.62 ± 0.59		38.86 ± 7.13		77.99 ± 10.22		72.54 ± 13.34	
	ns		ns		b		ns		ce		g	
Durum Wheat												
Divide	14.50 ± 2.12	1	27.33 ± 2.83	1	9.82 ± 2.13	1	47.11 ± 14.82	1	107.45 ± 37.41	1	39.00 ± 7.52	1
Mountrail	25.33 ± 5.27	2	21.33 ± 5.24	2	10.97 ± 3.76	1	38.09 ± 16.21	1	115.04 ± 39.22	2	46.91 ± 9.23	2
Silver	13.00 ± 2.34	1	29.33 ± 3.19	1	7.88 ± 2.24	1	49.13 ± 9.45	1	109.88 ± 21.73	1	38.10 ± 6.62	1
Mean	17.61 ± 3.88		26.00 ± 2.40		9.56 ± 0.90		44.78 ± 3.39		110.79 ± 2.24		41.34 ± 2.80	
	ns		ns		a		ns		de		h	
Barley												
Haybet	4.33 ± 1.28	1	18.33 ± 3.22	1	3.94 ± 1.11	1	40.64 ± 14.29	1	150.58 ± 36.78	1	135.08 ± 42.37	1
AC Metcalfe	12.83 ± 7.83	1	21.00 ± 1.00	1	5.15 ± 1.37	1	42.48 ± 13.09	1	128.12 ± 38.96	2	129.95 ± 39.18	1
Mean	8.58 ± 4.25		19.67 ± 1.34		4.55 ± 0.61		41.56 ± 0.92		139.35 ± 11.23		132.52 ± 2.57	
	ns		ns		b		ns		d		j	

Table 3.5. Shoot Zn concentration, shoot Zn content and shoot Fe content of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent the standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant). Shoot content was calculated by multiplying dry weights and concentration. Superscripted numbers denote significant difference at the 0.05 level between cultivars within each crop species.

Cultivar	Root to Shoot Zn Translocation Index (%) -Zn		Root to Shoot Zn Translocation Index (%) +Zn		Root to Shoot Fe Translocation Index (%) -Zn		Root to Shoot Fe Translocation Index (%) +Zn	
Spring Wheat								
Fortuna	26.50 ± 8	1	69.75 ± 6	1	23.94 ± 4	1	32.26 ± 3	1
Brennan	29.48 ± 6	1	66.66 ± 8	1	21.74 ± 3	1	42.75 ± 3	2
Chinese Spring	20.21 ± 5	2	70.38 ± 1	2	13.54 ± 0.17	2	23.77 ± 7	3
Mean	25.40 ± 3		68.93 ± 1		19.74 ± 3		32.93 ± 5	
	ns		a		c		ns	
Winter Wheat								
Ledger	24.30 ± 2	1	68.08 ± 8	1	15.58 ± 1	1	41.22 ± 6	1
Carter	45.88 ± 22	2	66.84 ± 10	1	29.36 ± 14	2	42.06 ± 4	1
Mean	35.09 ± 11		67.46 ± 1		22.47 ± 7		41.64 ± 0.42	
	ns		a		cd		ns	
Durum Wheat								
Divide	36.73 ± 3	1	48.26 ± 5	1	33.76 ± 4	1	39.05 ± 4	1
Mountrail	28.35 ± 4	2	58.80 ± 8	2	28.36 ± 2	1	41.11 ± 5	1
Silver	19.62 ± 2	3	61.35 ± 7	2	37.12 ± 3	2	41.64 ± 4	1
Mean	28.23 ± 5		56.14 ± 4		33.08 ± 3		40.60 ± 1	
	ns		b		d		ns	
Barley								
Haybet	19.61 ± 3	1	71.58 ± 8	1	27.53 ± 2	1	37.12 ± 2	1
AC Metcalfe	24.00 ± 6	2	70.45 ± 6	1	29.65 ± 1	1	53.86 ± 5	2
Mean	21.81 ± 2		71.02 ± 1		28.59 ± 1		45.49 ± 8	
	ns		a		cd		ns	

Table 3.6. Root to shoot Zn and Fe translocation indexes (%) of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent the standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant). Superscripted numbers denote significant difference at the 0.05 level between cultivars within each crop species.

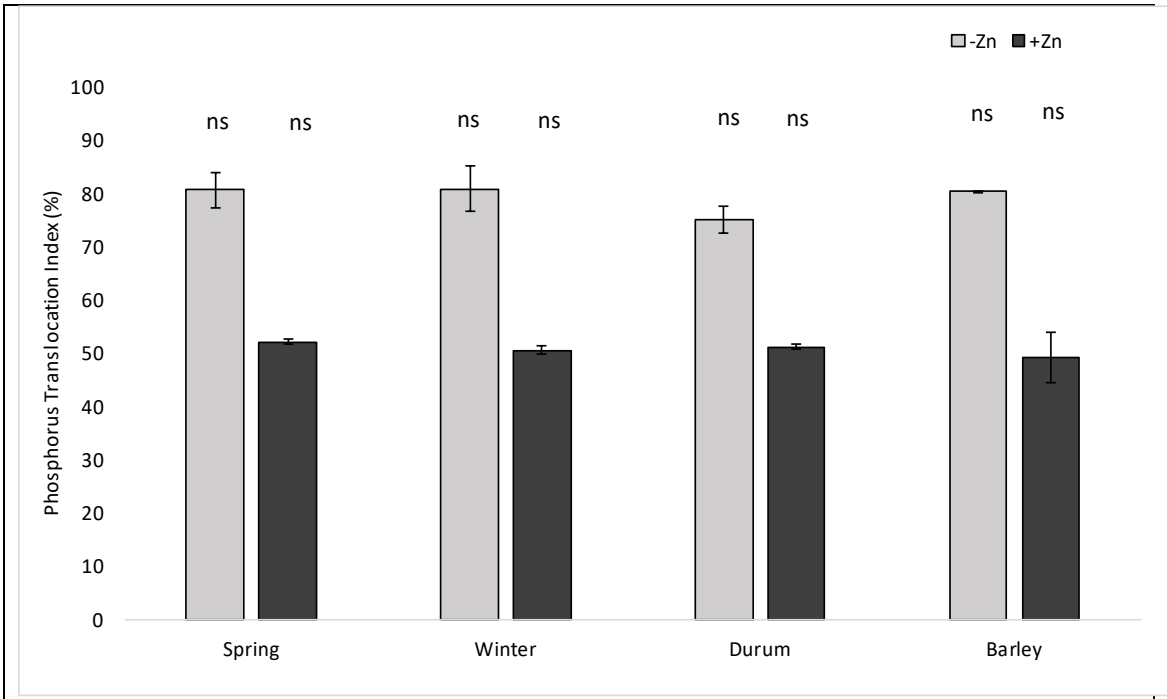


Figure 3.18. Mean phosphorus translocation index of wheat and barley crops after -Zn (no Zn) and +Zn (10^{-6} M) treatments. Vertical bars represent means ± standard error of two independent replications. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant).

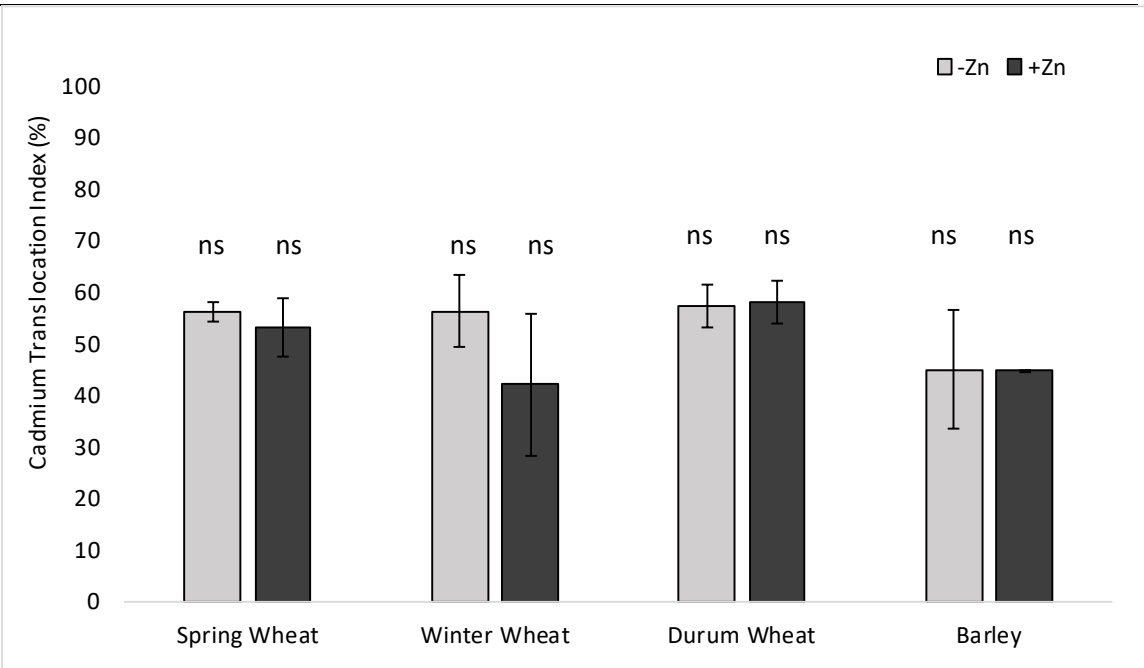


Figure 3.19. Mean cadmium translocation index of wheat and barley crops after -Zn (no Zn) and +Zn (10^{-6} M) treatments. Vertical bars represent means ± standard error of two independent replications. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant).

