Investigating the genetics and agronomic traits associated with elevated grain Zn concentration in wheat

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By

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This thesis is dedicated to my family Rashid, Junaid, Nadir, Asjid and Rubab whose moral support in many ways and over many years helped me to pursue my goals.

Abbreviations

PS : Phytosiderophores

NA : Nicotianamine

G x E : Genotype x Environment

EDXRF : Energy Dispersive X-ray Fluorescence

AAS : Atomic Absorption Spectrometry

CI : Confidence Interval

DH : Double Haploid

HPAN : HarvestPlus Advance Nursery

ICP-OES : Inductively Coupled Plasma–Optical Emission Spectrometry

LOD : Logarithm of the odds

QTL : Quantitative trait loci

XRF : X-ray Fluorescence spectrometry

MAS : Marker Assisted Selection

CIMMYT : International Maize and Wheat Improvement Center

SARDI : South Australian Research and Development Institute

TGW : Thousand Grains Weight

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Abstract

Biofortification of wheat and other food crops with Zn is an important and high-priority research issue. This project focused on the genetic and agronomic factors that control grain Zn concentration in wheat. Understanding this may make it possible to increase Zn in wheat for biofortification purposes. Specific aims were (i) to identify wheat genotypes that differ in grain Zn concentration. (ii) to understand agronomic traits that are relevant to Zn accumulation in grain (iii)) to conduct mapping studies to find genetic loci which confer high Zn concentration.

A number of genotypes of Berkut x Krichauff were grown to investigate Zn concentration and its relationship with morphological traits. This study confirmed a negative association of grain yield with Zn concentration. Low Zn lines which had low Zn concentration in the field, had high grain Zn concentration in the glasshouse which was considered to be due to dust contamination and a modified Kett mill, has been used to eliminate dust contamination but the results have shown that Fe concentration and ranking are greatly affected by soil contamination, whereas Zn concentration and ranking are not. A cleaning method was shown to be an important step prior to analysis for precise QTL analysis. The inconsistent results of low Zn lines were probably not a consequence of soil contamination. The results were due to inconsistent Zn levels in the grain itself. Because there was no correlation between field trials and glasshouse results, the focus of study was switched to the genetics of grain Zn concentration.

There was a need for increased knowledge of the genetic basis of Zn density in wheat and QTL analysis is considered a powerful strategy to do this. ICP-OES analysis is mostly used for QTL analysis which is very expensive. XRF was proposed as an alternate method for elemental analysis. Importantly, despite the greater errors of XRF results than ICP-OES results, XRF results allowed the identification of the same number of QTLs as ICP-OES, in the Berkut x Krichauff population in year 2007.

Berkut x Krichauff 2009, Excalibur x Kukri, and Kukri x RAC 875, populations were used for validation. In Berkut x Krichauff populations, two QTLs for Zn, located on 7A (LOD 4.9) and 5D (LOD 3.9), were identified and the QTL on 5D was a new one not reported in any published studies, whereas the QTL on 7A was also found in two other mapping populations and in four other studies reported in literature. These two new Zn QTLs were not co-located with grain size while in the other two populations, Zn QTLs were found but co-located with grain size and the Zn-dense trait was associated with a smaller grain size and hence a concentrating effect. This trait is not desirable for plant breeding as it is associated with a lower yield. The Zn QTLs not associated with grain size can be used to further develop genetic markers which plant breeders can use to speed up the breeding of genotypes with a higher level of grain Zn.

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Chapter 1 Literature review

1.1 Introduction

Zinc (Zn) is an important micronutrient for plants and animals owing to its main function in expression of genes and replication and development of cells (Tauris et al., 2009). A recent investigation has shown that 925 human proteins and 536 proteins present in *Arabidopsis thaliana* are bonded by Zn (Gladyshev et al., 2004). In plants, Zn is also especially important in membrane protection against oxidative damage through the detoxification of superoxide radicals $O_2^{\cdot -}$ to oxygen and hydrogen peroxide, blocking $O_2^{\cdot -}$ driven cell damage (Bowler et al.,1994; Cakmak 2000). It is also important for the establishment and maintenance of disease resistance mechanisms, photosynthesis/sugar formation, growth regulation and seed production (Agrios 2005; Marschner, 1995).

Zn deficiency is a major problem in less developed countries where diets are heavily based on cereals which are nutritionally quite low in Zn. The most common symptoms of Zn deficiency in humans include stunted growth, late maturation of bones, defective immune system and diarrhea (Ackland and Michalczyk 2006; *www.healthguidance.org/*.) Children and pregnant women are more at a risk of Zn deficiency particularly in developing countries where poverty leads to reduced diversity in their diets (Welch and Graham, 2004; Gibson, 2006). A further problem is that the presence of inhibitors like phytic acid in cereals reduce the bioavailability of Zn (Hurrell,2003) and during the milling and polishing processes, Zn present in the bran and embryo is also wasted and only small amounts of Zn present in the endosperm is available for absorption (Brinch-Paderson et al., 2007).

Recently, plant breeders have started to biofortify wheat with Zn (Ortiz-Monasterio et al., 2007). In order to enhance the quantity of Zn within the grain, a constant supply of Zn from root uptake and long distance transport to the grain are required (Palmgren et al,. 2008). Zhang et al. (2007) report that our knowledge of long distance Zn transport is still not well known and requires further research. The aim of this thesis is to study the mechanisms associated with genotypic variation in grain

Zn of wheat plants and will contribute to improving our knowledge of long – distance transport processes in the plant and the following review will highlight relevant literature on Zn transport processes and mechanisms that could operate within wheat to increase unloading of Zn into the grain.

1.1.1 The chemistry of Zinc

Zinc is a transition metal. Its atomic number is 30 and atomic mass is 65 (Barak and Helmke, 1993). It is the 23rd most abundant element in the earth's crust. Five stable isotopes of Zn are ^{64} Zn (48.63%), ^{66}Zn (27.90%), ^{67}Zn (4.90%), ^{68}Zn (18.75%H) and ^{70}Zn (0.62%) (*chemistry.about.com/od/element facts/a/**zinc**.htm*). Heavy and light isotopes have been used in many plants to enhance Zn fractions in roots and shoots (Weiss et al., 2005). Zn has 30 short-lived isotopes with atomic mass range of 54-83, and its longest-lived isotope is ^{65}Zn (t1⁄2 =244.26d) which is mostly used as a Zn tracer in plant transport studies.

The oxidation state of Zn in solutions is +2. A completely filled d shell of electrons makes it stable under physiological conditions. This is unlike $Fe^{2+}$and Cu^{2+} (Barak &Helme, 1993; Auld, 2001). Due to its small radius to charge ratio (i-e.0.83A, coordination number, CN=6), it behaves like a Lewis acid and forms covalent bonds with other elements such as sulfur, nitrogen and oxygen. As a result, Zn can link amino acid residues, for example, glutamic acid (Glu), aspartic acid (Asp), cysteine (Cys) and histidine (His) (Broadley et al., 2007).

Many soluble salts of Zn are present in the soil as halide, sulphates, nitrates, acetates, thiocyanates, perchlorates, fluorocynades, alkali metals, zincates and Zn-ammonia salts. It has some sparingly soluble salts also, for example, Zn-ammonium phosphate, Zn hydroxide and carbonate and insoluble organic compounds (Lindsay, 1979; Barak & Helmke, 1993).

1.1.2 Biochemical properties of Zn

Iron (Fe) is the most abundant transition metal found in living organisms and Zn comes after iron (Fe) (Broadley et al., 200). All six classes of enzymes, lyases, oxidoreductases, transferases, hydrolases, isomerases and ligases have Zn in them

(Webb, 1992). The binding properties of Zn^{2+} determine the function and reactivity of enzymes. The main binding sites of the Zn^{2+}-ligand are structural catalytic and co-catalytic (Auld, 2001; Maret, 2005). Four ligands are present at structural Zn sites and protein folding is ensured by Zn at structural Zn sites (e.g. alcohol dehydrogenases). In catalytic sites, Zn has its main role in catalytic functions such as, carbonic anhydrase. Zn^{2+} may be utilized in catalytic as well as regulatory and structural functions of co-catalytic sites such as in superoxide dismutases. The largest class of Zn–binding proteins in organisms is the finger domain containing proteins, which can regulate transcription directly through effects on DNA/RNA binding, and also through site-specific modification, regulation of chromatin structure, RNA metabolism and protein- protein interactions (Klug, 1999; Englbrecht et al., 2004).

There is another fourth kind of Zn^{2+}-ligand binding site which acts both as a catalytic and structural site. This happens when ligands from the surface of protein molecules, link to one Zn atom. Finger domain proteins are the biggest group of Zn-binding sites found in organisms. They can regulate transcription via site-specific changes (Klug, 1999; Englbrecht et al., 2004). Analysis of protein sequences has shown that 925 proteins in humans and 536 proteins in *Arabidopsis thaliana* require Zn for binding (Gladyshev et al., 2004).

1.1.3 Zinc is both an essential and a toxic element

All organisms require Zn for their normal growth. It is present as a cofactor in more than 300 enzymes which are involved in carbohydrates, fats, nucleic acid and protein metabolism (Coleman, 1998). In many proteins Zn has an important role, especially in transcription factors (Berg and Shi, 1996; Vallee and Fallachuk, 1993).

More than 2000 transcription factors need Zn as a co-factor (Prasad and Kuck, 2002). Zn is also involved in maintenance of cell and organ integrity by stabilizing molecular structures (Broadley et al., 2007). When Zn supply to plants is inadequate, growth and yield are affected severely and the quality of crops is also reduced. Zn deficiency in plants decreases growth, but excess Zn is toxic to biological systems through metal-based cytotoxic reactions (Nishizawa et al., 2011) and may show

similar symptoms as those of Cd or Pb toxicity symptoms (Chaney, 1993; Foy et al., 1978). The toxic effect of Zn may be due to the inactivation caused by certain reactions which can displace cofactors (i.e. metallic ions such as $Mn^{2+}$and Fe^{2+}). The mechanisms involved in Zn homeostasis in plants are still not fully known (Kramer et al., 2007; Hacisalihoglu et al., 2004; Broadley et al., 2007). Zn homeostasis which can control the changes in the availability of Zn is essential for plants because physiological differences between deficiency and toxicity are very small and narrow (Maret and Sandsteed, 2006). Zn toxicity has been reported by Keisling and others (1977) for peanuts in Georgia. Some crops may experience Zn toxicity in acid soils and species such as spinach and beet with a high Zn uptake capacity could be more sensitive to this excess (Chaney, 1993; Broadley et al., 2007). Bioaccumulation of Zn in plant tissues may present a health risk to wildlife and to humans (Sagardoy et al., 2009).

1.2 Zinc in Soil

Approximately 80 mg kg Zn is present in the lithosphere. Most of the soils have 10-300 mg kg^{-1} Zn while the average concentration of soil Zn is 50 mg kg^{-1} (Krauskopf 1972; Wedepohl 1972; Kiekens, 1995; Goldschmidt, 1954). In Australia, the range of total Zn concentrations is < 2-180 mg kg^{-1} with a mean Zn concentration of 34 mg kg^{-1} (Tiller, 1983). Bertrand et al. (2002) identified a range of total zinc concentrations of 4-41 mg kg^{-1} in alkaline, non-calcareous (< 2% CaCO3) arable soils in South Australia and Victoria, and 5-36 mg kg^{-1} in calcareous soils (>2% CaCO3) in the same regions. The Zn uptake by plants and all reactions taking place in the soil are controlled by speciation of Zn (Ellis and Knezk 1972; Singh and Sekhon 1977; Sidh et al.1977). The speciation and localization of Zn is related to its chemistry inherited from parent materials (Kabata-Pendias – 2000). Zn dissolves readily in the soil as compared to other micronutrients (Kabata-Pendias, 2001). Free and complex ions are present in soil solution, which include Zn^{2+}, $ZnCl^{+}$, $ZnOH^{+}$, $ZnHCO_3^{+}$, $[ZnCl_3]^{-}$, $[ZnCL4]^{2-}$, $Zn(OH)_3^{-}$, ZnO_2^{2-} and ZnO_2^{-} and plants mostly absorb Zn in the form of the Zn^{2+} ion (Marschner, 1995).

1.2.1. Total Zinc Concentration in Soils

During the weathering of rocks, primary minerals such as Zn^{2+} are absorbed back into solution and then Zn^{2+} ions are adsorbed by organic components of soils (Chesworth, 1991). The total concentration of Zn in soils is linked with the composition of parent rock material. Kabata-Pendias and Pendias (1992) showed that the concentration of Zn is higher in shale and clay sediments (80-120 mg kg^{-1}) than sandstone, limestone and dolomite sediments of sedimentary rocks.