CHAPTER FOUR – DISCUSSION

Discussion

My aim was to identify Zn and Fe transporters characterized in cereals, ZIP1 and IRT2 respectively, and have a better insight about grain Zn concentration and Zn deficiency tolerance among Montana wheat and barley cultivars. Zn deficiency tolerant cultivars, Fortuna, Brennan and Carter were shown to carry both of these genes (Table 3.1), suggesting ZIP1 and IRT2 may be the major genes responsible of assisting cereals in Zn deficiency tolerance. Although, deficiency intolerant Chinese Spring possessed both ZIP1 and IRT2 genes, expression of the respectable proteins may be lacking in Chinese Spring. Previous studies suggested the accumulation of ZIP1 proteins in the roots of susceptible wild wheat genotypes whereas expression was limited in deficiency tolerant genotypes (Durmaz et al., 2009). ZIP1 and IRT2 may be working synergistically, induced together under deficiency in the Zn efficient cultivars and/or IRT2 may be complementing Zn, unlike in rice, under Zn deficiency (Ishimaru et al., 2006). Therefore, further expression study is necessary in order to understand the relationship between ZIP1, IRT2 and Zn deficiency tolerance. DTZ staining results correlated with the previous studies, confirming aleurone layer and embryo were indeed parts of the grain where Zn is predominantly localized whereas endosperm was lower in Zn (Ajiboye, 2015; Choi, Graham, & Stangoulis, 2007; Ozturk et al., 2006; Shobhana et al., 2013; Velu, 2008). Color intensity differentiation between DTZ stained grains with Zn concentrations lower

than 25 mg kg^{-1} was ineffective, therefore, DTZ staining can be useful in rapidly determining grains with normal to high Zn concentrations. Only barley grain Hector, showed intense color formation in the endosperm, contrary to its low Zn concentration (23 mg kg^{-1}) (Figure 3.6). Hordein proteins which are homologs of wheat prolamins, are found to be major sink for Zn in barley grains (Menguer et al., 2017; Rahman, 1984; Uddin, 2014) Thus, Hector may have rich hordein reserves or low molecular weight amino acids in its endosperm. Spectral absorbance significantly correlated with increasing red color formation (Figure 3.8). However, color formation was not observable due to interference of grain matrix color with the red stain color. Therefore, DTZ staining of grain flour samples are not found to be as effective and reliable as grain DTZ staining (Figure 3.7). Zn specificity of dithizone staining showed only high levels of Cd may interfere and reduce the accuracy of Zn concentration reading (Figure 3.9). Previously, tissue dithizone staining of Cd hyperaccumulator *Biscutella laevigate* formed red color, however, the Cd concentration in the tissues were in extremely high concentration (12 m kg^{-1}) (Pielichowska, 2004). Slight pink color formation during Mn incubation is not related to DTZ but rather to the natural color of Mn^{2+}.

Generally, nutrients are localized in the protein bodies in the form of globoid crystals (Lott & Spitzer, 1980). Protein bodies, some of which contain phytic acid, are commonly present in the aleurone layer and embryo and found to be the major sites of Zn and Fe binding. However, the presence of protein bodies together with phytic acid is minimal in the endosperm of cereal grains. Although studies showed increase in

endosperm Zn concentration with the application of outsource Zn (Ajiboye et al., 2015), the increase was limited and Zn was not evenly distributed along the wheat grain (Ajiboye et al., 2015; Stomph, 2011). Accumulation of Zn in the crease tissues suggested that Zn is highly regulated in the wheat grain and it is still unknown whether poorer endosperm Zn concentration is transporter or sink related. Moreover, speciation of Fe and Zn was suggested to be different in cereal grains. Zn was found binding to sulfur containing peptides whereas phytic acid was the sink for Fe (Persson, 2009). Similar results were found in rice (Prom-u-thai et al., 2008). Grain protein levels was found to correspond to Zn concentrations in wild emmer wheat grains and common wheat grains (Morgounov et al 2007; Peleg et al 2008). High protein and high Zn relation correlates well with higher Zn concentrations found in pulses (Hemalatha 2007). In our study, concentrations of grain Zn and Fe were in the range of 15-49 mg kg^{-1} and 34-51 mg kg^{-1}, respectively (Table 3.2). Based on previous micronutrient diversity of wheat and barley grains (Cakmak et al., 2010; Zou et al., 2012; Rengel et al., 1999), our concentrations are in the normal range for modern wheat and barley cultivars. Spring wheat cultivars were generally higher in both Zn and Fe concentrations across all crop varieties. Data relates with the screening of spring and winter wheat cultivars in Central Asia in which spring wheat varieties were significantly higher in Fe concentration across all cultivars (Morgounov, 2007). Grain Zn, Fe, S, and P concentrations positively correlated with each other and not with Cd. As discussed above, S and P in grain were the sinks for Zn

and Fe, respectively. Therefore, positive correlation between grain Zn, Fe, S, and P concentrations may offer a successful breeding strategy for high Zn grains.

Hydroponics experiments revealed there was a considerable variation among spring wheat, durum wheat, winter wheat and barley cultivars in response to Zn deficiency. Two spring wheat cultivars (Fortuna and Brennan) and one winter wheat cultivar (Carter) were found to show higher tolerance to Zn deficiency whereas remaining cultivars (AC Metcalfe, Chinese Spring, Divide, Ledger, Haybet, Mountrail and Silver) were deficiency intolerant. Without Zn supply, shoot Zn content of all cultivars were below 20 $\mu g\ g^{-1}$ which is the Zn level threshold for normal plant growth (Genc, 2002).

The Zn efficiency of the cultivars ranged from 21% to 71%. Zn efficiency was not correlated with shoot dry weight, shoot Zn concentration, shoot Zn content, root to shoot Zn translocation index or grain Zn concentration. The results suggested that variation in Zn efficiency among these cultivars is hereditary and cultivars likely to have different Zn utilization processes at the cellular level. Zn supply decreased shoot Fe content in both winter and durum wheat. Similar negative relation between Zn and Fe were observed in a foliar Zn application study involving 80 wheat cultivars under field conditions (Saha et al., 2015). Zn translocation index suggested winter wheat cultivar Carter efficiently translocated Zn from root to shoot more than any other cultivar. Moreover, grain Fe concentration was found to negatively correlate with root to shoot Zn translocation yet positively correlate with root to shoot Fe translocation under

deficiency. Phosphorus translocation was observed to be significantly ($p < 0.05$) reduced when plants were supplied with Zn (Figure 3.18). However, there was no significant difference among cultivars within the same treatment group. Phosphorus and Zn elements are known to behave antagonistically, therefore, under higher P:Zn ratios Zn is suggested to be tied up to phytate in the root cells (Webb, 1990). Durum wheat has been associated with being the most Zn deficiency sensitive among all cereal species (Cakmak et al., 1997). In our study, we conclude the order of increasing Zn efficiency of respective cereals were to be durum wheat, barley, spring wheat and winter wheat. Many mechanisms have been proposed in order to explain Zn efficiency and Zn translocation variation in cereals. Root exudates, root uptake and shoot translocation, lack of D genome (in case of durum wheat), difference in cellular concentration of Zn binding substances, soil microbiome and weather conditions are attributed to variation among cereals (Cakmak et al., 1999; Genc & Mcdonald, 2004; Gupta, 2016; Marschner, 1998; Oburger et al., 2014). However, current knowledge does not propose the superiority of one mechanism over the other. Furthermore, Zn efficiency and grain Zn concentration should be taken into account as two different traits since our data indicates that initial grain Zn concentration is not related to Zn deficiency tolerance. Similar observations were found in wheat and rice plants (Genc, 2008; Jiang et al., 2008).

Our data suggests, Zn deficiency susceptible barley and durum wheat cultivars may be more suitable breeding candidates for higher grain Fe levels grown on Zn

deficient soils. On the other hand, spring wheat cultivars Fortuna and Brennan can be used for higher grain Zn and Fe concentrations as well as higher Zn efficiency on Zn deficient soils. Another factor to consider in this study, is the yield performance of the cultivars. When the combination of high Zn efficiency and a good yield is individually considered, Brennan and Carter were the leading cultivars (Heo et al., 2016; Berg, 2016; Ransom et al., 2016; Wichman, 2015). However, grain Zn concentration were low for both cultivars, 27 mg kg^{-1} and 17 mg kg^{-1} respectively.

CHAPTER FIVE – MICRONUTRIENT DIVERSITY OF MONTANA WHEAT AND BARLEY

Contribution of Authors and Co-Authors

Author: Eylul Kaya

Contributions: Collected the data, generated figures, performed statistical calculations, analyzed the data and wrote the manuscript in preparation for submission.

Co-author: Levent Ozturk

Contributions: Provided knowledge and collected one replication of the grain element analysis.

Co-author: Hikmet Budak

Contributions: Provided knowledge, assisted in interpretation of the data and preparation of the manuscript.

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MICRONUTRIENT DIVERSITY OF MONTANA WHEAT AND BARLEY

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Abstract

Zn deficiency is a commonly encountered issue especially in Northern regions of the United States. This issue not only manifests itself as lower grain Zn concentrations but also as deficiency-induced Cd toxicity in cereals, particularly in durum wheat. In this study, we found there is a great variation between different Montana cereal crops in response to Zn deficiency. The Zn efficiency of the cultivars ranged from 21% to 71%. Zn efficiency was not correlated with shoot dry weight, shoot Zn concentration, shoot Zn content, root to shoot Zn translocation index or grain Zn concentration. The results suggested that variation in Zn efficiency among these cultivars is hereditary and cultivars likely to have different Zn utilization processes at the cellular level. Grain Cd levels were higher than the required Cd concentration standard (0.2 mg kg^{-1}) for durum wheat cultivars except that of CDC Fortitude (0.11 mg kg^{-1}). We propose susceptibility of durum wheat cultivars to grain Cd accumulation are related to xylem translocation from root to shoot and phloem loading into grain and not root uptake. Differences in Zn

efficiency and grain Zn concentration among these cereal cultivars, indicate the potential to breed for cereal cultivars with increased Zn deficiency tolerance and elevated grain Zn concentrations.

Introduction

Micronutrients are essential for all living organisms because of its requirement for maintenance of healthy life. Plants use micronutrients in processes such as photosynthesis, cellular growth and development, which are directly associated with yield (Marschner, 2012). Intensive use of farmland causes reductions in micronutrients from the soil and heavily influences yield and nutritional quality of grain; consequently, this situation results in inadequate intake of dietary nutrients from plants (Jarrell et al. 1981). Industrial refining processes such as milling and polishing also decrease the essential micronutrient quality of plants, predominantly in cereals end products because of localization of micronutrients in grains. Human diets lacking adequate amount of essential micronutrients cause series of health complications including anemia, premature birth and stunted growth (Brown, 2001). In consideration of its importance for human health, it is essential to manipulate plant to maintain sufficient amount of micronutrients, particularly in cereals which is consumed worldwide as primary food source.

Deficiency of Zn (Zn) and iron (Fe) is the most prevalent micronutrient deficiency; particularly in countries with plant-based diets (Welch and Graham, 2002). In wheat and

barley grains, Zn and Fe are mainly deposited in higher concentrations in the aleurone layer, embryo and lower in endosperm within the seed (Ozturk et al., 2006). However, during refinement process, large proportions of the aleurone layer and the embryo are removed, causing a reduction of the nutritional value of cereal based food. Additionally, interest in crop productivity as a breeding strategy, surpassed nutritional value of cereal products and created a dilution effect over the years, resulting in micronutrient deficient cereal crops. Previous studies have shown a significant variation of concentration of Fe and Zn in grain across different wheat genotypes. Surprisingly, wild relatives of wheat such as *Triticum dicoccoides*, *Triticum monococcum* and *Triticum boeoticum* presented fairly higher concentrations of Fe and Zn in grain by reaching up to 92 mg kg^{-1} and 177 mg kg^{-1} in concentration, respectively, whereas modern wheat cultivars range from only 20 mg kg^{-1} to 35 mg kg^{-1} Zn (Cakmak, 2000; Monasterio and Graham, 2000; Rengel, 1999; Tiwari, 2009). Relatively, modern barley cultivars possessed similar grain Fe (11-60 mg kg^{-1}) and Zn (10-23 mg kg^{-1}) concentrations (Chen, 2007).

In addition, several studies have shown that final concentration of Zn in cereals grain is also dependent on environmental factors; such as soil characteristics, water availability and atmospheric carbon dioxide concentration, whereas environmental impacts have a minor effect on grain Fe concentration among different cereal genotypes (Nakandalage, 2016). For instance, screening of 175 wheat cultivars for grain Fe concentration was similar to previous studies yet screening of grain Zn concentration of different wheat genotypes showed greater variability among different trial sites (Oury,

2006; Morgounov, 2007; Zhao et al., 2009). In another study, Zn concentration of durum wheat grains were drastically reduced to 8 mg kg^{-1} when grown on potentially Zn deficient soils (Cakmak, 2010). Similar results were also found in barley (Xue et al. 2016). Furthermore, durum wheat was shown to be more susceptible to Zn deficiency among other cultivated wheat varieties. Discovery of genetic variation to Zn efficiency among cereal crops is a conventional breeding strategy to combat micronutrient deficiency and replenish Zn nutrition in cereal grains. Zn efficiency is a term to describe a plant's toleration to Zn deficiency characterized by higher yield and better seedling vigor (Graham, 1992). Studies suggested the order of increasing Zn efficiency of respective cereals were to be durum wheat, oat, bread wheat, barley, triticale and followed by rye (Cakmak et al., 1997). However, Zn efficiency of plants can be adversely affected by interactions between elements and/or metal transporters involved in uptake and transport of Zn. For instance, Zn homeostasis is controlled by metal transporters, some of which are; Zn-iron regulated transporter-like proteins (ZIP), Yellow Stripe Like (YSL) proteins and cation diffusion facilitator (CDFs) family of proteins (Colangelo and Guerinot, 2006; Gupta, 2016). These family of transporters differ in their substrate specificity and can transport Fe, Mn, Cd and Co as well (Hall and Williams, 2003; Williams, 2000). On the other hand, cadmium (Cd) has an antagonistic effect on Fe and Zn uptake from the roots, therefore, Cd can induce expression of genes in response to low or high Fe or Zn levels in plants (Detterbeck, 2016).

Biofortification programs aim to enrich micronutrient concentrations to produce more nutritious crops are on demand to alleviate micronutrient deficiencies. The HarvestPlus Challenge Program has targeted increasing grain Fe and Zn concentrations by at least 25 and 10 mg kg^{-1}, respectively, especially in developing countries (Graham, 2007). Therefore, special attention should be paid to micronutrient diversity of cereals not only because additional nutritional value but also increased seedling vigor and yield provided by these micronutrients (Boonchuay, 2013).

To our knowledge, there is no comprehensive study in the literature on micronutrient diversity of Montana cereal cultivars. In this paper, we report the genetic variation in Zn deficiency tolerance, root uptake and shoot transport of Zn among different genotypes of Montana wheat and barley cultivars. To further understand the levels of variability among cultivars in response to Zn deficiency and adequacy; a range of other micronutrient concentrations in plant shoot, root, and seed were also measured by ICP-OES. Seed Zn concentration and localization were performed on mature seeds, germinating seeds and whole grain flour by DTZ (diphenyl thiocarbazone) staining method (Ozturk et al., 2006).

Materials and Methods

Plant Material

A total of 17 wheat and 4 barley cultivars were obtained from Montana State University. Following preliminary Zn concentration data, 10 wheat and 2 barley cultivars

were ranked in agreement with high or low Zn concentration and were included for hydroponics studies.

Dithizone (DTZ) Staining

To localize Zn deposition in wheat and barley grains, a Zn chelating agent, 1,5-diphenyl thiocarbazone (Merck, Germany) solution was prepared by dissolving in 500 mg L^{-1} analysis-grade pure methanol. Individual mature seeds from each cultivar were imbibed in milli-Q water for an hour and excised longitudinally along the crease with a scalpel. Seeds were stained in DTZ solution for thirty minutes at room temperature. Samples were rinsed thoroughly with milli-Q water and blotted dry with Kimwipes. For DTZ staining of germinating seeds, ten individual seeds from each cultivar were surface sterilized with 75% ethanol for one minute and 30% sodium hypochlorite for twenty minutes and rinsed thoroughly with sterile distilled water for six times. Seeds were later placed on sterile filter paper (Whatman No:1) moistened with sterile distilled water and germinated in Petri dishes for 36 hours at 25°C in incubator. After germination, seeds were stained as described previously. Seed flour samples of each cultivar were obtained by grinding seeds for five minutes in a clean grinder. An amount of 0.2 grams of powder sample from each cultivar were placed in 24 well plates and stained with 200 µL of DTZ solution for thirty minutes. Stained cereal grains and flour samples were immediately visualized by AmScope SM-1BSX-64S stereomicroscope and photographed by a high-resolution camera. Quantification of dithizone staining was achieved via extraction of red color from the flour samples by methanol followed by centrifuging at 5000 g for ten

minutes. All extracts were then measured spectrophotometrically at 512 nm on a Genesys™ 30 Spectrophotometer (Fisher Scientific, Fairlawn, NJ). Zn specificity of dithizone was determined by incubating seeds of AC Metcalfe, a low Zn concentration (15 mg kg^{-1}) barley variety with 0.01 M, 0.05 M and 0.1 M of Cu, Cd, Fe, Mn and Zn sulfate salts for 20 hours. After incubation, seeds were washed thoroughly with sterile milli-Q water and dried at 50°C until the seeds were completely dry. Ground seeds were stained with DTZ as previously described.

Hydroponics Experiment

Detection of Zn deficiency tolerance among wheat and barley cultivars were performed by surface sterilization of seeds as mentioned previously and grown on sterile filter paper (Whatman No:1) for seven days at 25°C in incubator. After seven days, a group of seedlings were transferred to continuously aerated 10 L of hydroponics nutrient solution, containing adequate Zn (1 µM $ZnSO_4$) whereas the other group were transferred to Zn deficient nutrient solution. The composition of the nutrient solution was as follows: 0.88 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM $MgSO_4$, 0.1 mM KCl, 100 µM Fe-EDTA, 1.0 µM H_3BO_3, 1.0 µM $MnSO_4$, 0.2 µM $CuSO_4$ and 0.02 µM $(NH_4)_6Mo_7O_{24}$. Plants were grown in a growth chamber under controlled conditions (light/dark regime 16/8 h, temperature 24°C /22°C and photon flux density of 100 µmol m^{-2} s^{-1}) at the Plant Growth Center, MSU. Nutrient solutions were renewed every 4 days. After 21 days of growth in the growth chamber, shoots and roots were separated at the soil level and rinsed with deionized water thoroughly to remove adsorbed elements on the tissue surface. Roots

and shoots were dried at 35°C until constant dry weights and weighed for determination of dry matter production. Zn efficiency of the cultivars were determined according to the ratio of shoot dry weight at Zn deficient condition to shoot dry weight at Zn sufficient condition. Root to shoot Zn translocation index was calculated as the ratio of total shoot Zn content to total Zn content (root and shoot) per plant (Rengel & Graham, 1996). Study included nine wheat (Fortuna, Brennan, Chinese Spring, Ledger, Carter, Divide, Mountrail and Silver) and two barley (AC Metcalfe and Haybet) cultivars subjected to two treatments (-Zn and +Zn). Experiment was designed in a complete randomized design with a total of three replications. Experiment was run twice.

Total Mineral Analysis

The dried shoot and root samples from the hydroponics experiment were ground and approximately 0.5 g ground samples were digested using a 6 mL nitric acid / 12 mL hydrogen peroxide mix in a closed-vessel microwave system and the concentrations of Al, B, Ca, Cd, Cu, Fe, K, Mg, Mn, P, S and Zn were determined using a Perkin Elmer 5400 inductively coupled plasma optical emission spectrometry (ICP-OES) analyzer (Zarcinas, 1987). Additionally, whole grain samples from each cultivar were ground and analyzed. The whole grain analysis was performed on 22 grains with three replicates randomly collected from different plants of the same cultivar. Data provided courtesy of AGVISE Laboratories (Northwood, ND). Element content of the plant parts were calculated as follows: Content = Dry Weight x Concentration

Data Analysis

Statistical analysis was performed using the SPSS statistical software (version 1.0.0.642). Data from two experiments were combined and significant differences between means were determined using the least significant difference (LSD) mean separation procedure at the 5% level. Single correlation coefficients among the studied were obtained using Pearson correlation analysis.

Results

Localization and Mobilization of Zn in Cereal Grains

Wheat and barley grains were excised along the crease and stained with diphenyl thiocarbazone (DTZ) to study the deposition of Zn in grain, when bound to Zn, DTZ forms a pink/red colored complex and is highly selective for this metal. DTZ staining of matured seeds showed that Zn was found to be present mostly in the embryo and aleurone/sub-aleurone layer in wheat as well as in barley seeds. Pink/ red color formation intensified as the Zn concentration increased (Figure 5.1). However, color intensity was not significant when the Zn concentration was lower than 25 mg kg^{-1} and there was no observable difference among grains with Zn concentrations higher than 35 mg kg^{-1}. As for the dithizone staining of whole grain flour, the color intensity was correlated with increasing Zn concentration (Figure 5.2). Similar to that of stained mature seeds, ground flour with Zn concentrations higher than 35 mg kg^{-1} were notably red. Spectrophotometric quantification of flour samples indicated there was a

significant correlation between seed Zn concentration and the spectral absorbance of the dithizone stained flour extracts (Figure 5.3). Study of Zn mobilization in grain is achieved via germinating of mature seeds of barley cv. Haybet, for over 36 hours and staining with DTZ. Figure 5.4 shows the remobilization of Zn from grain to newly forming radicle and coleoptile indicated with dense red color. However, red color was most dense in the embryo especially scutellum being the most concentrated with Zn. Zn specificity of dithizone staining was performed on low Zn concentration (15 mg kg^{-1}) AC Metcalfe seeds. Clearly, only increased Cd levels did interfere with the dithizone and formed an orange/red color (Figure 5.5). However, red color was not as distinct as Zn, therefore, much higher Cd levels can only interrupt the accuracy of the DTZ staining method.

Figure 5.1. Localization of Zn in matured seeds of **(A)** *T. aestivum* L. cv Chinese Spring (top) and *T. aestivum* L. cv Brennan (bottom), **(B)** *T. aestivum* L. cv Norris (top) and *T. aestivum* L. cv Carter (bottom), **(C)** *T. turgidum* L. cv Havasu (top) and *T. turgidum* L. cv CDC Fortitude (bottom), **(D)** *Hordeum vulgare* L. cv Haybet (top) and *Hordeum vulgare* L. cv AC Metcalfe (bottom) shown here after 30 minutes of DTZ staining at room temperature. Concentrations represent the highest and lowest seed Zn levels.