A minute quantity of total Zn is soluble or available; therefore the total concentration of Zn cannot be taken for the evaluation of plant available Zn. Zn reacts with organic acids, humic substances and dissolved organic carbon and forms complex compounds (Barak and Helmke, 1993). Plants can adsorb Zn in various forms, such as complex ions and Zn-chelates (Tiffin, 1972; Loneragan, 1975; Weinberg, 1977).

1.2.2 Zn in the Soil Solution

Soil solution contains a very minute quantity of the total zinc content of a soil solution (Barber, 1984; Alloway, 2004). Kabata-Pendias and Pendias (1992) explained that in the soil solution the soluble Zn is present in the range of 4-270 µg/L (ppb) which seems very low when the average total concentration of Zn is approximately 50-80 mg kg^{-1} (ppm).

When the solubility of zinc hydroxide ($Zn(OH)_2$), zinc oxide (ZnO) and zinc carbonate ($ZnCO_3$) is compared with soil Zn, these minerals are about 10^5 times more; Zn is mostly found as Zn^{2+} below pH 7.7, but above pH 7.7, $ZnOH^+$ predominates and above pH 9.11 $Zn(OH)_2$ predominates (Kiekens, L.1995).

Soluble compounds are formed when Zn reacts with sulphate, chloride, nitrate and phosphate ions. The most important forms that enhance total concentration of zinc in solution are sulphate ($ZnSO_4$) and phosphate ($ZnPO_4$). The use of acid fertilizers, for example, $ZnSO_4$, increases Zn availability to plants (De Datta , 1981; Longnecker and Robson, 1993).

1.2.3 Factors affecting the Availability of Zinc in Soils to Plants

The severity of Zn deficiency is determined by the concentration of Zn ions in the soil. Tiller et al. (1972) reported that less than 14% of Zn in acid soils is available in Australia. Vale (1982) reported that 0.3-0.5 % of total Zn is available in acid soils of the USA and Portugal. There are many factors which may affect Zn availability to plants. The nature and quantity of Zn in the soil is related with soil texture, pH, calcium carbonate organic matter (Sharma *et al.*, 2004). As soil solution is an immediate reservoir of available Zn to plants, these factors control the Zn present in the soil solution and also its sorption from and desorption into the soil. Factors include total soil Zn content, soil pH, organic matter content, redox conditions, microbial activity in the rhizosphere, soil moisture status and concentrations of other nutrients (Alloway, 2004; Brennan, 2005). Soils having a low amount of plant available zinc are frequently sandy and highly leached acid soils and zinc deficiency in their crops in common (Alloway, 2004).

1.3 Zinc in Plants

Plants require very small amounts of Zn, just like other essential micronutrients, for their metabolism (Marschner, 1995). Zn deficient soils are common in the world, and some arable lands being also calcareous and alkaline in nature with very high soil pH, reducing the Zn availability to the plants (Alloway, 2004). Many crop species are very sensitive to Zn deficiency for example cotton, legumes (particularly subterranean clover), flax, hops maize, sorghum, wheat, barley, grapes, citrus and many fruit tree species (Chapman 1966; Wellace 1966; Bould et al.1983).

In order to survive in Zn deficient soils, some sophisticated mechanisms have evolved in adapted plants to ensure adequate Zn uptake, utilization and storage in grains (Curie and Briat, 2003).

1.4 Short distance Transport (the absorption of Zn by roots)

The uptake of nutrients is a dynamic and complicated phenomenon that takes place through plant roots. The absorption of nutrients is dependent on the ion

concentration at the root surface (rhizoplane), root biomass, and the Zn requirement by plants (Fageria et al. 1991).

Zn uptake from soil and its transport within the plant are continuous processes, so the rate change becomes a limiting factor. Generally, Zn is transported to the root surface by means of three processes, mass flow, diffusion and root interception (Mengel and Kirkby, 1987; Salisbury and Ross, 1992). The transport of Zn by mass flow has little significance as compared to the other two processes owing to the low concentration of Zn in the soil solution. Many physiologists have studied the processes which are responsible for Zn uptake in roots (Rains et al.1964;Pitman 1976; Kochian 1991; 1993; Reid et al .1996; Williams et al.2000; Sattelmacher 2001).

Zn uptake by plants may be an active or a passive process (Kabata-Pendias, 2000). Transpiration is involved in the passive transport of ions from soil solution to roots by mass flow, which depends on the concentration of Zn present in soil solution and the moisture content of the soil. Diffusion and root interception also contribute to movement of Zn and other micro- and macronutrients (Barber, 1984; Jungk, 1991; Fageria et al., 1991). The active process of Zn uptake takes place under Zn deficiency and involves root exudates, which can be similar to what happening under Fe deficiency (Marschner, 1995).

For example in Fe- deficient soils, higher plants may absorb insoluble Fe by means of two strategies (Römheld and Marschner 1986). In nongraminaceous monocots and dicots (Strategy I plants), phenolic compounds are significant components of root exudates in response to Fe deficiency (Römheld and Marschner, 1986; Susın et al., 1996; Curie and Briat, 2003; Hell and, Stephan, 2003). It has been suggested that these phenolic compounds increase Fe availability in the rhizosphere soil as a supplement to the plasma membrane-bound ferric reductase by chelating and reducing insoluble Fe (Dakora and Phillips, 2002). Moreover these physiological responses are linked with morphological and anatomical changes of the root such as transfer cell development and root hair formation (Marschner, 1995). Strategy II is a characteristic of graminaceous monocots including cereals (Römheld and Marschner

1986). Strategy II plants synthesize and release non-protein amino acids known as phytosiderophores (PS) which form chelates with Fe^{3+} ions ((Nishizawa et al., 2011) and these are less positively charged and soluble and are re-absorbed by the roots (Scholz et al.,1988).

Under Zn deficiency, phytosiderophores are also released from the roots of graminaceous species (Cakmak et al., 1996). Low amounts of PS release were observed under adequate supply of Zn and Fe but higher amounts were released under deficiency conditions (Erenoglu et al., 2000; Awad et al., 2000).

Zn is mostly absorbed as Zn^{2+} ions but it is also transported in the hydrated and chelated form (Loneragan 1975; Kabata-Pendias and Pendias 1984; Bell et al 1991; Marschner 1993; Kabata-Pendias, 2001). For symplastic transport, Zn is transported from the epidermis and cortex through a cytoplasmic continuum (plasmodesmata). Zn from this region can be pumped into the stelar apoplast (Lasat and Kochian, 2000). The tonoplast and plasma membrane uptake are mainly responsible in regulating this symplastic pathway. Zn can also be transported by the apoplastic pathway into the stele. The endodermis is the main barrier in passive movement of ions into the stele through the apoplastic pathway with the casparian band considered an effective barrier in this passive transport, but the casparian band is not fully developed at the root apex so it makes the barrier leaky (White, 2001; White et al., 2002b).

When entering the stele, Zn ions are unloaded into xylem vessels (Kochian, 1991). The transpiration stream then takes these ions toward the shoot. This radial transport of ions has certain restrictions. Pores present in the cell wall and root epidermal and cortical cells have a lining of pectin and hemicelluloses. These substances have galacturonic and glucuronic acids which possess higher cation capacity and may restrict the uptake of Zn^{2+} ions through roots (Clarkson, 1988). The transport of Zn ions needs complexation in order to avoid any oxidative damage to root cells which can occur due to interaction of free ions with oxygen intermediates (Briat et al.1995). Nicotianamine may help in the radial transport of Zn^{2+} by forming Zn-nicotianamine complexes (Sattelmacher, 2001; Scholz et al.1988).

A number of genes are linked with the transport of Zn ions in both strategy I and II plants (Eide et al. 1996; Zhao and Eide, 1996; Grotz et al. 1998; Eckhardt 2000; Vert et al. 2001; Waters et al. 2002). Gross et al., (2003) have identified forty three putative candidate genes in rice, belonging to five gene families (viz. OsYSLs, OsZIPs, OsNRAMPs, OsFROs and Ferritin). The expression of some of these genes has also been studied at different developmental stages and tissues (Doyle et al. 1998; Shigaki et al. 2001). Popayan and Kochian (2004) has described one metal transporting ATPase (*TcHMA4*) in *Thlaspi carerulescens,* a Zn hyper-accumulator plant. This protein works in the plasma membrane of the root and may cause efflux of Zn from xylem parenchyma to the xylem vessels in *Thalaspi*. The NAM transcription factor genes of wheat have also been shown to regulate Zn remobilization and its transport from vegetative tissues to the grain (Water et al., 2008).

Table 1.1 Genes involved in Fe and Zn uptake in different plants

Genes	Plants	Function	References
AtNAS$_{1,2,3}$	*Arabidopsis*	Encodes for nicotianamine synthase	Suzuki et al.(1999)
CLN	Tomato	Encodes for nicotianamine synthase	Ling et al. (1999)
AtHMA$_2$	*A. thaliana*	Catalyze Zn^{2+} efflux	Eren and Ariuell, (2004)
AtHMA$_4$	*A. thaliana*	Load Zn into xylem	Verret et al.(2004)
HuNAS$_1$	Tobacco	Encodes for nicotianamine synthase	Takahashi et al.(2003)
OsNAS$_3$ *OsNAS*$_4$	Rice Rice	Increase leaf and seed Zn concentration	Lee at al. (2009) Johnson et al. (2011)
NAM-B$_1$	Wheat	Encodes for NAC transcription factor	Uauy et al. (2006)
OsZIP$_4$ *OsZIP*$_5$	rice rice	Encoding Zn transporters Catalyze Zn^{2+} influx to root cells	Ishimaru et al. (2007) Lee et al. (2010)
TcZNT$_5$ *TcZNT*$_5$	*A. thaliana* *A. thaliana*		Wu et al. (2009)
AtMTP$_1$, *AtMTP*$_3$	*Arabidopsis*	Encoding protein that transport Zn^{2+}	Arrivault et al. (2006) Gustin et al. (2009)
HuIDS$_3$ *HuNAS*$_1$	rice rice	Encoding dioxyginase Encodes for nicotianamine synthase	Suzuki et al. (2008)
NRAMP$_{1,3,4}$ *NASHOR*$_{1,2}$ *NAAT* *OSNAS*$_{1,2,3}$	*Arabidopsis* Barley Barley Rice	Encodes for nicotianamine synthase	Cohen et al.(1998) Herbik et al. (1999) Takahashi et al.(1999) Higuchi et al.(2001b)

1.5 Long Distance Transport

1.5.1Transport within the xylem

Nutrients and water move towards transpiring organs in the transpiration stream. The driving force for xylem transport is root pressure and the water potential gradient between roots and shoots (Kochian 1991; Marschner, 1995). The rate of water transport during the transpiration process determines the concentration of Zn inside the xylem (Marschner, 1995). Inside the xylem stream, Zn does not move in the form of free ions but is bound to different ligands such as citrate and malate (Wolterbeek et al., 1984). Once Zn reaches the leaf, it can take different pathways to move from xylem to a specific cell. Zn can also continue to travel from cell to cell symplastically, where Zn is taken up and used. In the xylem stream of tomato and soybean, citric acid was found which acts as a ligand for Zn transport and Zn moves in the xylem as a Zn-citrate or Zn-malate complex (White et al.1981 a, b).

Solutes may reach the leaf through the transpiration stream and Leigh and Tomos (1993) described three possible pathways by which Zn is transported from the xylem to leaf cells. Firstly, through an apoplastic pathway in which water and Zn move together through the leaf apoplast passing the bundle sheath, mesophyll and then the epidermis. Secondly, through a partly symplastic pathway, Zn and water pass into the symplast at the bundle sheath cells. Water continues to move towards the sites of evaporation through a symplastic pathway, but ions may be secreted back to the mesophyll apoplast and move towards the epidermis. Thirdly, through the vein extension pathway which provides a diffusional pathway for ions to the epidermis. Every pathway transports Zn ions to particular cell types inside the leaf (Leigh and Tomos, 1993). As mentioned above, negatively charged carboxyl ions are present in the walls of xylem vessels which bind Zn^{2+} ions and hinder their transport inside the xylem (White et al.1981; Kochian, 1991).

Zn uptake of the root and leaf may be similar due to the ionic transfer of Zn from the apoplast to symplast (Schmidt, 2003). It is reported that the acidification of the xylem apoplasm occurs in leaf cells for Fe uptake and that also helps in Zn uptake

(Schmidt, 2003). Phytosiderophores present in the xylem, can be helpful in Zn uptake by chelating Fe and Zn in leaf tissues (Welch, 1995).