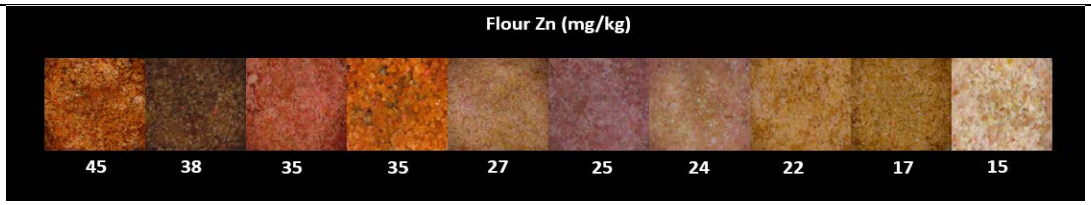

Figure 5.2. DTZ staining of whole grain flour of wheat and barley cultivars. Cultivars are; Chinese Spring, Haybet, Fortuna, Divide, Silver, Brennan, Mountrail, Ledger, Carter and AC Metcalfe respectively. Numbers below represent Zn concentration of whole grains.

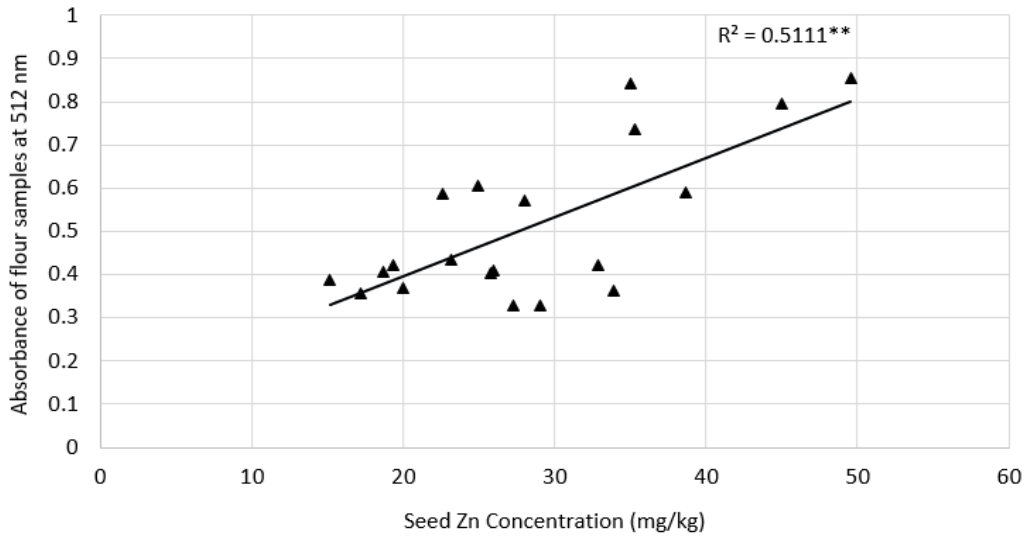

Figure 5.3. Relationship between Zn concentration and spectral absorbance of DTZ stained whole grain flour samples of Montana wheat and barley cultivars. Each triangle represents the average of spectral absorbance and average seed Zn concentration of three independent replications. (**, $p < 0.01$)

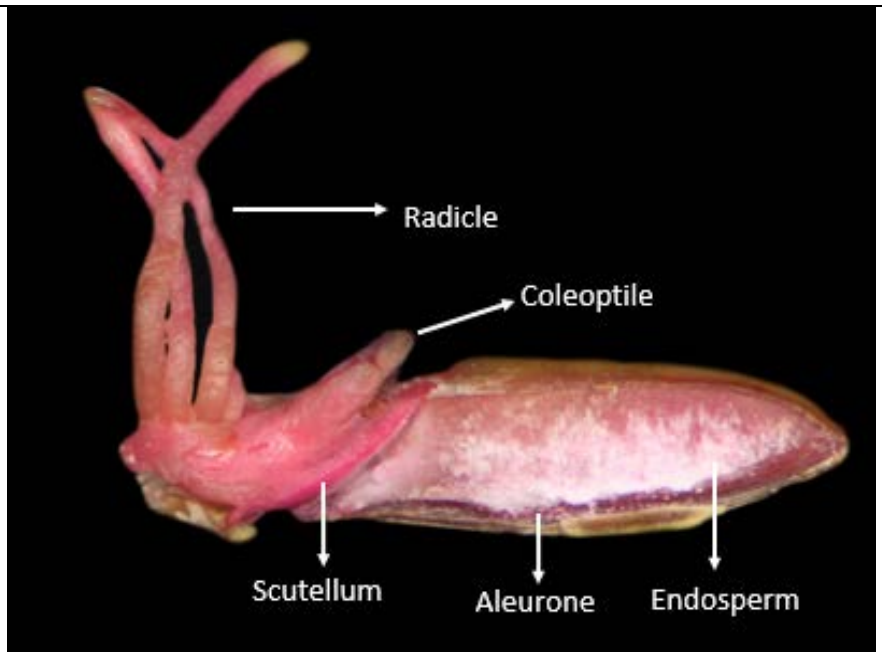

Figure 5.4. Mobilization of Zn in *Hordeum vulgare* L. cv Haybet after germination of 36 hours and DTZ staining. Labels represent seed parts.

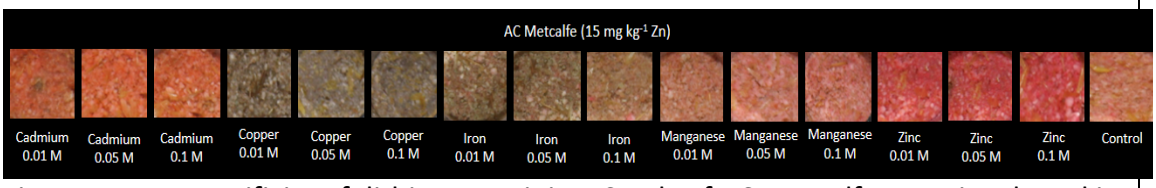

Figure 5.5. Zn specificity of dithizone staining. Seeds of AC Metcalfe were incubated in three concentrations (0.01 M, 0.05 M, 0.1 M) of Cd, Cu, Fe, Mn and Zn solutions in order to demonstrate the interference of other metals in red color formation by DTZ staining.

Whole Grain Micronutrient Diversity

Average seed Zn concentration of wheat and barley grains ranged from 15 to 49 mg kg^{-1} whereas Fe concentrations ranged from 34 to 51 mg kg^{-1} (Table 5.1). The highest grain Zn concentrations were detected in spring wheat with an average of 33 mg kg^{-1} and the lowest in winter wheat (20 mg kg^{-1}). There was a significant difference in seed Zn concentration between the means of winter wheat and durum wheat cultivars ($p < 0.05$) (Figure 5.6). On average, spring wheat grain Zn concentrations were 39% higher than that of winter wheat grains. Moreover, mean of durum wheat Cd levels were significantly higher than the mean of other crops. Grain Fe and P concentrations were the highest in Chinese Spring. Grain Zn, grain Fe and grain P concentrations across all cultivars significantly correlated (Figure 5.7), however, no correlation was detected for grain Cd concentrations and rest of the elements ($r = 0.36$, $p = 0.12$). Moreover, sulfur, a constituent of proteins involved in Fe and Zn binding in grains, correlated significantly with both of these elements (data not shown).

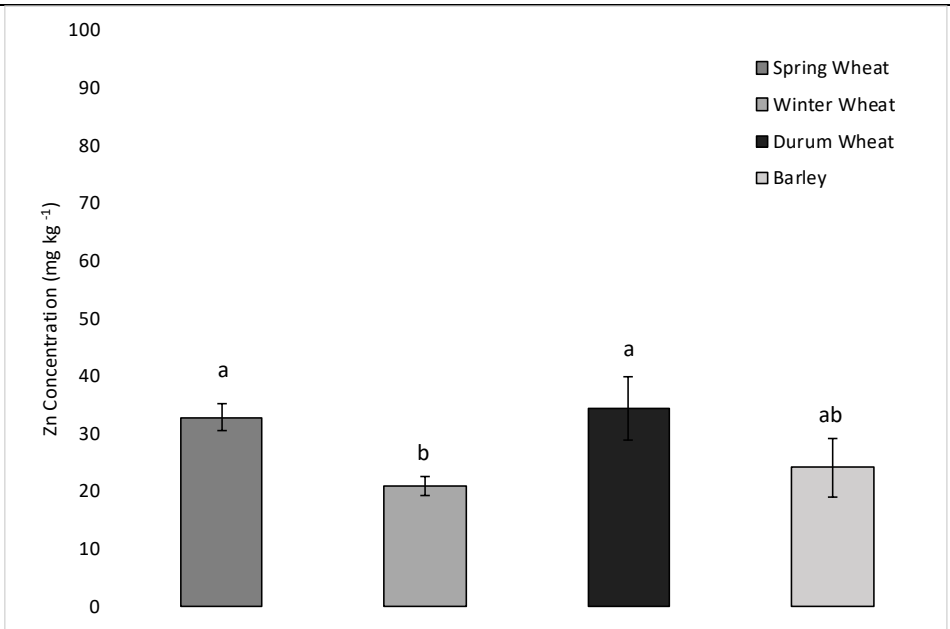

Figure 5.6. Average seed Zn concentrations of wheat and barley crops. Vertical bars represent ± standard error of three independent replications. Letters denote significant difference at the 0.05 level between means of crop varieties.

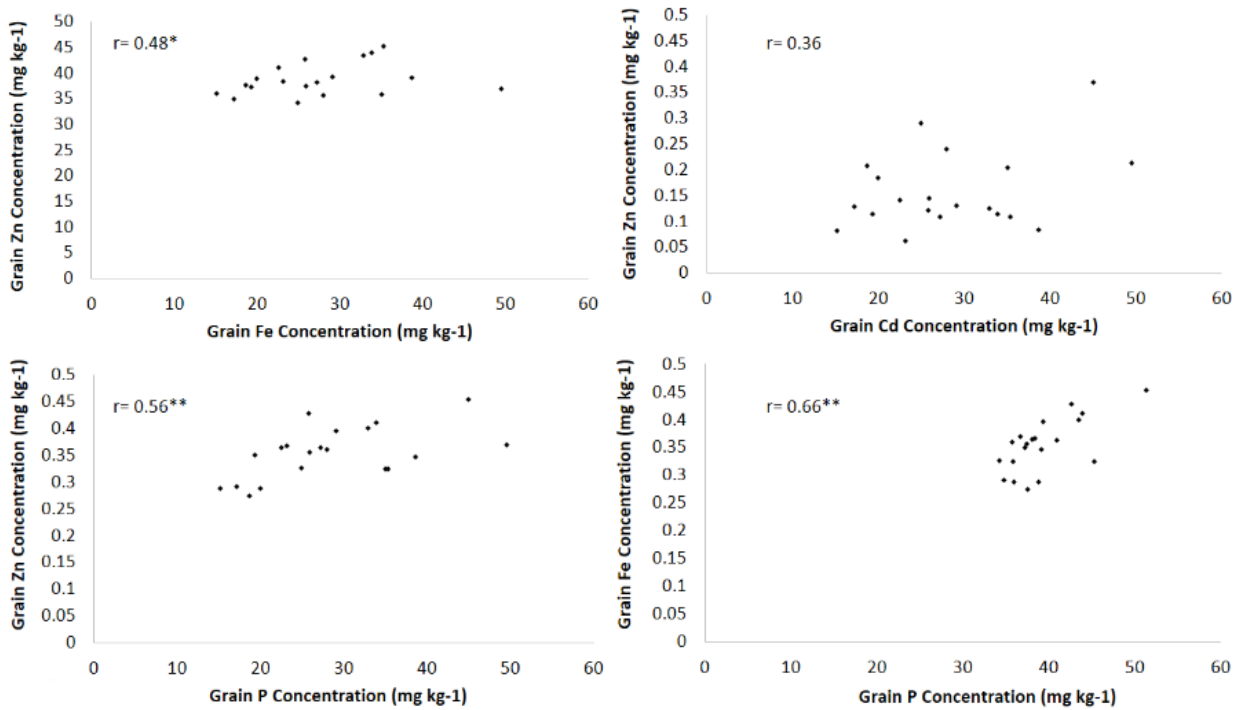

Figure 5.7. Relationships between grain element concentrations. * and ** indicates significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Cultivar	Seed Zn Concentration (mg kg-1)	Seed Fe Concentration (mg kg-1)	Seed Cd Concentration (mg kg-1)	Seed P Concentration (mg kg-1
Spring Wheat				
Brennan	25.94 ± 3.6	37.49 ± 2.0	0.14 ± 0.03	0.36 ± 0.01
Corbin	27.25 ± 4.3	38.08 ± 1.5	0.11 ± 0.02	0.36 ± 0.03
Vida	29.09 ± 3.5	39.32 ± 2.6	0.13 ± 0.04	0.40 ± 0.03
Mott	32.89 ± 2.5	43.48 ± 2.8	0.13 ± 0.02	0.40 ± 0.02
Mc Neal	33.87 ± 3.6	43.89 ± 0.4	0.11 ± 0.04	0.41 ± 0.01
Fortuna	35.33 ± 1.3	45.3 ± 3.3	0.11 ± 0.03	0.41 ± 0.001
Chinese Spring	45 ± 1.2	51.33 ± 0.1	0.37 ± 0.02	0.45 ± 0.01
Mean	32.77 a	42.7 e	0.16 d	0.39 g
Winter Wheat				
Carter	17.18 ± 1.4	34.83 ± 4.7	0.13 ± 0.04	0.29 ± 0.02
Warhorse	18.67 ± 2.3	37.61 ± 5.0	0.21 ± 0.02	0.27 ± 0.01
Judee	20 ± 0.6	38.84 ± 1.9	0.18 ± 0.01	0.29 ± 0.003
Ledger	22.56 ± 1.3	41 ± 4.2	0.14 ± 0.03	0.36 ± 0.01
Norris	25.82 ± 1.2	42.67 ± 2.1	0.12 ± 0.02	0.43 ± 0.02
Mean	20.84 b	38.99 ef	0.16 d	0.33 h
Durum Wheat				
CDC Fortitude	19.33 ± 0.9	37.25 ± 5.4	0.11 ± 0.02	0.35 ± 0.01
Mountrail	24.93 ± 3.3	34.27 ± 3.9	0.29 ± 0.07	0.33 ± 0.02
Silver	27.97 ± 3.5	35.71 ± 1.1	0.24 ± 0.08	0.36 ± 0.03
Tioga	30.33 ± 0.33	38.67 ± 0.88	0.41 ± 0.07	0.34 ± 0.00
Divide	35.04 ± 6.8	35.81 ± 1.2	0.2 ± 0.07	0.32 ± 0.02
Havasu	49.56 ± 2.3	36.72 ± 1.4	0.21 ± 0.02	0.37 ± 0.01
Mean	31.36 a	36.40 f	0.24 c	0.34 h
Barley				
AC Metcalfe (**Malting**)	15.15 ± 2.3	36 ± 3.9	0.08 ± 0.03	0.29 ± 0.02
Hector (**Feed**)	23.18 ± 7.5	38.37 ± 0.6	0.06 ± 0.02	0.37 ± 0.01
Haybet (**Feed**)	38.67 ± 4.4	39.12 ± 2.2	0.08 ± 0.03	0.35 ± 0.04
Mean	25.33 a	37.83 f	0.07 d	0.33 h

Table 5.1. Seed Zn, iron, cadmium and phosphorus concentrations of Montana wheat and barley cultivars. ± values represent standard error of three independent replications per grain. Letters denote significant difference at the 0.05 level between means of crop varieties.

Zn Efficiency of Montana Cereals

Zn efficiency and shoot Zn translocation was determined by supplying each cultivar adequate Zn (1 µM $ZnSO_4$) and deficient Zn (no Zn) levels for 21 days. Deficiency symptoms were visible around 14th day. Deficiency symptoms were observed as stunted growth and interveinal chlorosis and necrosis which are the typical symptoms appear under Zn deficiency. Mean shoot heights of spring wheat, durum wheat, winter wheat and barley cultivars were not significantly different when subjected to Zn deficiency.

However, significant difference between treatment groups was observed and adequate Zn supply increased mean shoot heights of barley and durum wheat cultivars more than other crops (data not shown). Zn efficiency was determined by considering tolerance index and leaf deficiency symptoms. According to Zn efficiency, there was no significant difference among crops, but when considered individually, winter wheat cultivar Carter (71%) and spring wheat cultivars Fortuna (65%) and Brennan (35%) were recorded as Zn deficiency tolerant; whereas Chinese Spring, Ledger and all durum wheat and barley cultivars were recorded as susceptible. Among all cultivars, Chinese Spring (21%) was the most deficiency intolerant cultivar (Table 5.2). Although, Zn efficiency of Brennan was less than any of the durum wheat cultivars, leaf symptoms of chlorosis and necrosis was not as pronounced as the susceptible cultivars. Therefore, Brennan was determined to be tolerant to Zn deficiency. There was a significant difference in shoot and root dry weight between -Zn and +Zn treated plants. Furthermore, average leaf number was higher than those without supply, although the difference between Zn treatments was insignificant (data not shown).

Cultivar	Shoot Dry Weight (g plant^{-1}) -Zn	Shoot Dry Weight (g plant^{-1}) +Zn	Zn Efficiency (%)
Spring Wheat			
Fortuna	0.82 ± 0.06	1.35 ± 0.31	65
Brennan	0.58 ± 0.05	1.66 ± 0.22	35
Chinese Spring	0.39 ± 0.12	1.84 ± 0.56	21
Mean	0.6 ± 0.13	1.62 ± 0.14	40
	ns	bc	ns
Winter Wheat			
Ledger	0.57 ± 0.15	1.04 ± 0.02	39
Carter	0.71 ± 0.10	1.36 ± 0.14	71
Mean	0.64 ± 0.07	1.2 ± 0.16	55
	ns	bd	ns
Durum Wheat			
Divide	0.7 ± 0.14	1.68 ± 0.21	42
Mountrail	0.6 ± 0.12	1.77 ± 0.13	34
Silver	0.66 ± 0.08	1.61 ± 1.11	41
Mean	0.65 ± 0.03	1.69 ± 0.05	39
	ns	b	ns
Barley			
Haybet	0.93 ± 0.08	2.28 ± 0.09	41
AC Metcalfe	0.59 ± 0.17	2.01 ± 0.53	29
Mean	0.76 ± 0.17	2.14 ± 0.13	35
	ns	a	ns

Table 5.2. Shoot dry weight, Zn tolerance index of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant)

Shoot Tissue Element Concentrations

Plant shoots included in the hydroponics study was analyzed for elements by ICP/OES. Generally, Zn supply significantly increased shoot Zn concentration and content (shoot dry weight x shoot Zn concentration) in all crop varieties. According to Table 5.3, spring wheat cultivars were relatively higher than rest of the crop varieties in shoot Zn concentration and content. Although significant increase among treatments were detected, no significant difference was observed between neither of the crop

varieties for shoot Zn concentration or content. Moreover, there was no significant correlation between Zn efficiency and/or shoot Zn concentration and shoot Zn content with or without Zn supplementation; suggesting an increase in Zn deficiency tolerance was not reflected by the increase in Zn concentration or Zn content. Highest shoot Zn concentration increase was observed in barley cultivar Haybet, followed by winter wheat cv. Carter. Percent increase in shoot Zn content was not correlated with percent increase in shoot Zn concentration. Therefore, Zn deficiency tolerant cultivars cannot be differentiated from the susceptible ones by increased Zn concentrations or contents. As Table 5.3 illustrates, deficiency susceptible cultivars harbor higher shoot Fe concentrations in Zn deficient conditions than tolerant ones while doing the opposite during Zn sufficient conditions, suggesting ZIP transporters in these cultivars may have specificity for Fe as well. However, shoot Fe contents of barley cultivars were not affected by the Zn supply, therefore Fe specificity of ZIP transporters may not be the only parameter involved in Fe uptake difference.

Cultivar	Shoot Zn Concentration (mg kg^{-1}) -Zn	Shoot Zn Concentration (mg kg^{-1}) +Zn	Shoot Zn Content (µg plant^{-1}) -Zn	Shoot Zn Content (µg plant^{-1}) +Zn	Shoot Fe Content (µg kg^{-1}) -Zn	Shoot Fe Content (µg kg^{-1}) +Zn
Spring Wheat						
Fortuna	13.00 ± 1.00	34.33 ± 1.72	10.34 ± 0.22	46.50 ± 3.14	94.63 ± 27.69	97.20 ± 17.21
Brennan	17.33 ± 9.00	23.17 ± 0.83	8.01 ± 2.89	39.37 ± 5.13	70.05 ± 7.65	112.00 ± 23.95
Chinese Spring	36.50 ± 28.00	38.00 ± 4.00	10.05 ± 5.52	47.56 ± 8.66	41.57 ± 11.20	98.78 ± 35.25
Mean	22.28 ± 7.22	31.83 ± 4.46	9.47 ± 0.73	44.48 ± 2.57	68.75 ± 15.33	102.66 ± 4.69
	ns	ns	a	ns	c	f
Winter Wheat						
Ledger	15.67 ± 8.67	36.00 ± 4.33	7.20 ± 1.97	45.99 ± 0.97	67.77 ± 28.05	59.20 ± 6.08
Carter	8.17 ± 2.84	28.50 ± 3.83	6.03 ± 2.77	31.72 ± 2.62	88.21 ± 1.39	85.87 ± 27.43
Mean	11.92 ± 3.75	32.25 ± 3.75	6.62 ± 0.59	38.86 ± 7.13	77.99 ± 10.22	72.54 ± 13.34
	ns	ns	b	ns	ce	g
Durum Wheat						
Divide	14.50 ± 2.12	27.33 ± 2.83	9.82 ± 2.13	47.11 ± 14.82	107.45 ± 37.41	39.00 ± 7.52
Mountrail	25.33 ± 5.27	21.33 ± 5.24	10.97 ± 3.76	38.09 ± 16.21	115.04 ± 39.22	46.91 ± 9.23
Silver	13.00 ± 2.34	29.33 ± 3.19	7.88 ± 2.24	49.13 ± 9.45	109.88 ± 21.73	38.10 ± 6.62
Mean	17.61 ± 3.88	26.00 ± 2.40	9.56 ± 0.90	44.78 ± 3.39	110.79 ± 2.24	41.34 ± 2.80
	ns	ns	a	ns	de	h
Barley						
Haybet	4.33 ± 1.28	18.33 ± 3.22	3.94 ± 1.11	40.64 ± 14.29	150.58 ± 36.78	135.08 ± 42.37
AC Metcalfe	12.83 ± 7.83	21.00 ± 1.00	5.15 ± 1.37	42.48 ± 13.09	128.12 ± 38.96	129.95 ± 39.18
Mean	8.58 ± 4.25	19.67 ± 1.34	4.55 ± 0.61	41.56 ± 0.92	139.35 ± 11.23	132.52 ± 2.57
	ns	ns	b	ns	d	j

Table 5.3. Shoot Zn concentration, shoot Zn content and shoot Fe content of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant). Shoot content was calculated by multiplying dry weights and concentration.