1.5.2 Phloem Transport

In the phloem, Zn is present in low concentrations (Pearson et al., 1996). Zn concentration in phloem sap range from 3 to 170μM Zn (Robson and Pitman, 1983) and Zn is thought to be transported complexed with nicotianamine (NA) or small proteins (Welch, 1995; Curie et al., 2009; Waters and Sankaran, 2011).and its flow rate is strongly dependent on the rate of phloem loading and unloading in the source and sink areas (Wolswinkel, 1990). Phloem loading occurs down the whole phloem transport pathway, which is hypothesized to be either apoplastic or symplastic (Atwell et al., 1999). The intercellular transfer continues from vascular parenchyma cells to phloem within the symplastic loading step. During apoplastic phloem loading, a membrane transporter is needed for transporting a metal-chelator complex by apoplastic and symplastic interfaces.

Inside the phloem stream, due to a high pH (9.5-8.5), Zn moves as a complex (Longnecker and Robson, 1993). Zn can form a bond with NA (von Wiren et al., 1999). Mori et al. (1991) reported that phytosiderophores present inside the phloem can also bind Zn and Fe. Due to differences of the physiological characteristics of sink tissues, phloem unloading of Zn is different in these tissues. Within the root and young leaves, Zn unloading can be symplastic and at the maternal-filial interface in developing seeds, it can be apoplastic or a combination of both (Patrick, 1997; Atwell et al., 1999). It was suggested that phloem is discontinuous within the floral axis (Jenner, 1986). Pearson et al. (1995) proposed that in wheat Zn is unloaded from the maternal phloem into the apoplast at the point where xylem is discontinuous and after that is taken up and transferred into the vascular system of the grain. Due to the symplastic discontinuity inside wheat grains ((Lucas et al., 1993), Zn unloading from phloem into grains is comparatively more difficult than other sink tissues. A Zn transporter is required for passing through this barrier. In

developing grains, NA and its transporter may be responsible for Zn phloem unloading (Koike et al., 2004; Tauris et al., 2009).

Additionally, a surplus of Zn supply has a negative effect on phloem loading and transport of other mineral nutrients (Herren and Feller.1996; Welch et al., 1999) which shows that it has the capability to induce local deficiencies.

1.5.3 Zn loading into seeds

The grain loading of Zn occurs during seed development. At this developing stage, the Zn content of vegetative parts fall off quickly due to remobilization of Zn into developing seeds and a reduction in Zn uptake and root activity takes place, this coincides with a competition between sinks for carbohydrates (Marschner,1995).

The Zn concentration in the seeds may depend on the quantity of Zn uptake during seed development and also on the rate of Zn remobilization through the phloem into developing seeds (Garnett and Graham, 2005). Pearson et al. (1995) reported that Zn loading capacity is relatively low, even in the presence of higher external Zn supply (i.e. in a culture solution with a 10-fold difference of Zn concentration, only 2.3-fold Zn loading into seeds was observed). Different types of vegetative parts showed variations in Zn concentration with Zn remobilization taking place from flag leaves (as compared to other lower leaves) into developing seeds (Pearson et al., 1994; Wu et al.,2010).

Pearson et al. (1998) used the radioactive isotope of ^{65}Zn to study the route of Zn transport from vegetative tissues into the wheat grains during seed development. The tracer study revealed that Zn transport first had to load into the phloem from the peduncle and rachis (Herren and Feller, 1994; Pearson et al., 1995). Up to 11 times more Zn accumulated in the pericarp than endosperm after 6 h of monitoring (Pearson et al., 1996a). Initially Zn is transferred to the pericarp and crease tissues through the crease phloem and then into the embryo via a phloem only strand (Zee and O'Brien 1970b; O'Brien et al., 1985; Pearson et al., 1998; Tauris et al., 2009). In more detail, Zn moves from transfer cells to nucellar projection and from there into the endosperm cavity (Thorne, 1985). The aleurone cells bordering the endosperm

cavity at the endosperm side take Zn and transfer it (Ellis and Kenezk 1972) into the endosperm (Stomph et al., 2011) and a large amount of Zn still remains present in the aleurone layer at seed maturity (Ozturk et al., 2006; Liu et al., 2007; Hansen et al., 2009; Persson et al., 2009; Cakmak et al., 2010a, b; Lombi et al., 2011; Stomph et al., 2011). Phytates present in the aleurone may absorb Zn and stop further transport into the endosperm and embryo (Marschner, 1995).

Our knowledge of grain Zn loading into developing seed is still limited (Welch and Graham, 2004). For a better understanding of Zn loading into grains, there is a need to focus on the route of Zn transport from vegetative tissues to grains and to better understand the rate of transfer within the xylem and phloem (Wolswinkel, 1999).

1.5.4 Source- sinks relationship

A positive correlation between Zn concentration and protein has been found in many plant species such as wheat (Peterson et al 1986; Morgounov et al 2007), triticale (Feil and Fossati 1995), maize (Banziger and Long 2000), soybean (Raboy et al 1984), and wild emmer wheat (Peleg et al 2008). This positive correlation indicates that grain proteins represent a sink for Zn (Cakmak et al 2010). Zhang et al. (2012) reported the effect of source and sink manipulation on accumulation of Zn and protein in wheat grains in a field experiment and ear culture and found that Zn and protein accumulation in grains can be affected by the source–sink relationship of carbohydrate and nitrogen. In the embryo, Zn is mainly concentrated in the protein bodies that contain ≤ 600 mg kg^{-1} of Zn (Mazzolini et al 1985). Given that Zn plays a vital role in protein synthesis (Cakmak et al 1989; Marschner 1995), an increase in protein biosynthesis due to increased nitrogenous applications may also enhance Zn sink strength.

Ozturk et al. (2006) and Stomph et al. (2009) concluded that the highest amount of Zn accumulation in wheat seed was during the early stage of seed development and at the same stage the highest protein synthesis takes place (Greene 1983; Martre et al 2003). Proteins are highly dependent on Zn ions to maintain their activities and numerous proteins require Zn is for a catalytic and a structural role (Anzellotti and

Farrell 2008). Zn is the most critical micronutrient which affects protein synthesis in plants (Cakmak et al., 1989). Cakmak et al. (2004) reviewed many studies showing the concentrations of protein and Zn have a positive correlation. Similarly, phytate also acts as an important sink in the grain for Zn. Aleurone layer and embryo fractions of grain are high in phytate and have high Zn concentrations (Lott and Spitzer 1980; Lin et al 2005).

1.6 Screening for Zn Efficiency

Zn efficiency is defined here as the ability of a genotype to grow and yield well in soils too Zn-deficient for a standard genotype (Graham, 1984). This ability may be achieved through greater Zn uptake, root to shoot transport, remobilization or utilization. Lower Zn requirement may be one of the mechanisms of Zn efficiency (Genc et al., 2004). Zinc efficient genotypes can have better Zn uptake from soil, better transport from root to shoot, better utilization of Zn within plant tissues and may or may not have high Zn in the grain (Hart et al., 1998). Rengel and Graham (1995) reported that there is no correlation between Zn uptake rate and dry matter production in many wheat genotypes. Similar results were found when Turkish wheat cultivars under different growing conditions were studied by Cakmak et al. (1997) and they concluded that for evaluation of Zn efficiency in different genotypes, tissue Zn concentration is not a dependable parameter. Erenoglu et al. (1999) also compared Zn uptake in rye and wheat and found that no positive correlation was found between efficiency and the uptake rate in bread wheat.

Zn efficient cultivars have a lot of advantages, for example, lower need of fertilizers, better seedling vitality, and resistance to soil-borne diseases, greater yield and increased nutritional status of seed grains (Graham and Rengel, 1993; Bouis, 1996; Graham and Welch, 1996).

Generally, three methods are utilized for screening of Zn efficiency. These methods are field screening, solution culture and pot screening. The parameter used in the field screening to measure the Zn efficiency is grain yield. In pot screening, usually pots filled with Zn deficient soils having a basic nutrient solution added in it, are

used, in comparison to high-Zn control treatments (Thongbai et al., 1993; Nair and Prabhat, 1977; Rengel and Graham, 1995a, b).

Genotypes are mostly chosen for Zn utilization efficiency based on reduction in shoot dry matter. Pot screening requires a lot of space. It is usually conducted in growth chambers, where the environment is kept under control because in order to induce Zn deficiency, low temperature treatment is essential (Cakmak et al., 1997b).

When the Zn efficiency ratios of different genotypes grown under Zn deficiency conditions are compared with optimal yield potential, it should be kept in the mind that genotypes showing very little response to supplied Zn are highly Zn-efficient (Kalayci et al., 1999).

1.6.1 Factors regulating zinc efficiency of cereals

1.6.1.1 Root morphology

Higher plants have a genetically controlled, metabolism-dependent process of Zn uptake. Zn-efficient genotypes might be able to keep up stability of structure and function of root-cell plasma membranes in a better way than Zn-inefficient cultivars, both under Zn deficiency conditions (Rengel, 1995). More recent studies in wheat (Genc et al., 2006) and rice (Gao et al., 2005) propose that Zn uptake is the most important mechanism under Zn deficiency. Plants having finer roots with diameter < 0.3 mm may exploit a larger volume of soil and therefore can more efficiently scavenge the minute quantities of static Zn ion as compared to plants having thicker roots. The wheat cultivar Excalibur is a Zn efficient genotype of wheat, which develops finer roots than Gatcher, a Zn-inefficient cultivar (Sillanpaa, 1982). Additionally, plants having a longer root system are likely to be more Zn efficient, because a deep rooting system can explore Zn more efficiently in the subsoil (Grewal et al., 1997). Longer and thinner roots having a greater percentage of thinner roots in the total biomass are two traits of Zn–efficient genotypes. Zn^{2+} ions can reach absorption sites of the root more easily in plants with greater root surface area (Marschner, 93). Rengel and Wheal (1997) reported a decrease in Zn uptake in

wheat genotypes with a lower ratio of finer roots (diameter < 0.2 mm) in the entire root biomass.

It is recommended that the capability of Zn-efficient cultivars to develop a larger ratio of fine roots (< 0.2 mm in diameter) having greater surface area to volume ratio might be interrelated to higher efficiency of the wheat genotypes (Dong et al., 1995). Genetic and environmental factors influence the root and root hair growth. Different plant species (Dittmer, 1949) and even genotypes of the same species (Gahoonia et al., 1997) have differences in length and compactness of root hairs and this can exploited in breeding for Zn-efficiency.

Root hairs grow rapidly in the presence of limited moisture content (Mackay and Barber, 1985) and low amounts of nutrients, (i.e. P, Fe and nitrate) (Foehse and Junk, 1983; Muller and Schmidt, 2004) while deficiency of Ca usually inhibits the growth of root hairs (Tanaka and Woods, 1973). Very little information is available about the effect of Zn on the growth of root hairs except that one investigation showed a 0.7-fold increase in root hair density under Zn deficiency in *Arabidopsis thaliana* (Ma et al., 2001).

1.6.1.2 Rhizosphere

The rhizosphere is the region of soil adjacent to the root where vigorous changes take place in chemistry and microbiology compared to the non-rhizosphere soil (Brown, 1975; Darrah, 1993). Rhizosphere soil is different from the bulk soil in the concentration of nutrients, microbial activities, in the quantity of root exudates, and the soil pH (Dinkelaker et al., 1992; Marschner, 1995).

Plant growth depends on the amount of nutrients available in the rhizosphere, which have different levels of thickness according to the length of root hairs (Dinkelaker and Marschner, 1992). The chemical and physical properties of soil control the amount of nutrients present in the rhizosphere and also interactions occurring between plant roots and adjoining microbes in the adjacent soil (Shen et al., 1997). Rengel et al. (1997) reported that Zn deficient wheat plants can change the soil chemical and biological properties of the rhizosphere to scavenge more Zn.

1.6.1.3 Changes in rhizosphere pH and redox potential

Plants can alter the pH of their rhizosphere and thereby manipulate nutrient uptake (Darrah 1993). By decreasing pH, the availability of Zn is increased through desorption of Zn^{2+} from soil colloids. Availability of some micro- and macronutrients, such as P, Fe, Mn, B and Mo may also show a response to changes in rhizosphere pH, but their relationships are more complicated as compared to Zn. The availability of Zn is declined by increasing soil pH from 5.5 to 7.0 (Moraghan and Mascagni, 1991; Armour and Brennan, 1999). The most significant root-induced modifications occurring in rhizosphere pH can be the cation/anion uptake ratio, the discharge of root exudates and the emission of carbon dioxide, when roots and micro-organisms respire (Marschner, 1993).The extent of pH change in relation to the bulk soil is dependent on some plant factors and soil properties such as the initial pH and buffering capacity of the soil pH (Marschner and Romheld, 1983).