Root to Shoot Zn, Iron, Phosphorus and Cadmium Translocation Index

Root to shoot Zn translocation index was calculated as the ratio of total shoot Zn content to total Zn content (root and shoot) per plant. As expected, all cultivars translocated more Zn to the shoot when supplied with Zn (Table 5.4). Zn deficiency tolerant cultivar Carter showed the highest Zn translocation index (45%) than any other

cultivar under deficiency. However, no relation was found in regards to Zn efficiency and Zn translocation. Zn supply enhanced root to shoot Zn translocation of all wheat cultivars up to two-fold whereas the increase was up to three-fold for barley cultivars. Deficiency susceptible durum wheat cultivars translocated less Zn to the shoots when supplied with Zn. On the contrary, durum wheat and barley cultivars translocated more Fe to the shoots under Zn deficiency. Interestingly, grain Fe concentration and Fe translocation index under Zn deficiency was significantly correlated ($p=0.019$), suggesting grains with lower Fe levels translocate more Fe to edible parts under Zn deficiency. However, the relation is dependent on the grain Zn levels. Cultivars with higher grain Zn concentrations grown under Zn sufficient condition translocated significantly lower Fe to the shoots ($p= -873$). Moreover, grain Zn, Fe, P and Cd concentrations were found to inversely affect Zn translocation under deficiency.

On the other hand, cultivars with lower grain Zn concentrations demonstrated greater ability in translocating Zn to the shoots when Zn supplied. P translocation index was adversely affected under Zn sufficient conditions (data not shown). Whereas, Zn levels did not show any effect on Cd translocation (data not shown). Barley cultivars retained the lowest Cd translocation index under both Zn deficient (45%) and sufficient conditions (44.6%).

Cultivar	Root to Shoot Zn Translocation Index (%) -Zn	Root to Shoot Zn Translocation Index (%) +Zn	Root to Shoot Fe Translocation Index (%) -Zn	Root to Shoot Fe Translocation Index (%) +Zn
Spring Wheat				
Fortuna	26.50 ± 8	69.75 ± 6	23.94 ± 4	32.26 ± 3
Brennan	29.48 ± 6	66.66 ± 8	21.74 ± 3	42.75 ± 3
Chinese Spring	20.21 ± 5	70.38 ± 1	13.54 ± 0.17	23.77 ± 7
Mean	25.40 ± 3	68.93 ± 1	19.74 ± 3	32.93 ± 5
	ns	a	c	ns
Winter Wheat				
Ledger	24.30 ± 2	68.08 ± 8	15.58 ± 1	41.22 ± 6
Carter	45.88 ± 22	66.84 ± 10	29.36 ± 14	42.06 ± 4
Mean	35.09 ± 11	67.46 ± 1	22.47 ± 7	41.64 ± 0.42
	ns	a	cd	ns
Durum Wheat				
Divide	36.73 ± 3	48.26 ± 5	33.76 ± 4	39.05 ± 4
Mountrail	28.35 ± 4	58.80 ± 8	28.36 ± 2	41.11 ± 5
Silver	19.62 ± 2	61.35 ± 7	37.12 ± 3	41.64 ± 4
Mean	28.23 ± 5	56.14 ± 4	33.08 ± 3	40.60 ± 1
	ns	b	d	ns
Barley				
Haybet	19.61 ± 3	71.58 ± 8	27.53 ± 2	37.12 ± 2
AC Metcalfe	24.00 ± 6	70.45 ± 6	29.65 ± 1	53.86 ± 5
Mean	21.81 ± 2	71.02 ± 1	28.59 ± 1	45.49 ± 8
	ns	a	cd	ns

Table 5.4. Root to shoot Zn and Fe translocation indexes (%) of wheat and barley cultivars after -Zn (Zn deficient) and +Zn (Zn sufficient) treatments. ± values represent standard error of two independent replicates. Letters denote significant difference at the 0.05 level between means of crop varieties (ns= not significant).

Discussion

In recent years, substantial effort has been paid to biofortification programs to enrich and increase the micronutrient status, especially Zn and Fe, of cereal crops. In this study, our aim was to investigate the Zn deficiency tolerance and micronutrient diversity of Montana wheat and barley cultivars.

DTZ staining results correlated with the previous studies, confirming aleurone layer and embryo were indeed parts of the grain where Zn is predominantly localized

whereas endosperm was lower in Zn (Ajiboye, 2015; Choi, Graham, & Stangoulis, 2007; Ozturk et al., 2006; Shobhana et al., 2013; Velu, 2008). Color intensity differentiation between DTZ stained grains with Zn concentrations lower than 25 mg kg^{-1} was ineffective, therefore, DTZ staining can be useful in rapidly determining grains with normal Zn concentrations. We found Zn is highly mobile during germination and can be re-translocated to newly emerging root and coleoptile in less than 36 hours (Figure 5.4). Spectral absorbance significantly correlated with increasing red color formation (Figure 5.3). However, color formation was not observable due to interference of grain matrix color with the red stain color. Therefore, DTZ staining of grain flour samples are not found to be as effective and reliable as grain DTZ staining (Figure 5.2). Zn specificity of dithizone staining showed only high levels of Cd may interfere and reduce the accuracy of Zn concentration reading (Figure 5.5). Previously, tissue dithizone staining of Cd hyperaccumulator *Biscutella laevigate* formed red color, however, the Cd concentration in the tissues were in extremely high concentration (12 m kg^{-1}) (Pielichowska, 2004). Slight pink color formation during Mn incubation is not related to DTZ but rather to the natural color of Mn^{2+}.

Generally, nutrients are localized in the protein bodies in the form of globoid crystals (Lott, 1980). Protein bodies, some of which contain phytic acid, are commonly present in the aleurone layer and embryo and found to be the major sites of Zn and Fe binding. However, the presence of protein bodies together with phytic acid is minimal in the endosperm of cereal grains. Although studies showed increase in endosperm Zn

concentration with the application of outsource Zn (Ajiboye et al., 2015), the increase was limited and Zn was not evenly distributed along the wheat grain (Ajiboye et al., 2015; Stomph, 2011). Accumulation of Zn in the crease tissues suggested that Zn is highly regulated in the wheat grain and it is still unknown whether poorer endosperm Zn concentration is transporter or sink related. Moreover, speciation of Fe and Zn was suggested to be different in cereal grains. Zn was found binding to sulfur containing peptides whereas phytic acid was the sink for Fe (Persson, 2009). Similar results were found in rice (Prom-u-thai et al., 2008). Grain protein levels was found to correspond to Zn concentrations in wild emmer wheat grains and common wheat grains (Morgounov, 2007; Peleg et al., 2008). High protein and high Zn relation correlates well with higher Zn concentrations found in pulses (Hemalatha, 2007).

In our study, concentrations of grain Zn and Fe were in the range of 15-49 mg kg^{-1} and 34-51 mg kg^{-1}, respectively (Table 5.1). Based on previous micronutrient diversity of wheat and barley grains (Cakmak, 2010; Kumar 2016; Rengel, 1999), our concentrations are in the normal range for modern wheat and barley cultivars. Spring wheat cultivars were generally higher in both Zn and Fe concentrations across all crop varieties. Data relates with the screening of spring and winter wheat cultivars in Central Asia in which spring wheat varieties were significantly higher in Fe concentration across all cultivars (Morgounov, 2007). Grain Cd levels in durum wheat cultivars were significantly higher than other crops. The Codex Alimentarius Commission standardized maximum Cd levels in wheat and rice grains not to exceed 0.2 mg kg^{-1} (Codex, 2001).

Cultivars Chinese Spring (0.38 mg Cd kg^{-1}), Divide (0.20 mg Cd kg^{-1}), Havasu (0.21 mg Cd kg^{-1}), Mountrail (0.29 mg Cd kg^{-1}), Silver (0.24 mg Cd kg^{-1}), Tioga (0.41 mg Cd kg^{-1}) and Warhorse (0.21 mg Cd kg^{-1}) exceeded the standard. Increased Cd accumulation in durum wheat grains is a commonly encountered phenomenon (Zook, 1970). We found strong positive correlation between grain Cd levels and shoot Zn concentration and root Cd content under Zn deficiency for all crops. However, similar relations were not observed under Zn supply. Root to shoot translocation index significantly positively correlated with durum wheat grain Cd levels under both Zn deficiency (r= 0.998*) and Zn sufficiency (r= 0.992*) contrary to other crops. Data suggests Cd can interfere with Zn root uptake under Zn deficiency in cereals and Zn supply decreases tissue Cd concentration in both shoot and root (data not shown). However, cadmium root to shoot translocation is not affected by Zn supply in any of the crops including durum wheat, suggesting Cd and Zn may only interfere and behave antagonistically with each other within the root cells. Field studies have demonstrated soil Zn addition helps reduce crop Cd accumulation (Choudhary, 1995; Hart et al., 2005). It is likely that susceptibility of durum wheat to grain Cd accumulation of these three cultivars are related to xylem translocation from root to shoot and phloem loading into grain and not root uptake. Strong positive correlation between grain Cd and root to shoot translocation of durum wheat cultivars may offer a rapid screening and identification of Cd accumulating durum wheat cultivars in a short term hydroponics study (Archambault, 2001).

Grain Zn, Fe, S, and P concentrations positively correlated with each other and not with Cd (Figure 5.7). As discussed above, S and P in grain were the sinks for Zn and Fe, respectively. Therefore, this positive correlation may offer a successful breeding strategy for simultaneous grain Zn and Fe concentration improvement. Previously, association of grain protein concentration, Zn and Fe was identified between QTL's from recombinant inbred lines (RILs), derived from a cross between durum wheat and wild emmer (Peleg, 2009). Moreover, colocalization of Fe and Zn QTL's was also observed in hexaploid wheat (Crespo-Herrera, 2016).