Numerous investigators have related pH changes taking place in the rhizosphere with various types of nitrogen supply (Jones and Darrah, 1993; Wang and Zabowski, 1998). Ammonium treated plants absorb more cations as compared to anions, due to which the rhizosphere becomes acidic and on the other hand when nitrate is absorbed in greater amounts (than cations), alkalinization can occur (Marschner and Römheld, 1983; Haynes, 1990). Numerous reports show that the pH of the rhizosphere is different from bulk soil pH by up to 2 pH units (Riley and Barber, 1971; Hedley et al.1982; Dinkelaker et al.1993a, b; McGrath et al., 1998). Conversely, only a small number of studies have shown variations in nutrient composition of rhizosphere and bulk soil solution (Lorenz et al., 1994; Wang and Zabowski, 1998).

1.6.2 Breeding for Zn efficiency

The main purpose of plant breading is to increase the productivity of plants through the development of crops having greater yield and improved agronomic performance. Problems related to nutritional status of crops are mostly ignored by plant breeders. Addition of fertilizers to soil having micronutrient deficiency, brought about by high pH, is not always an effective solution (Cakmak and Braun,

2001). Moreover, Zn deficiency produced by constraints of soil, dryness of fertile top soil and disease, may be corrected by fertilizers, but it is not often successful (Graham and Rengel, 1993). Therefore, development of Zn-efficient genotypes by breeding is ideally the genetic solution, because these genotypes have the ability to grow successfully, even under Zn-deficient conditions.

Crops have shown genotypic differences for mineral ions like Zn. The average concentration of Zn in wheat grains was 25 mg kg^{-1}, ranging from 14 to 42 mg kg^{-1}. The concentration of Fe in wheat has a range of 8-24 mg kg^{-1} **(** Ruel and Bouis ,1998) . Rice has also similar concentration ranges for Fe and Zn (Bouis, 1995). Genotypic differences exist in Zn-efficient plants (Cakmak et al., 1998) and plants having greater efficiency for Zn uptake could be selected.

Mostly screening is done in controlled environments. Soil-filled pots grown in glasshouse can be used to assess the efficiency of genotypes (Cakmak et al., 1997b; Genc and McDonald, 2004), but here instead of grain yield, seedling growth is taken as the selection criterion and this is because Zn has a significant function during the initial growth of the plant, particularly in soils having poor availability of Zn (Welch1999).

Lombnaes and Singh (2003) used a soil-free hydroponics system for the study of micronutrients, a different approach, to find the tolerance of Zn deficiency found in wheat and barley. Barley and wheat exhibited different tolerance to Zn deficiency, with barley being consistently more tolerant than wheat. One appropriate approach for fast screening of several genotypes in a short period, is evaluation of Zn deficiency symptoms present on the leaves, along with the Zn-efficiency ratio (Cakmak and Braun,2001; Genc et al.,2003).

1.6.3 Influence of high Zn reserve in seed on Zn efficiency

There is significant genotypic variation for seed Zn accumulation in several staple crops including rice, wheat, maize and bean (Graham et al., 1999; Gregorio et al., 2000; Mantovi et al., 2003 Moraghan and Grafton, 1999; Stomph et al.,2009). For example, the Zn concentration of wheat cultivars ranged from 25 to 64 mg kg^{-1} (Frossard et al., 2000), and barley seed Zn varied from 0.6 to 0.9 µg/seed (Genc et al., 2002b).

All plant genotypes have capability of absorbing Zn and Fe from the soil and then these nutrients are accumulated in the grains even in nutrient deficient soils (Graham and Welch, 1996). The physiological mechanisms involved in control of trace element accumulation are still not fully understood (Welch and Graham, 2004). Many crops such as rice, maize, bean and wheat have shown genotypic variation for accumulating Zn in the grain (Graham et al., 1999; Gregorio et al., 2000; Mantovi et al., 2003 Moraghan and Grafton, 1999).

Zn concentrations ranged from 26 to 40 mg kg^{-1} in one study with 27 modern wheat cultivars (Peterson et al. 1986); in another study with 132 modern genotypes the range was from 25 to 53 mg kg^{-1}(Graham et al., 1999) and in another study with 57 modern genotypes, the range of Zn concentrations was from 29 to 46 mg kg^{-1} (Ficco et al., 2009). However, the range was much lower than that seen by Cakmak et al. (2004) who studied 825 wild emmer accessions and obtained grain Zn concentrations over a range of 14–190 mg kg^{-1} in the greenhouse and high Zn concentration in the seeds was not due to small size of seeds (i.e. it was not a consequence of a concentration effect) there is no yield data for these accessions which is unfortunate. When wheat seeds having high Zn were grown in greenhouse, the plants had a better growth rate as compared to those plants which were grown from seeds having low Zn content (Rengel and Graham 1995a;1995b).

Similarly seeds with medium and high Zn content produced wheat plants having better grain production than those which were grown from low Zn seeds (Cakmak and Braun, 2001). Genc et al. (2000) have shown that the high amount of Zn present in the seeds decreased the deficiency symptoms and increased growth rate, particularly in Zn deficient soils. Moreover, high Zn concentration in seed has also shown a positive influence on tillering (Lotfollahi et al., 2007). When sufficient nutrients were not provided to the seedling via the seed, plants became more susceptible to disease (Grewal et al., 1997; Streeter et al., 2001).

1.6.4 Genotype x Environment interactions

The ultimate goal of plant breeders is to develop high yielding cultivars with wide adaptability. However, attaining this aim is complicated because of genotype x environment interactions (G x E). Identification of yield contributing traits and knowledge of (G x E) and yield stability are important for breeding new cultivars with improved adaptation to the environmental constraints prevailing in the target environments (S. D. Tyagi and M. H. Khan www.soygenetics.org/).

G x E interactions have been defined as the failure of genotypes to attain the identical relative performance in multi environments (Baker, 1988).The large G x E environmental interactions usually impairs the accuracy of yield estimation and decreases the association between genotypic and phenotypic values (Nachit et al., 1992). G x E means that the difference between genotypes is not equal in a set of environments (Woolaston, 1987). Mohammed (2009) suggested that additive main effects and multiplicative interaction (AMMI) modelling can be a powerful tool in diagnosing G x E patterns. Different responses of genotypes in various environments make selecting the superior genotypes hard in plant breeding programs (İlker et al., 2009).

G x E is not only a problem; it is also an opportunity (Simmonds, 1991). Evaluation of genotypic performance in different environments gives valuable knowledge for identifying their adaptation and stability (Crossa, 1990).

The effects of climate and soil properties on grain yield are important factors for the development of new durum wheat cultivars. So, wheat breeders must try to choose wheat lines responsive to various environments for high quality grain yield and yield components (Sakin et al., 2011). Environmental factors in the Mediterranean countries have strong influences on yield and quality of durum wheat (Rharrabti et al., 2003). G x E associated with Zn concentration in wheat seed is very high (Cakmak et al., 2004). Oury et al., (2006) reported Zn concentration in bread wheat generally ranged from 15 to 35 mg kg^{-1}, but increased to 43 mg kg^{-1} in some genetic resources. These variations in Zn were also partly due to a dilution effect and high G x E existed.

Murphy et al. (2011) evaluated 18 spring wheat (*Triticum aestivum* L.) cultivars on three organic farms in Washington State for Zn concentration and grain yield in 2008 and 2009 and found GEI for grain yield and Zn concentration. They found no consistent correlation between grain yield and Zn concentration across years and locations. Velu et al. (2012) evaluated Harvest Yield Trials at nine locations for agronomic traits, yield potential and grain Zn concentration and found significant GEI for grain Zn and grain yield. They noticed that variances linked with environment were greater than genotypic effects and showed that there is no negative correlation between the grain yield and Zn concentration.

1.7 Dust Contamination of Seeds

Both Fe and Zn are micronutrients, present in grain at very low levels (generally between 10 and 50 mg kg^{-1} dry weight). Major seed crops of interest in the biofortification strategy include wheat, rice, maize, pearl millet and beans (Nestel et al., 2006). For wheat, Fe and Zn are present in grain at very low levels (generally between 10 and 50 mg kg^{-1} dry weight) and a major obstacle in wheat breeding is that analysis of Fe and Zn in grain is often confounded by the presence of soil derived contamination and Fe is more an issue than Zn (Pfeiffer and McClafferty, 2007: Mikko and Sillanpaa, 1982).

Soil borne micronutrients adhere to the outer epidermal surfaces (Djingov and Kuleff, 1994; Hall, 1995; Jones, 2001). Soil typically contains around 4% Fe (Sparks, 2003), far more than is ever found in grain, so traces of soil on grain lead to erroneously high levels of grain Fe concentration. Soil contamination is mostly considered as a problem for the plants grown and collected from windy areas (Cherney and Robinson, 1983; Jones, 1991, 2001).

Other common sources of Fe and Zn contamination are residues from grain handling equipment or threshers, and residues from human hands or even gloves used during the preparation of seed samples (Sager and Mittendorfer, 1997; Jones, 2001). There are two main methods used to identify Fe and Zn contamination in wheat. Firstly, major sources of contamination may be seen as concentration ‘spikes’, which are generally not reproduced in replicated analysis. Secondly, since Al is even more

abundant in soil (Ibia, T.O., 2002; Rauch and Pacyna 2009) than Fe but is not found in clean plant tissue, and is easily analyzed by ICP, it can be used as an indicator of soil contamination. Typically, soil contamination of grain is considered to be acceptably low in grain analysis reports where Al is present at <5 mg kg^{-1} (Pfeiffer and McClafferty, 2007).

Washing experiments of wheat and maize grains have been conducted to remove the iron contaminations which were successful to some extent (Kevin Pixley, CIMMYT; James Stangoulis). Some researchers fear that washing of samples can leach out the micronutrients from the grains and other plant tissues, giving distortive results (Djingov and Kuleff, 1994; Hall, 1995; Jones, 2001). There is very little information available in literature on dust contamination of Zn which is probably because of very little Zn in soil (the global average is only 48 $\mu g g^{-1}$, compared to 33,000 $\mu g g^{-1}$ Fe and 62,000 $\mu g g^{-1}$ Al (Rauch and Pacyna 2009). For this thesis, Zn contamination will need to be considered as this may give erroneous results that can lead to false positives.

1.8 XRF and its application

X-Ray Fluorescence (XRF) has been shown to provide a fast, economical alternative to ICP analysis of biological samples for elemental content. XRF is fast and has unique capabilities such as simple method of sample preparation and a broad elemental spectrum without standards. (Va zquez et al., 2003; Margui et al., 2005). Dried samples can also be used in XRF, without digestion in corrosive and toxic acids (Noda et al., 2006). XRF provides a cost effective alternative to ICP-OES for the analysis of grain Zn in biofortification breeding programs. However, XRF has not previously been validated for use in mapping Zn QTL.

The use of X-ray fluorescence (XRF) for elemental analysis of plant tissue has increased in recent years (Kaymak et al. 2010). Initially, X-ray fluorescence analysis (XRF) was applied on geochemistry and manufacturing, later on it was considered a suitable technique for analysis of organic and inorganic samples. Recently, With recent improvements in sensitivity, XRF has been used for the analysis of different

food materials such as beverages (Nielson et al., 1988), spices (Al- Bataina et al., 2003), milk based products (Perring et al., 2005; Perrin,L. and Andrey, 2003), tea (Salvador et al.,2002) potato starch (Noda et al., 2006), medicinal plants (Queralt et al.,2005) and bread improvers (Ekinci et al., 2002). XRF has been shown to be a useful method for the analysis of Fe, Zn and Cu in plant materials (Paltridge et al., 2012 a, b).

Recently, energy dispersive x-ray fluorescence (EDXRF) can also be used on whole grain samples of rice and pearl millet (Paltridge et al. 2012a), and wheat (Paltridge et al. 2012b), and that technology is both convenient and economical for screening Fe and Zn concentration in rice, pearl millet and wheat biofortification programs. Secondly, the expense of micronutrient analysis is a major constraint to mapping micronutrient density traits; Paltridge et al., (2012 a, b) showed the suitability of XRF data for use in nutrient analysis and in this thesis, it is tested for its application in mapping Zn QTLs.

1.9 QTL Mapping

Molecular markers have been used in marker assisted selection (MAS) approaches (Ribaut and Hoisington, 1998). MAS can be useful for the selection of polygenic traits, like grain Zn and Fe and also for the combination of these with other traits such as stress tolerance, yield and quality (Schachtman and Barker, 1999). On the basis of presence of QTL related to traits of interest, genotypes can be selected in the process of MAS. This sort of choice is more precise than conventional approaches. It gives information of chromosomal position of target loci and can be used for improvement of micronutrient concentration by marker–assisted selection (MAS) (Collard et al. 2005; Ghandilyan et al. 2006; Tiwari et al.2009). However, only a few QTL studies of micronutrient traits have been reported in the literature (Wissuwa, 2005).

Before MAS can be employed, QTL must be identified and validated. A major Zn QTL was identified in *Triticum monococcum* wheat population on chromosome 5 with 3.76 to 5.22% of the variation in Zn explained (Ozkan et al. 2006). Distelfeld et al. (2007) found one QTL on 6B chromosome in wheat *Triticum turgidum* ssp

diccoccoides associated with Zn concentration. In another study, Shi et al. (2007) conducted QTL studies on the DH population of Hanxuan10 x Lumai 14 and found 7 QTLs linked to Zn concentration on 4A, 4D, 5D and 7A chromosomes and 7 QTLs for Zn content on 1A, 2D, 3A, 4A, 4D, 5A and 7A which explains 4.6 to 14.6% of phenotypic variance. One QTL study was conducted in the DH population of RAC875-2 and Cascades in which 4 QTLs linked with zinc efficiency and grain zinc concentration were identified on chromosomes 3D, 4B, 6B, and 7A (Genc et al. 2008). The large variation in population structure, such as plant morphology, maturity, yield and seed size was not adequately reported in these papers and they may affect Zn QTL. A full list of reported Zn QTL is reported in table 1.2.

Tiwari et al. (2009) identified one significant and one suggestive QTL in a DH population derived from a cross between T. *boeoticum* accession pau5088 and T. monococcum accession pau14087 on chromosomes 7A and 7A and this was linked to grain Fe and Zn concentration. Peleg et al. (2009) carried out QTL studies in the population of 152 recombinant inbred lines (RILs) developed from a cross between durum wheat (cultivar Langdon; LDN hereafter) and wild emmer wheat (accession#G18-16) and found 6 QTLs on chromosomes 2A, 5A, 6B, 7A, 7B and 2A. More information related to genetic loci which control accumulation of seed Zn is essential step required to increase the Zn concentration of seeds.

A similar study was conducted by Wissuwa et al. (2006) for deficiency tolerance of Zn in rice and in this study visual symptoms of Zn deficiency were taken as the basis for QTL and for plant Zn concentration no QTLs were reported. One study identified

Table 1.2 List of reported Zn QTLs in different double haploid wheat populations

Publication	QTLs	Chromosome	Population	Range of Zn	% variation
Shi et al. 2008: Identification of quantitative trait locus of zinc and phosphorus density in wheat (Triticum aestivum L.) grain	4	4A ,4D, 5A and 7A	DH Population of cross between Hanxuan10 and Lumai 14	25.9-50.5	5.3 - 11.9%
Genc. et al. 2009: Quantitative trait loci analysis of zinc efficiency and grain zinc concentration in wheat using whole genome average interval mapping	4	3D, 4B, 6B, 7A	DH Population of cross between RAC875-2 and Cascades	29.0-42.7	
Tiwari et al.2009: Mapping of Quantitative Trait Loci for Grain Iron and Zinc Concentration in Diploid A Genome Wheat	1	7A	Population derived from a cross T. boeoticum accession *Tb*5088/T. monococcum accession *Tm1*4087	19.0 - 64.2	18.8%
Peleg et al. 2009 Quantitative trait loci conferring grain mineral nutrient concentrations in durum wheat *3* wild emmer wheat RIL population	6	2A, 5A, 6B, 7A, 7B, 2A	population of 152 F6 RILs was developed by single-seed decent from a cross between durum wheat (cultivar Langdon; LDN hereafter) and wild emmer wheat (accession #G18-16) (Peleg etal.2008b)	48.5-114.7	1-23%

QTL for micronutrients in rice (Stangoulis et al.2006) in which most of attention was paid to the relationship between phytate and the micronutrients Fe and Zn. A significant Zn QTL was reported in rice on chromosome 12 and this appears to be conserved with two other groups also reporting the presence of this QTL in different environments and different populations (Anuradha et al., 2012).

To complete this type of analysis, one must see if contamination is an issue and this will be studied within this thesis. Furthermore, selection of genotypes out of the Berkut x Krichauff DH population for mechanistic studies is undertaken.

2.0 Conclusion

The transport of Zn from soil solution to grains occurs in the form of a continuous process through various physiological pathways. Some of these processes are well understood whereas some mechanisms still need more research. Some substances serve as transporters and help in the translocation of Zn within the plant. Nicotianamine is an important chelator which helps in intracellular transport of Zn. The highest amount of Zn accumulates in the grains during early stages of seed development.

Genetic variations exist in grain Zn which is promising for breeding program. There is very little knowledge available about grain genetics of Zn which makes it difficult to adopt any breeding method. XRF can be used as an alternate method to ICP-OES, not only for micronutrient analysis but also for the identification of QTLs. The identification of Zn QTLs in grains can be helpful in understanding the genetics of grain Zn concentration which can facilitate marker-assisted selection (MAS) and implementation of a proper breeding method. The aim of this thesis is to conduct mapping studies to find genetic loci conferring high Zn concentration.

This thesis is set out in six chapters, followed by a reference section. Chapter 2-5 describe how experiments were conducted whereas chapter 6 presents general discussion of the thesis.

Chapter 2: Study of phenotypic traits associated with grain Zn concentration in wheat

2.1 Introduction

The Zn concentration in wheat grain is relatively low when compared to many other foods while milling and polishing of grain further reduces the concentration of this element (Cakmak et al. 2002; Poletti et al. 2004; Welch and Graham 2004). In developing countries where wheat is a major dietary staple (e.g. India), the low level of Zn in wheat is thought to contribute toward micronutrient malnutrition and poor health (Graham et al. 2001).

The easiest way to increase seed Zn in wheat is through agronomic biofortification (Cakmak, 2008); however, this approach is not considered a sustainable long term strategy in many of the world's poorest communities due to poor access to high quality fertilizers and the cost of this commodity. Biofortification by plant breeding is more cost-effective and sustainable (Pfeiffer and McClafferty 2007).

Breeding for Zn-dense wheat requires the existence of significant genotypic differences in Zn concentration (Cakmak et al., 2004). However, several studies report little genotypic variation in Zn concentration in cultivated wheat lines and some argue that the variation is inadequate to develop Zn-dense wheat varieties (Cakmak et al., 2004; White and Broadley, 2005), although recent efforts at CIMMYT have shown significant gains in Zn among elite lines specifically bread for higher levels of Zn within the grain (Wolf Pfeiffer, personal communication). Significant genotype x environment (G x E) interactions, particularly related to variation in available water and Zn, have confounded efforts to study genotypic variation in Zn concentration (Banziger and Long, 2000).

Another factor impeding progress towards breeding high Zn wheat is that high grain Zn concentration can be associated with low grain yield (Murphy et al. 2008; McDonald et al., 2008). The negative correlation between increased grain yield and decreased grain Zn content may indicate that total Zn accumulation by different

wheat genotypes is relatively similar, but partitioned to more grain in higher yielding varieties, leading to lower grain Zn concentrations (Garvin et al. 2006).

High grain Zn can also be associated with desirable morphological traits such as reduced plant height (Morgounov et al. 2007) and high kernel weight (Genc et al., 2009), though other authors report no significant relationship between kernel weight and Zn concentration (Garvin et al. 2006). However, there is little published information regarding traits and mechanisms that contribute to high grain Zn (White and Broadley 2005).

A double haploid (DH) wheat mapping population was available for this study which had been generated by crossing the Australian cultivar Krichauff with the cultivar Berkut, developed by the International Maize and Wheat Improvement Centre (CIMMYT, Mexico). This population already has been used in different studies (McDonald et al. 2008 ; Huynh et al. 2008; Nguyen et al. 2011).The population had been grown in the field in South Australia by the South Australian Research and Development Institute (SARDI). SARDI recorded information on grain yield and thousand grain weight (TGW), but not other morphological traits or Zn concentration. Because of the wide nature of the cross, it was considered likely that the population would have genotypic variation for both morphological traits and grain Zn concentration.

A hypothesis was proposed that Zn concentration is associated with grain yield and thousand grain weight and results obtained from field would be reproduced in the greenhouse. To test this hypothesis, several high and low Zn lines were taken on for a greenhouse study of Zn concentration and morphology, to see if results obtained in the field would be reproduced in the glasshouse environment, and for a more detailed study of phenotype in this second environment.

2.2 Materials and Methods

Seed of the Berkut x Krichauff DH population was provided by SARDI. The population, which comprises 154 doubled haploid lines plus the two parents, was

grown at Roseworthy (34.31^{o}S, 138.44 ^{o} E) Agricultural Campus, South Australia during the 2007 season in three replicates.

2.2 Experimental design

2.2.1 Elemental analysis and associations between Zn concentration and yield and TGW

To investigate the concentrations of Zn in this population, approximately 0.6-0.8 g of oven dried wheat seeds were weighed into 75mL Pyrex tubes and 11 ml mixture of HNO_3/$HCLO_4$ was added. This mixture was wrapped with polyethylene and kept overnight in a fume cupboard for pre-digestion. Tubes were then removed from the cupboard and digested to reduce the sample volume to around 1ml $HClO_4$. Then samples were vortexed after adding water and analyzed (Wheal et al. 2011). Quality control and quality assurance was assessed using control sample data generated when performing routine analyses. Averaged results were compared to published values from either NIST certified values (mean ± 95%CI) or ASPAC consensus values (median ± MAD) (Wheal et al.2011).

Due to the high cost of analysis, data was obtained for all individuals from the first replication only. This information was used to examine the correlation between Zn concentration and yield and TGW in the entire Berkut x Krichauff population.

2.2.2 Detailed study of phenotype in selected high and low Zn individuals

Based on Zn concentration results from replication 1, 15 high Zn lines and 2 low Zn lines, as well as the two parents, were chosen for reanalysis in replications 2 and 3 from Roseworthy in 2007. Data from all three replicates were then used to select 4 lines with high Zn and two lines with low Zn for further study of phenotype.

These genotypes were grown in the glasshouse at Flinders University, Adelaide, from June to November 2009. Plants were grown in 19 cm wide pots in Debco ® soil mix (Green Wizard). To investigate if associations between phenotype and Zn concentration were affected by Zn level in the soil, the experiment was conducted with two Zn treatments, without applied Zn and with zinc sulphate ($ZnSO_4.7H_2O$)

applied at 1g per kg of soil. Zn sulphate was applied in solution. Six seeds of each genotype were sown into each pot to a depth of 1 cm. At 15 days after sowing, plants were thinned to leave two strong plants in each pot. There were four replications of each treatment in the experiment. Nearly 0.23g of urea fertilizer equating to 75 kg ha^{-1}N which is related to typical farmer practice, was added to the soil at three weeks after germination. Plants were watered regularly to maintain the moisture in soil.

At maturity, each plant was measured for tiller number, number of spikes, plant height, biological yield (total weight of shoots and grain), grain yield, harvest index and thousand grain weight (TGW). Shoots and grains were dried in the oven at 65^{o}C for 48 h before weighing.

2.3 Results

2.3.1 Screening the full population

Zn concentrations in replication 1 of the Berkut x Krichauff DH population ranged from 17 to 46 mg kg^{-1}, and had a mean value of 25 mg kg^{-1}but parents had almost similar Zn concentration. The standard errors of Zn concentration measurements by ICP-OES have been reported to be ±3ppm at 95 % of the CI. Across the population, there was a weak negative correlation between Zn concentration and yield (r^2 = 0.24; correlation coefficient =0.491; Fig 2.1). The grain yield in replication 1 ranged from 352 to 3337 kg h^{-1} and had a mean of 1997 kg ha^{-1}. There was no correlation between Zn concentration and TGW (Fig 2.2), with TGW ranging from approx. 20 to 40 g. There was a weak negative correlation between Zn concentration and yield (r^2 = 0.25) (Fig 2.2). Finally, there was a positive correlation between grain yield and TGW in the population (r^2 = 0.25; Fig 2.3).