Hydroponics experiments revealed there was variation among spring wheat, durum wheat, winter wheat and barley cultivars in response to Zn deficiency, although not statistically significant (Table 5.2). Two spring wheat cultivars (Fortuna and Brennan) and one winter wheat cultivar (Carter) were found to show higher tolerance to Zn deficiency whereas remaining cultivars (AC Metcalfe, Chinese Spring, Divide, Ledger, Haybet, Mountrail and Silver) were deficiency intolerant. Without Zn supply, shoot Zn content of all cultivars were below 20 $\mu g\ g^{-1}$ which is the Zn level threshold for normal plant growth (Genc, 2002).

The Zn efficiency of the cultivars ranged from 21% to 71%. Zn efficiency was not correlated with shoot dry weight, shoot Zn concentration, shoot Zn content, root to shoot Zn translocation index or seed Zn concentration. The results suggested that variation in Zn efficiency among these cultivars is hereditary and cultivars likely to have different Zn utilization processes at the cellular level. Previous studies demonstrated

lower efficiency index for durum wheat cultivars (ranged 57%-33%) when compared to wild emmer (ranged 47%-19%) (Durmaz et al., 2009). In a recent comprehensive study including 106 RIL's derived from a cross between durum wheat and wild emmer, the Zn efficiency range was between 31% to 91%. Similar to our results, a relation was absent between Zn efficiency and shoot Zn concentration (Velu et al., 2017).

Zn supply decreased shoot Fe content in both winter and durum wheat. Similar results were observed in a study involving 80 wheat cultivars (Saha et al., 2015). Zn translocation index suggested winter wheat cultivar Carter efficiently translocated Zn from root to shoot more than any other cultivar (Table 5.4). Moreover, grain Fe concentration was found to be negatively correlated with root to shoot Zn translocation yet positively correlated with root to shoot Fe translocation under deficiency. Although, data suggests high Fe grains may translocate less Zn to xylem and eventually to the grain under deficiency, the positive correlation between grain Fe and grain Zn concentrations is in contrast with this hypothesis.

Durum wheat has been associated with being the most Zn deficiency sensitive among all cereal species (Cakmak et al., 1997). In our study, results suggest the order of increasing Zn efficiency of respective cereals were to be durum wheat, barley, spring wheat and winter wheat. Many mechanisms have been proposed in order to explain Zn efficiency and Zn translocation variation in cereals. Root exudates, root uptake and shoot translocation, lack of D genome (in case of durum wheat), difference in cellular concentration of Zn binding substances, soil microbiome and weather conditions have

been attributed to variation among cereals (Cakmak et al., 1999; Genc & Mcdonald, 2004; Gupta, 2016; Marschner, 1998; Oburger et al., 2014). However, current knowledge does not propose the superiority of one mechanism over the other. Furthermore, Zn efficiency and grain Zn concentration should be taken into account as two different traits since our data indicates that initial grain Zn concentration is not related to Zn deficiency tolerance. Therefore, genotypic differences in Zn efficiency cannot be explained by Zn concentration in shoot or grain. Similar observations were found in wheat and rice plants (Genc, 2008; Jiang et al., 2008). Our data suggests, Zn deficiency susceptible barley and durum wheat cultivars may be more suitable breeding candidates for higher grain Fe levels grown on Zn deficient soils. On the other hand, spring wheat cultivars Fortuna and Brennan can be used for higher grain Zn and Fe concentrations as well as higher Zn efficiency on Zn deficient soils. Recent field studies under different environmental conditions demonstrated heritability of grain Zn and grain Fe concentrations in spring wheat genotypes (Velu, 2012), contributing to the sustainability of biofortified wheat varieties.

Another factor to consider when breeding for Zn efficient genotypes, is the yield performance of the cultivars. When the combination of high Zn efficiency and a good yield is individually considered, Brennan and Carter were the leading cultivars (Heo et al., 2016; Berg, 2016; Ransom et al., 2016; Wichman, 2015). However, grain Zn concentration were low for both cultivars, 27 mg kg^{-1} and 17 mg kg^{-1} respectively. However, it should be kept in mind that simultaneous improvement of yield and grain

Zn concentrations are not always feasible and in some occasions negative correlations were observed (Velu, 2016).

In conclusion, the observed relative differences in Zn efficiency and grain Zn concentration among these cereal cultivars indicate that a potential to breed for cereal cultivars with increased Zn deficiency tolerance and elevated grain Zn levels.

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CHAPTER SIX – GENERAL DISCUSSION AND FUTURE DIRECTIONS

General Discussion and Future Directions

Zn deficiency is a commonly encountered issue especially in Northern regions of the United States. This issue not only manifests itself as lower grain Zn concentrations but also as deficiency induced Cd toxicity in cereals particularly in durum wheat. There is a lack of information on Zn efficiency and grain micronutrient diversity for Montana cultivars. In this study, we found there is a great variation between different Montana cereal crops in response to Zn deficiency. The Zn efficiency of the cultivars ranged from 21% to 71%. Zn efficiency was not correlated with shoot dry weight, shoot Zn concentration, shoot Zn content, root to shoot Zn translocation index or grain Zn concentration. The results suggested that variation in Zn efficiency among these cultivars is hereditary and cultivars likely to have different Zn utilization processes at the cellular level. Grain Cd levels were higher than the required Cd concentration standard for all durum wheat cultivars. We propose susceptibility of durum wheat cultivars to grain Cd accumulation are related to xylem translocation from root to shoot and phloem loading into grain and not root uptake. Moreover, the existence of ZIP1 and IRT2 genes may offer over-expression of these genes to increase xylem translocation and grain phloem loading of Zn. A short-term solution to alleviate Zn deficiency in Montana wheat and barley cultivars would be the addition of Zn to common NPK fertilizers. Studies have shown that the addition of Zn to N fertilizer, in particular, can increase the grain Zn

concentrations above the targeted amount (40 mg kg^{-1}) (Velu, 2014). Alternaively, milling methods may be improved in order to minimize nutrition loss in cereal-based food. A long-term approach would be the breeding approach; for example, breeding of high grain Zn content Montana cultivars with wild relatives of wheat, such as *Triticum diccoccoides*. It has previously been demonstrated that *Triticum diccoccoides* provides the genetic resource to modern wheat cultivars for grain Zn and Fe content improvement (Cakmak, 2004). Furthermore, breeding of Zn efficient and Zn inefficient cultivars can be used to identify QTL's and DNA markers linked to Zn efficiency to improve Montana wheat and barley cultivars with high deficiency tolerance.

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APPENDICES

APPENDIX A

ZIP1 AND IRT2 MRNA SEQUENCES

ZIP1 and IRT2 mRNA Sequences

Tricitum aestivum Zn transporter ZIP mRNA (complete cds)

```
LOCUS       AY864924 1083 bp mRNA linear PLN 01-FEB-2005
DEFINITION Triticum aestivum Zn transporter ZIP mRNA, complete cds.
ACCESSION   AY864924
VERSION     AY864924.1
SOURCE      Triticum aestivum (bread wheat)
ORGANISM  Triticum aestivum
          Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
          Spermatophyta; Pooideae; Triticodae; Triticeae; Triticinae; Triticum.
AUTHORS   Zhao,Y.L., Zhou,R.H. and Jia,J.Z.
TITLE     Diversity, evolution and gene expression of Zn transporters from wheat
JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 1083)
FEATURES             Location/Qualifiers
     source          1..1083
                     organism="Triticum aestivum"
                     mol_type="mRNA"
                     db_xref="taxon:4565"
     CDS             1..1083
                     codon_start=1
                     product="Zn transporter ZIP"
                     protein_id="AAW68439.1"
                     translation="
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                     KLKLVAIASILTAGAAGVLVPVLGRSMAALRPDGDIFFAVKAFAAGVILATGMVHILP
                     AAFDGLTSPCIYKGGGDRNGFPFAGLVAMSAAMATMVIDSLAAGYYRRSHFSKARPLD
                     NIDIPGDEEGRADHPHVHAHGHSHGDAIVVSSPEEAAIADTIRHRVVSQVLELGILVH
                     SVIIGVSLGASVRPSTIKPLVGALSFHQFFEGIGLGGCIVQANFKVRATIIMATFFSL
                     TAPVGIVLGIAISSSYNVHSSTAFIIEGVFNSASAGILIYMSLVDLLAKDFNNPKLQT
                     NTKLQLMTYLALFLGAGMMSMLAIWA"
```

ORIGIN
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61 caggccgcgg cggccagcgg cgggttcgag tgcacgaccg ccacggacgg ggcggacaag
121 cagggcgcga cgaagctgaa gctggtcgcc atcgcgtcca tcctcaccgc cggggcggct
181 ggcgtgctgg tgccggtgct cggacgctcc atggccgcgc tgcgccccga cggcgacatc
241 ttcttcgcgg tcaaggcgtt cgccgctggc gtcatccttg ccactggcat ggtgcacatc
301 ctgccggcgg cgtttgacgg gctcacctcc ccgtgcatct acaaaggtgg cggggacagg
361 aacggcttcc cttttgcggg acttgtggcc atgtctgcag ccatggccac aatggtgata
421 gactcgctgg ctgctgggta ctaccgccgg tctcacttca gcaaggcacg cccacttgac
481 aacatcgaca tacccggaga tgaggaaggg agggccgatc atccacatgt gcacgcgcat
541 ggccattcac atggtgacgc aattgttgtc agctcaccgg aggaggctgc catagctgac
601 acaatccggc acagggtggt atctcaggtt ctagagctgg gaatcttggt gcattcagtg
661 ataattggtg tgtcattagg agcatctgtg aggccatcca ccatcaagcc tctggtcggt
721 gccctcagct tccatcaatt ctttgaaggc ataggcttgg gtggttgcat tgtacaggct
781 aatttcaagg taagggcaac catcatcatg gcaacgtttt tctccctgac cgcacccgtg
841 ggcatcgtgc tagggattgc gatatcgtct agctataatg tgcatagctc tactgccttc
901 attattgagg gagtcttcaa ctcagcctcg gcagggattt taatctacat gtccctggtg
961 gaccttctag caaaagattt caataaccca aagctacaga caaatacaaa gcttcagctg
1021 atgacatatc ttgcactttt cctaggtgca gggatgatgt ccatgcttgc catatgggca
1081 tag

Oryza sativa Japonica Group OsIRT2 mRNA (complete cds)