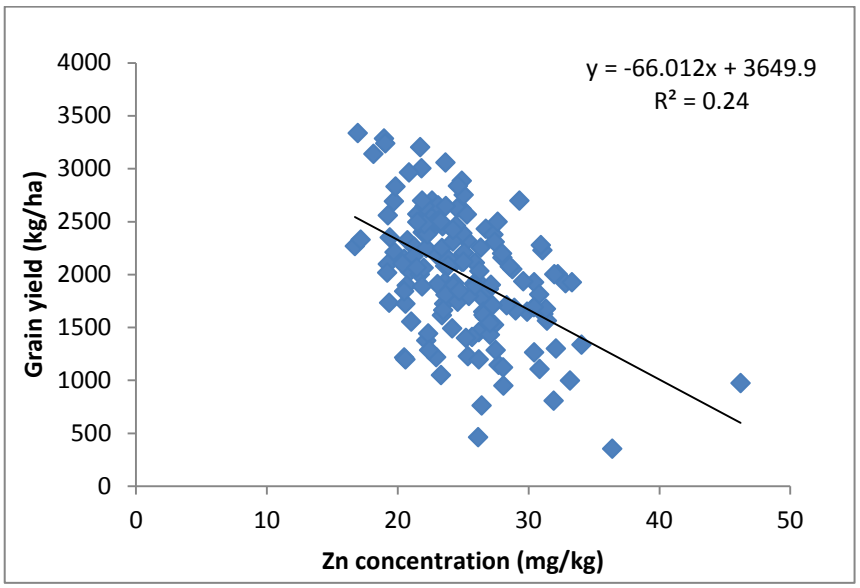


Fig 2.1 Relationship between Zn concentration and **grain yield** in replication 1 of Berkut x Krichauff, 2007 ($p < 0.01$)

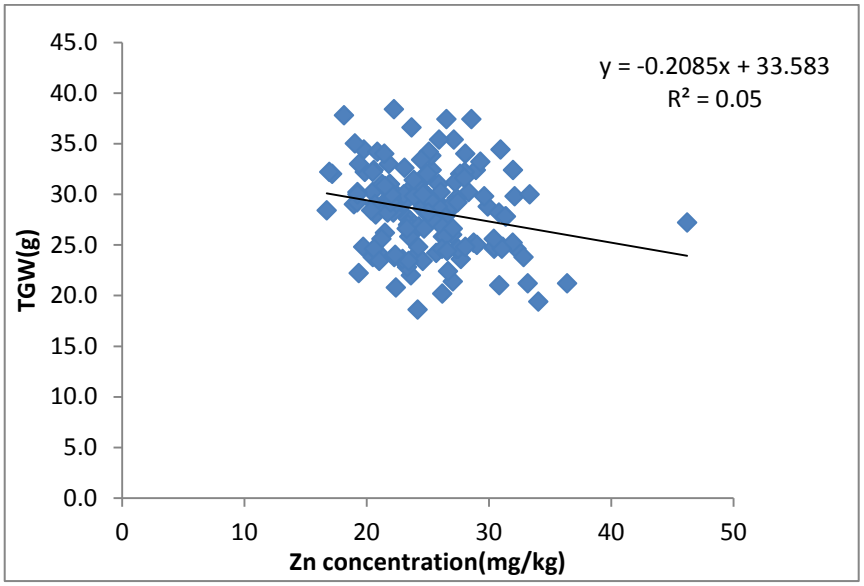


Fig 2.2 Relationship between Zn concentration and **TGW** in replication 1 of Berkut x Krichauff, 2007 ($p < 0.05$)

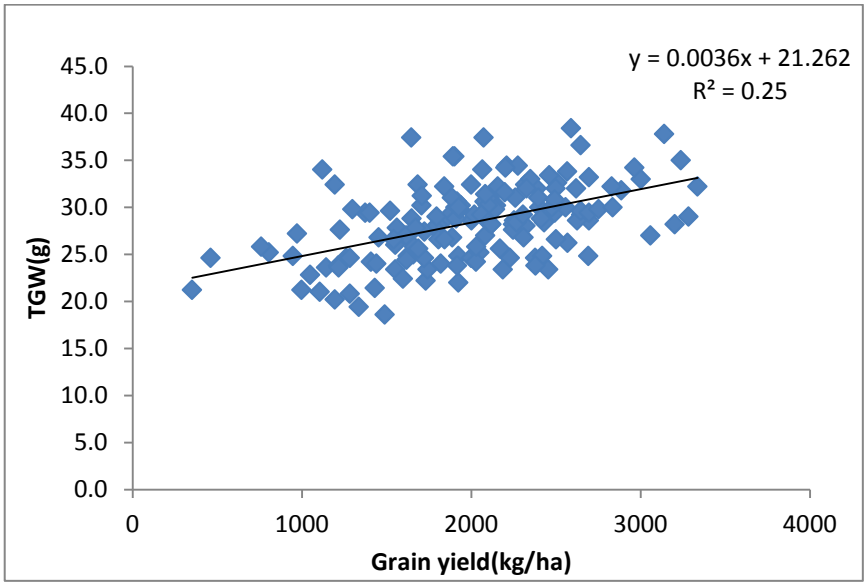


Fig 2.3: Relationship between grain yield and TGW in replication 1 of Berkut x Krichauff, 2007 ($p < 0.001$)

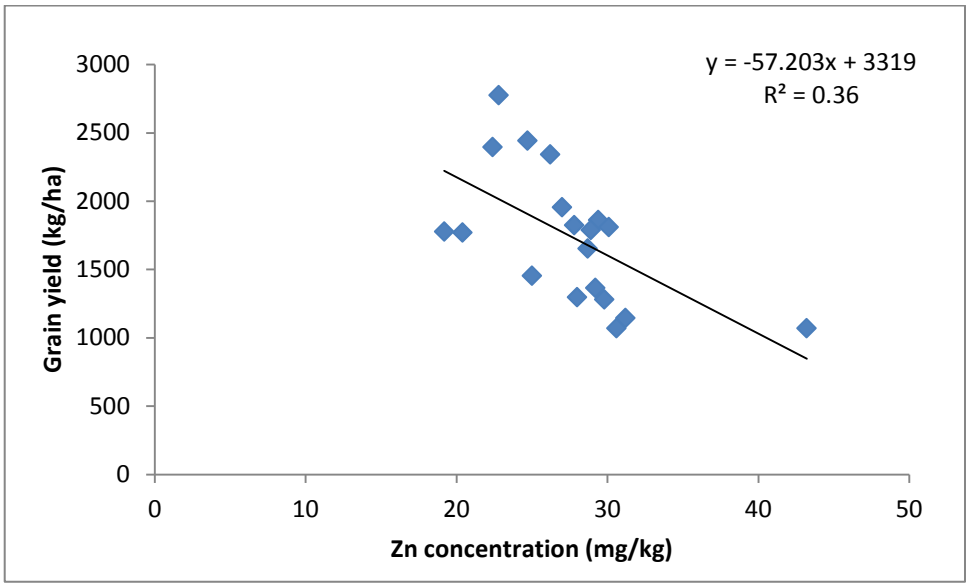
2.3.2 Selection of lines for study in glasshouse environment

Based on the Zn concentration results from replication 1, the fifteen highest Zn lines were selected, together with the two lowest Zn lines, and the two parents, for further analysis by ICP-OES, allowing the calculation of mean Zn concentration across the three replicates. Significant variation was observed for Zn concentration across these 17 lines. The two lines selected to be low based on replication 1 were also relatively low in replicate 2 and 3, though in replicate 2, one of the low lines, line 153, did show a relatively high level of Zn. Some of the high lines (e.g., lines 57, 28) showed consistently higher Zn across the three replicates than was present in parents or in the 'low' lines.

Table 2.1: Mean and standard deviation values of grain Zn concentration (mg kg^{-1}), grain yield (kg ha^{-1}) and TGW (g) in 9 double haploid genotypes

Classification	DNA No.	Zn Conc.		Grain Yield		TGW	
		mean	std.	mean	std.	mean	std.
High	28	29.4	1.8	1861	342	25.7	2.4
High	33	28.7	4.9	1653	505	29.1	1.4
High	35	28.0	3.7	1296	420	21.2	0.9
High	36	31.2	3.6	1143	304	27.5	2.0
High	37	27.0	3.8	1955	584	23.9	0.8
High	49	30.1	2.8	1808	139	22.6	1.6
High	57	43.2	3.7	1068	189	28	0.8
High	60	24.7	5.8	2441	761	29.8	1.7
High	84	30.6	5.6	1068	622	25.9	5.0
High	85	26.2	4.2	2341	113	33.8	1.6
High	99	29.2	4.2	1363	609	21.3	3.9
High	103	28.9	2.4	1787	103	26.2	1.5
Low	121	19.2	2.2	1775	668	27.5	4.0
High	130	29.8	3.4	1279	355	23.1	2.4
High	151	25.0	6.1	1453	579	30.1	1.1
High	152	27.8	4.8	1824	107	28.0	2.1
Low	153	22.4	8.1	2395	1160	30.6	2.4
Parent	Berkut	20.4	1.3	1769	257	28.9	4.6
Parent	Krichauff	22.8	1.8	2775	136	29.1	1.0

Information on grain yield and TGW for replications 2 and 3 was obtained from SARDI, allowing the relationships between these traits and grain Zn to be studied across all three replicates. The same relationships were observed in this data set (i.e. with information on 19 lines across 3 replicates) as was observed in all lines in replication 1, with a negative correlation evident between grain yield and Zn concentration and no correlation between grain yield and TGW (Figs 2.4 and Fig.2.5).



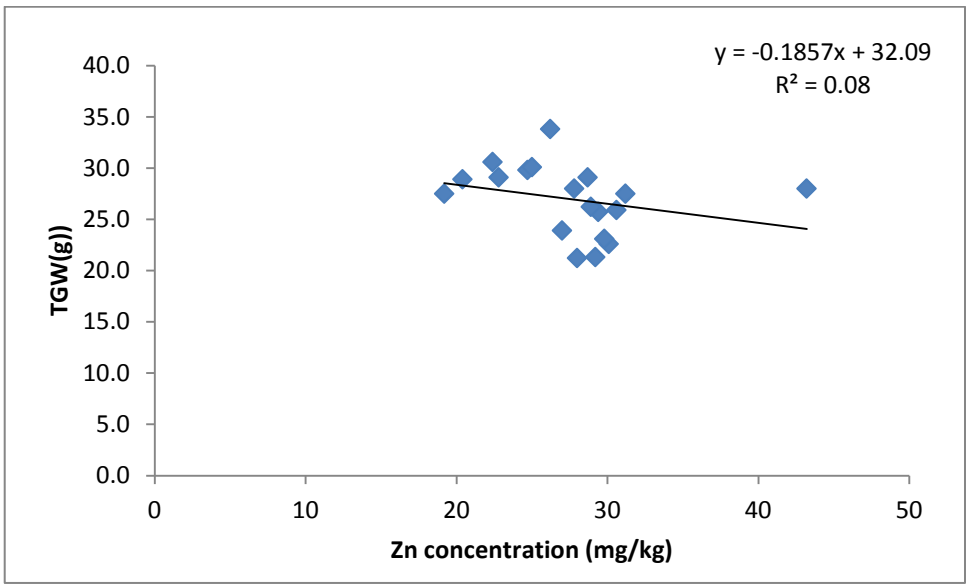


Fig 2.4: Relationship between Zn concentration, grain yield ($p < 0.001$),

Zn concentration and TGW in the means of replication 1, 2 and 3 of Berkut x Krichauff, 2007 ($p > 0.1$)

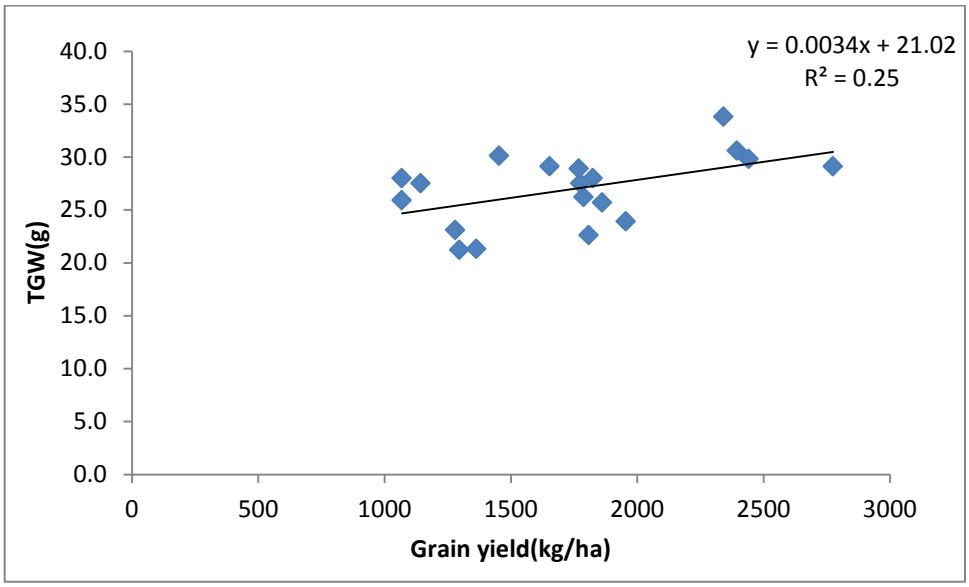


Fig 2.5: Relationship between grain yield and TGW in means of replication 1, 2 and 3 of Berkut x Krichauff, 2007 ($p < 0.01$)

For a more detailed study of traits associated with high or low grain Zn in the glasshouse, a further subset of DH lines were selected. Lines with a wide range of average Zn concentrations were chosen. Specifically, low Zn lines DH 121 and 153, as well as the parents Berkut and Krichauff, all in the range 19 to 23 mg Zn kg^{-1}; medium Zn lines (DH 28 and 35) comprised lines with 28-29mg Zn kg^{-1}, and the lines with the highest average Zn across the three replications were DH 36, 84 and 57, with Zn concentrations of 31, 31 and 43 mg kg^{-1}respectively. Because of the negative relationship between yield and grain Zn, it was not surprising that the lowest Zn lines were relatively high yielding (1775-2775 kg ha^{-1}), that medium Zn lines showed middle-range yields (1296 and 1861 kg ha^{-1}), and that the highest Zn lines had lowest yields (1068-1143 kg ha^{-1}).