LOCUS AB126086 1113 bp mRNA linear PLN 15-FEB-2008
DEFINITION Oryza sativa Japonica Group OsIRT2 mRNA for iron regulated transporter-like protein, complete cds.
ACCESSION AB126086
VERSION AB126086.1
SOURCE Oryza sativa Japonica Group (Japanese rice)
ORGANISM Oryza sativa Japonica Group
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Oryzoideae; Oryzeae; Oryzinae; Oryza; Oryza sativa.
AUTHORS Ishimaru,Y., Suzuki,M., Tsukamoto,T., Suzuki,K., Nakazono,M., Kobayashi,T., Wada,Y., Watanabe,S., Matsuhashi,S., Takahashi,M., Nakanishi,H., Mori,S. and Nishizawa,N.K.
TITLE Rice plants take up iron as an Fe3+-phytosiderophore and as Fe2+
JOURNAL Plant J. 45 (3), 335-346 (2006)
PUBMED 16412081
REFERENCE 2 (bases 1 to 1113)
AUTHORS Ishimaru,Y. and Nishizawa,N.K.
JOURNAL Submitted (13-NOV-2003) Contact: Yasuhiro Ishimaru University of

Tokyo, Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Science; Bunkyo-ku, Tokyo, Japan

FEATURES Location/Qualifiers

gene 1..1113
gene="OsIRT2"

CDS 1..1113
gene="OsIRT2"
note="IRT-like protein;
ZRT, Zn regulated transporter"
codon_start=1
product="iron regulated transporter-like protein"
protein_id="BAD18964.1"
translation="
MMMSSSQTPVRIAFVFLVILAATDAHSDHRTPPPACGGAAVGGE
CHSVARALRLKLIAIPAILAASVAGVCLPLFARSVPALRPDGGLFAVVKAFASGVILG
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RASSAVAHHGDHGHCHAHALGQADVAALSTTEAADQGSGDVEAGNTTKAQLLRNRVIV
QVLEMGIVVHSVVIGLGMGASQNVCTIRPLVAALCFHQMFEGMGLGGCILQAGYGGRT
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ADFMGPKLQGNVRLQLAASLAILLGAGGMSVMAKWA"

ORIGIN

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  61 gccgcgactg atgcgcacag cgaccaccga actccgccgc cggcgtgcgg aggcgcggcc
 121 gtgggagggg aatgccacag cgtggccagg gcgctccgcc tgaagctgat cgccatcccg
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 361 ctgcccagga agccatggtc ggagttcccg ttcgcggcgt tcgtcgccat gctcgccgcc
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 841 gtcttcttct tctccaccac gacgccgttc gggatcgcgc tggggctcgc gctgaccagg
 901 gtgtacagcg acagcagccc gacggcgctg gtcgtcgtcg gcctgctcaa cgcggcgtcg
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1021 aagctgcagg gcaacgtccg tctccagctc gccgcgtccc tcgccatcct cctcggcgcc
1081 ggcggcatgt ccgtcatggc caagtgggcg tga
```

APPENDIX B

ZIP1 AND IRT2 ASSOCIATED SEQUENCES IN *T. AESTIVUM*

ZIP1 and IRT2 Associated Sequences in *T. aestivum*

ZIP1 Associated Sequence in *T. aestivum* Chromosome 3

>lcl|chr3A:740881659-740883099 (1240 kb)
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CGTACGTACTGGCCAAATATACCTAGAAACTCCTCTGCATATATATCGTGCTGGCCTTGAAGAAA
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OsIRT2 Associated Sequence in *T. aestivum* Chromosome 4

>lcl chr4D 7797354-7799147 (1794 bp)
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TCGTCGAGCTCCTCGCCGCCGACTTCATGGGGCCCAAGCTGCAGGGCAGCGTCAGGCTCCAGCT
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APPENDIX C

ZIP1 AND IRT2 SEQUENCING RESULTS

ZIP1 and IRT2 Sequencing Results

ZIP1 Sequence of Chinese Spring

ATTGCCAGGCCTTGGCTGGCTAGCTTACCTGCTTCTTCCATGCAGGGCGCAGGCGGCCATGGGC
GGCGTCGACCACGGTGACGGCGATGAGGAGGACGAAGATGGCCACCACGGCGACGCGGGCG
TCGGCGGGGGGCTCCGGTCCAGGGGGCTCATCGCCGTGAAGGTGTGGTGCCTGGTGATCCTGC
TGGTGTTCACCTTCCTGGGCGGCGTGTCCCCCTACTTCTACCGCTGGAACGAGGCCTTCCTCCTC
CTCGGCACCCAGTTTGCCGCCGGCATCTTCCTCGGCACGGCCCTCATGCACTACCTCGCGGACGT
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GCTTCCTCCTCACCATGCTCAGCGACGTCGTCATCGTCGCCGTCGCCAACAGGCAGAGGGTCAA
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GCGGTGGCGCACGCGCACCCTATGCTCATGACGGCGACATCGTCCTTCGAGGACGCGATCCTCC
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CTCCTCCGGATGATCCCCAAACGCCCATTCCTCCTGACGGTGGCTCTACTCGCTAAGCGTTCCG
CAGTGTCAAGACCGAGGTAGGGGGTGGGGCATCGGCATTC

IRT2 Sequence of Chinese Spring
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CAGCTTGGGCCCCATGAAGTCGGCGGCGAGGAGCTCGACGAGCGCCATGTAGTGCAGCAGCC
CCGCGGAGGCGGCGTTGAGCAGGCCGACGACGATGAGCGCGGTGGGGCTGTTGTCCTTGTAC
ACCTTGGTGAGCGCCAGGCCGAGCGCGATCCCGAAGGGCGTGGTGGTGGAGAAGAAGAAGAC
CAGCCCGGCCTTCATCCTGGTGCCGTACTCGGCCTGGAGGATGCAGCCGCCGAGGCCCATCCCC
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APPENDIX D

HYDROPONICS EXPERIMENT CULTIVAR PHOTOS

APPENDIX D

Hydroponics Experiment Cultivar Photos

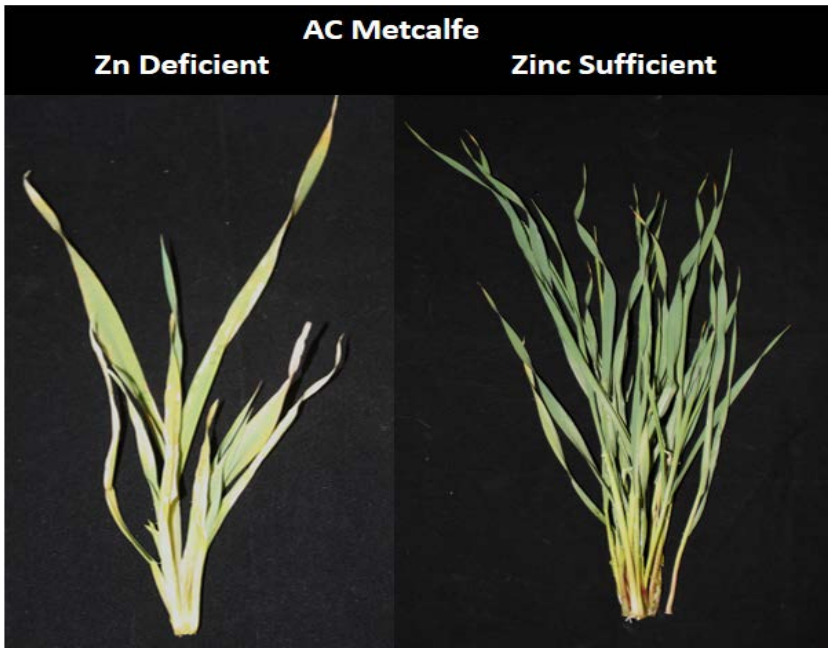

Figure Appendix D.1 Physiological response of spring wheat cultivar AC Metcalfe when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.2 Physiological response of spring wheat cultivar Brennan when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.3 Physiological response of spring wheat cultivar Carter when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.4 Physiological response of spring wheat cultivar Chinese Spring when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.5 Physiological response of spring wheat cultivar Divide when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.6 Physiological response of spring wheat cultivar Fortuna when subjected to Zn deficient and Zn sufficient environments.

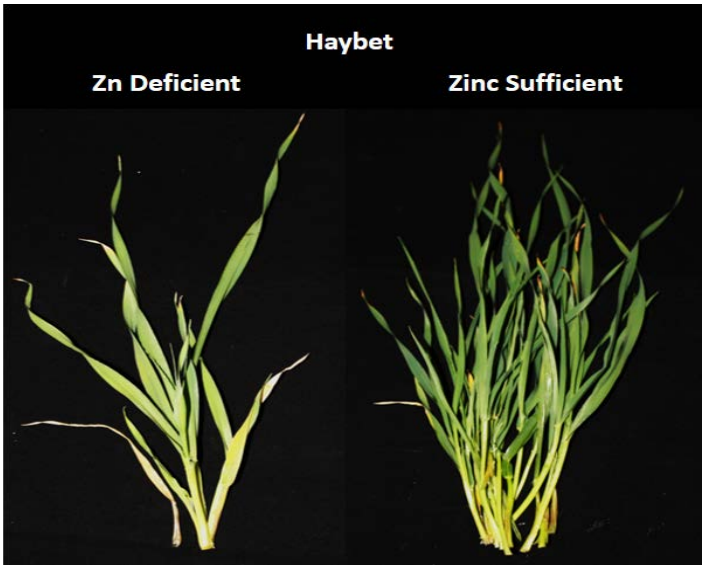

Figure Appendix D.7 Physiological response of spring wheat cultivar Haybet when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.8 Physiological response of spring wheat cultivar Ledger when subjected to Zn deficient and Zn sufficient environments.

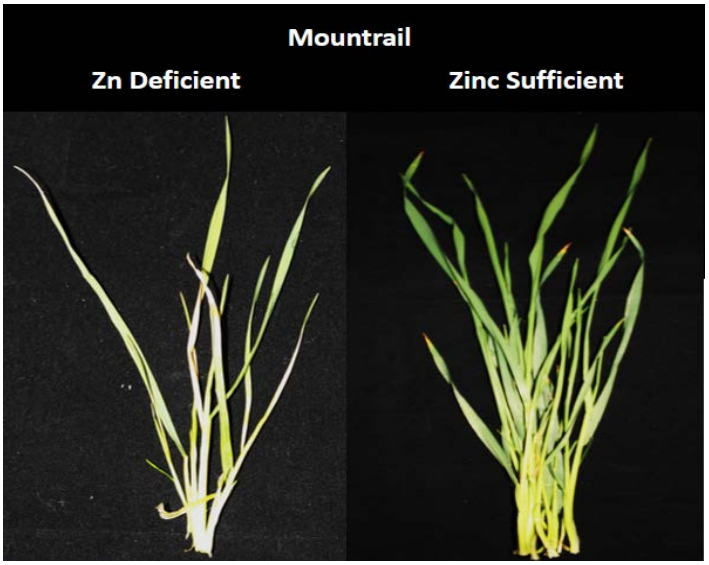

Figure Appendix D.9 Physiological response of spring wheat cultivar Mountrail when subjected to Zn deficient and Zn sufficient environments.

Figure Appendix D.10 Physiological response of spring wheat cultivar Silver when subjected to Zn deficient and Zn sufficient environments.