2.3.3 Greenhouse study

Grain Zn concentration

Mean grain Zn concentrations of lines selected for the glasshouse study, in the field, and at both Zn treatments, appear in Table 2.2 and Figure 2.6. For all genotypes, grain Zn concentrations were higher in the glasshouse than in the field, even in the absence of supplemental Zn. In the presence of supplemental Zn, Zn concentrations were even higher. Among glasshouse grown plants, supposedly high Zn lines produced grain with similar Zn concentration as the lines which had been low Zn in the field, at both Zn treatments (i.e., there were no clear differences in Zn concentration between the 'high' and 'low' Zn lines). The one exception to this was line DH 57, which showed the highest Zn concentration in the field as well as in both Zn treatments. When field Zn concentration was plotted against glasshouse Zn concentration without supplemental Zn (r^2=0.34, Fig 2.6) or with supplemental Zn (Fig 2.7), there was no significant correlation between field and glasshouse Zn concentration.

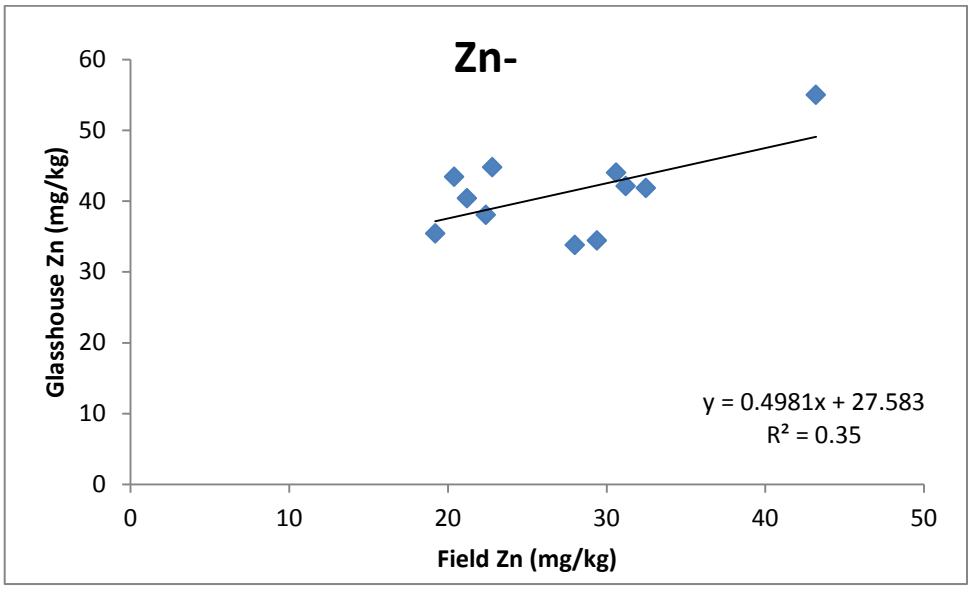


Fig 2.6: Relationship between field Zn concentration and glasshouse Zn concentration without supplemental Zn

Given this result, there appeared little point in studying the phenotypes of all lines initially categorized as ‘high’, and comparing that phenotype to ‘low’ lines. Instead, the focus of this study leads to describing the general phenotypes of all lines at both Zn levels and to investigate if DH57 displayed any exceptional phenotypic traits that could contribute to its exceptional Zn concentration.

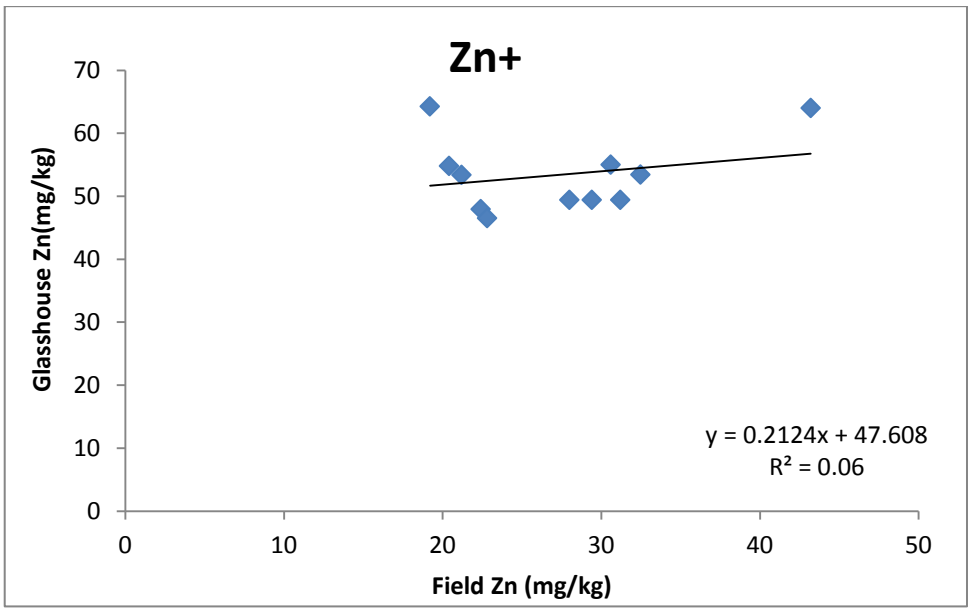


Fig 2.7: Relationship between field Zn concentration and glasshouse Zn concentration with supplemental Zn

Table 2.2 Mean of 4 replicates and standard deviation values of grain Zn concentration (mg kg^{-1}) in double haploid genotypes from the Berkut x Krichauff DH population

Genotypes	(field study)		Pot study		Pot study		Absolute increase in Zn concentration	
	mean	std.	mean	std.	mean	std.		
	(2007)		Zn0		Zn+			
	Zn (mg kg^{-1})		Zn (mg kg^{-1})		Zn (mg kg^{-1})		Zn (mg kg^{-1})	% increase in Zn conc.
DH 28 (high)	29	1.8	32	3.4	49	6.6	17	53
DH 35 (high)	28	3.7	33	3	49	2.8	16	48
DH 36 (high)	31	3.6	42	4.3	49	0.6	7	17
DH 57 (high)	43	3.7	55	2.7	64	3	9	16
DH84 (high)	31	5.6	44	3.5	55	4.2	11	25
DH121 (low)	19	2.2	35	2.8	64	2	29	83
DH153 (low)	22	8.1	38	0.8	48	2.9	10	26
Berkut	20	1.3	43	1.7	55	2.5	12	28
Krichauff	23	1.8	45	1.9	47	3.1	2	4

Effect of applied Zn on other phenotypic traits, and examination of DH57

In total, 13 phenotypic traits were examined besides grain Zn concentration. Interestingly, applied soil Zn led to increased tiller number, grain yield, total spike weight, dry shoot matter, biological yield and spike number, but decreased height, TGW, weight per spike and HI. They also showed higher shoot Zn content (as well as concentration). Thus, the generalization may be made that plants with supplemental Zn had more, shorter, tillers, more dry shoot matter, more spikes and more grain, containing higher concentrations of Zn and higher Zn content (Table 2.3).

Regarding DH57, the line with highest Zn concentration, it appeared similar to all other lines in tiller, height, TGW, spike weight, dry shoot matter, biological yield, spike number and HI. Without supplemental Zn, the line did however display the lowest weight/spike (Table 2.3), yet with supplemental Zn the line produced the highest yield. DH57 was highest in Zn concentration in both environment but it was not clear why.

Yield data were recorded as grams per pot (of two plants) in this study. It is worth considering whether the yields per plant seen in this study were generally high or low, relative to what is seen in field crops of wheat. According to CIMMYT, typical field crops may have about 200 plants/m2, and produce 4 t/ha, equating to a density of 2,000,000 plants/ha producing 4000 kg per ha, or 2 g per plant. Thus the yields produced in this pot study were higher than are generally seen in the field.

Table 2.3 Means of four replicates for 14 traits in 7 DH lines of the Berkut x Krichauff mapping population plus the two parents with and without applied soil Zn. (Units of parameters are: height (cm), grain yield (g/plan)t, TGW, spike wt., DSM, biological yield, wt/spike (g), grain Zn concentration, shoot Zn concentration in mg kg^{-1},grain Zn content in µg/grain

Traits	Zn tmt	DH28 (H)	DH35 (H)	DH36 (H)	DH57 (H)	DH84 (H)	DH121 (L)	DH153 (L)	Berkut	Krichauff
Tiller no.	Zn-	4±0.6	9±0.6	4±0.6	7±1.5	5±1.4	4±0.8	5±0.6	4±1.3	5±1.0
	Zn+	7±0.6	12±0.5	5±0.5	11±1.3	10±0.6	10±1	11±1.3	8±1.3	10±2.1
Height	Zn-	83±4	75±1	93±4	77±2	83±3	71±1	80±3	83±3	81±2
	Zn+	71±4.6	72±3.5	82±3.5	63±4.4	77±2.1	67±1.7	72±1.7	75±1.2	73±2.5
Grain yield	Zn-	12±1	13±2	9±1	14±2	12±1	11±2	14±2	11±2	10±1
	Zn+	13±0.4	14±2.4	9±0.9	20±2.5	13±1.1	16±2.4	18±2.4	18±2.1	16±3
TGW	Zn-	48±2	46±1	44±2	38±2	42±4	34±1	43±4	39±2	38±4
	Zn+	33±4	23±3	30±5	31±2	41±4	28±3	36±3	41±4	33±4
Spike wt.	Zn-	19±1	22±3	14±2	19±3	16±3	16±2	20±3	15±4	14±1
	Zn+	20±2	22±3	16±3	25±4	22±3	23±3	27±4	27±3	22±3
DSM	Zn-	23±3	20±2	25±5	14±2	10±1	13±2	12±3	10±3	13±1
	Zn+	26±1	25±2	32±4	18±5	15±1	17±2	19±1	17±1	17±3
Biol. yield	Zn-	43±4	42±5	38±7	33±4	26±3	28±4	31±5	25±6	27±2
	Zn+	46±3	46±3	46±4	41±4	37±3	40±5	45±4	43±3	39±4
No. spikes	Zn-	4±0.6	9±0.6	4±0.6	7±1.5	5±1.4	4±0.8	5±0.6	4±1.3	5±1
	Zn+	7±0.6	12±0.5	5±1	11±1.3	8±3.4	10±1	11±1.3	8±1.3	9±3
wt/spike	Zn-	3.3±0.4	3.2±0.5	4±0.4	2.4±0.6	4±0.4	3.9±0.4	4±0.8	4.2±0.5	3±0
	Zn+	2.9±0.3	1.7±0.3	3.1±0.6	2.2±0.6	2.4±0.4	2.5±0.6	2.5±0.2	3.3±0.4	2.5±0.7
H. Index	Zn-	0.27±0.03	0.33±0.02	0.24±0.04	0.41±0.03	0.46±0.04	0.39±0.01	0.44±0.02	0.43±0.03	0.37±0.02
	Zn+	0.28±0.02	0.30±0.05	0.19±0.03	0.48±0.06	0.36±0.01	0.40±0.03	0.39±0.04	0.42±0.04	0.40±0.06
Grain Zn conc.	Zn-	32±3.4	33±3	42±4.3	55±2.7	44±3.5	35±2.8	38±0.8	43±1.7	45±1.9
	Zn+	49±6.6	49±2.8	49±0.6	64±3	55±4.2	64±2	48±2.9	55±2.5	47.±3.1
Grain Zn cont.	Zn-	1.4±0.2	1.4±0.3	1.8±0.2	1.9±0.3	1.9±0.2	`1.2±0.1	1.6±0.2	1.7±0.1	1.7±0.1
	Zn+	1.6±0.3	1.1±0.2	1.6±0.3	2±0.2	2.2±0.2	1.8±0.2	1.7±0.2	2.4±0.3	1.5±0.2
Shoot Zn conc.	Zn-	32±4	29±3	36±4	34±4	28±4	31±4	24±3	22±6	28±4
	Zn+	46±4	38±5	53±4	41±3	48±8	56±4	47±4	34±5	39±11
Shoot Zn cont.	Zn-	5±0.7	5±0.5	6±0.9	5±0.4	4±0.7	5±0.9	4±0.5	3±0.8	4±0.7
	Zn+	7±0.7	6±0.8	9±0.6	6±0.6	7±1.1	9±0.7	7±0.8	5±0.7	6±1.7

There was genotypic variation for all traits (Table 2.4). Variation was observed for Zn concentration, grain yield and TGW across these 9 lines grown in glasshouse at both Zn levels but no relationship between Zn concentration and grain yield was

observed in these 9 lines grown in glasshouse at low Zn treatment (Fig2.8) and high (at supplemental) Zn treatments (Fig2.9).

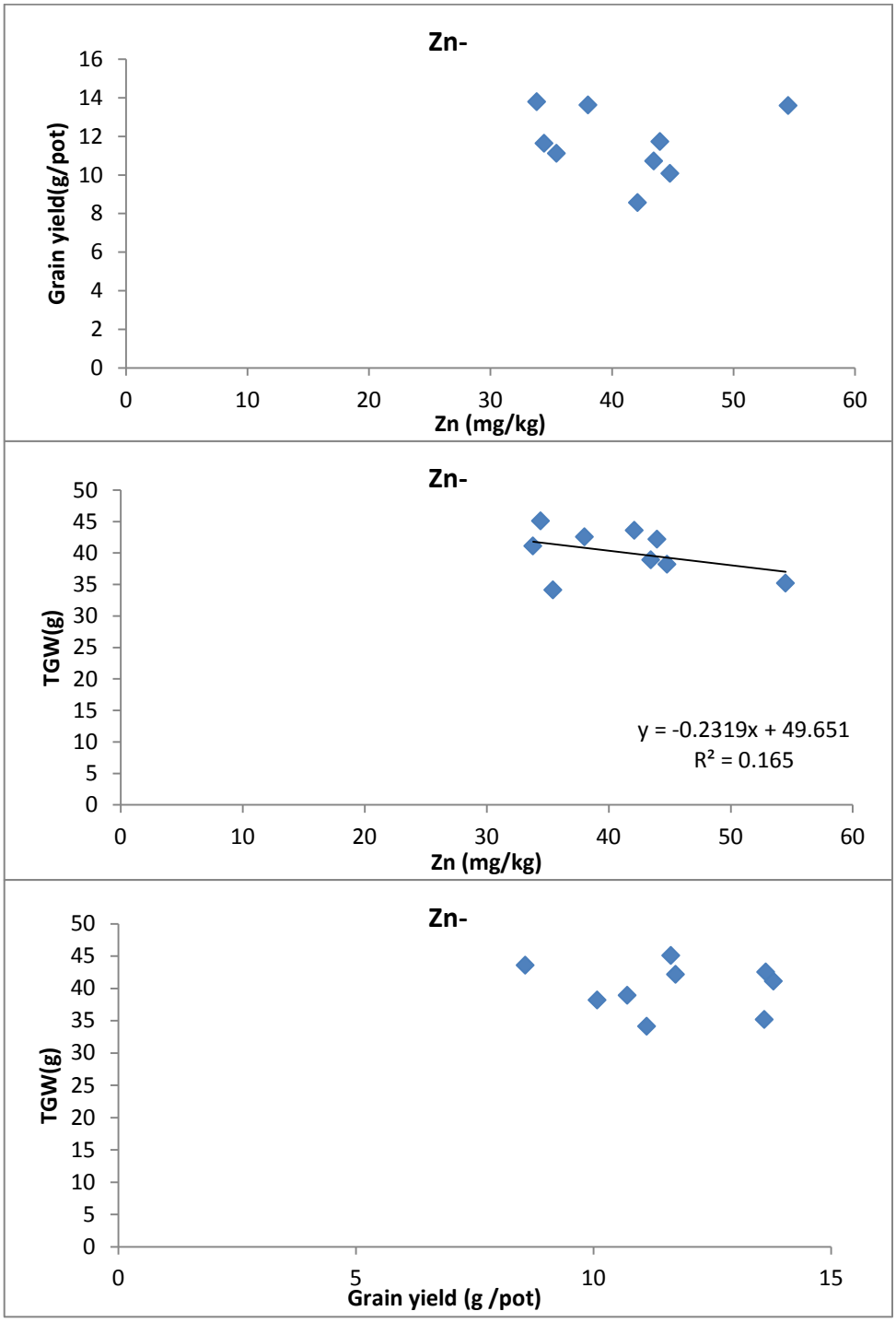


Fig 2.8: Relationship between Zn concentration, grain yield and TGW in DH lines grown at adequate level of Zn in glasshouse

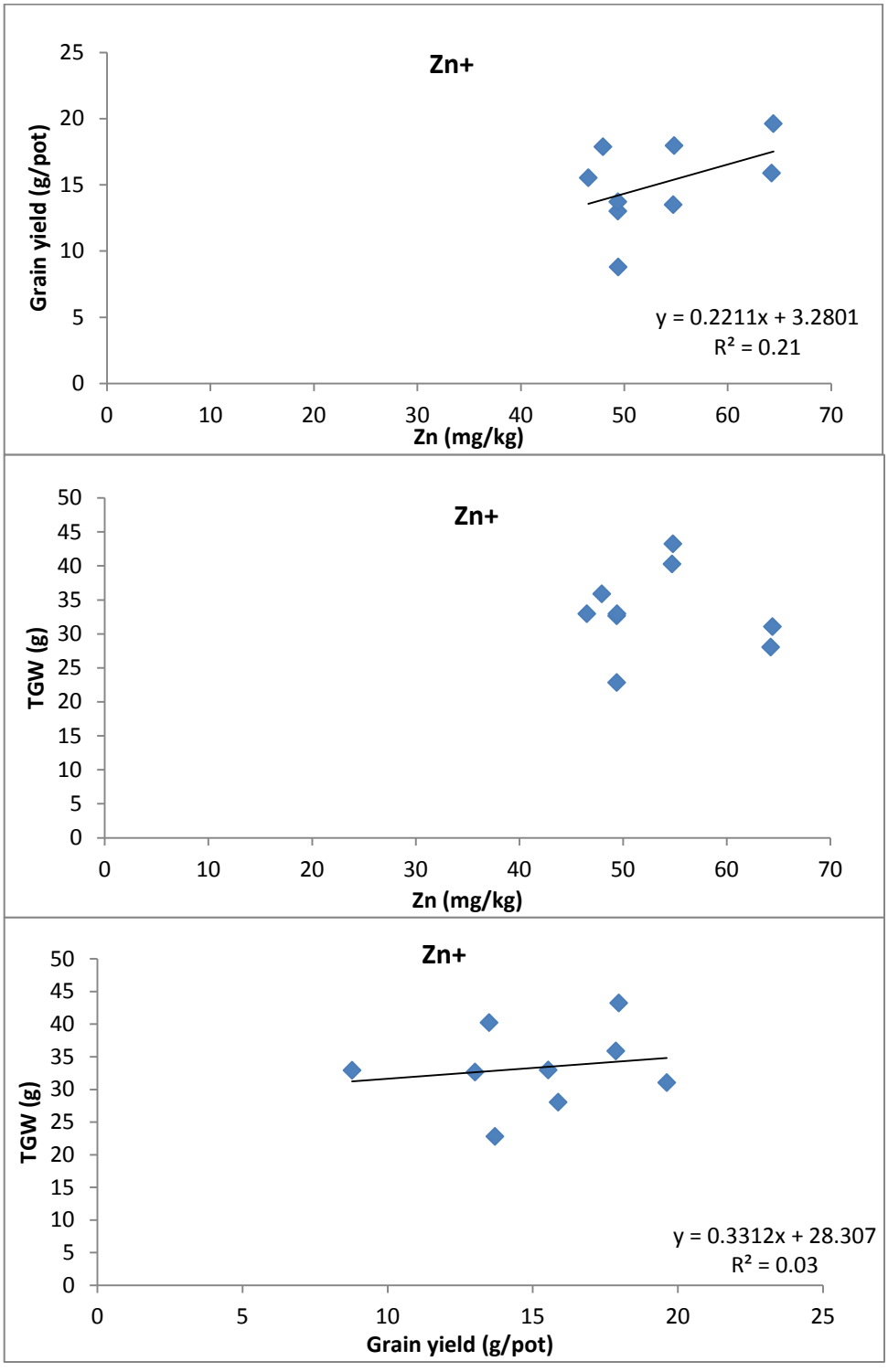


Fig 2.9: Relationship between Zn concentration, grain yield and TGW in 9 DH lines grown at high level of Zn in glasshouse

Grain Zn concentration had a significant negative correlation with dry shoot matter (DSM), biomass and significant positive correlation with grain Zn content and harvest index, and no significant correlation with grain yield, tiller number, TGW, plant height at adequate (i.e. without supplemental) Zn treatment (Table 2.4a). Grain Zn concentration showed a significant negative correlation with dry shoot matter (DSM), height, and significant positive correlation harvest index and had no significant correlation with grain yield, tiller number, , biological yield and grain Zn content(Table 2.4b).

Grain Zn content

Genotypes showed highly significant variations for Zn content at both Zn levels. Zn content ranged from 1.2 to 1.9µg/seed at Zn- treatment (without supplemental Zn) and from 1.1 to 2.4 µg/seed at Zn+ treatment (with supplemental Zn) DH 57 and DH 84 had the highest Zn content of 1.9 µg/seed and DH 121 had the lowest Zn content of 1.2 µg/seed (Table 2.3). At Zn+ level genotypes behaved differently. Zn content of DH 35 and DH36 and Krichauff had decreased while Zn content of DH 57, 84, DH121, Berkut was increased. Berkut had the highest Zn content of 2.4 µg/seed at Zn+ level (Table 2.3)

Table 2.4a: Pearson correlation among different traits measured in 9 double haploid wheat genotypes grown at adequate (i.e. without supplemental) Zn treatment

	Grain yield	Tiller no.	TGW	Spikes wt.	DSM	Biomass	Plant height	No. of spikes	Weight/ spike	Harvest Index	Grain Zn conc.	Grain Zn content	Shoot Zn conc.	Shoot Zn content
Grain yield	1													
Tiller no.	.553**	1												
TGW	-.018	-.281	1											
Spikes wt.	.852**	.523**	.151	1										
DSM	-.017	.122	.289	.311	1									
Biomass	.413*	.341*	.259	.689**	.883**	1								
Height	-.499**	-.426**	.407*	-.308	.284	.015	1							
No. of spikes	.553**	1.000**	-.281	.523**	.122	.341*	-.426**	1						
wt./Spike	-.215	-.543**	.319	-.183	-.139	-.211	.313	-.543**	1					
Harvest Index	.348*	.081	-.259	-.060	-.907**	-.691**	-.384*	.081	.093	1				
Grain Zn conc.	.021	.145	-.319	-.228	-.370*	-.410*	.142	.145	-.311	.387*	1			
Grain Zn content	-.087	-.316	-.100	-.265	-.502**	-.498**	.091	-.316	.188	.485**	.445**	1		
Shoot Zn conc.	-.228	.121	-.108	-.181	.395*	.189	.124	.121	-.349*	-.409*	.258	-.108	1	
Shoot Zn content	-.251	.077	-.111	-.196	.383*	.171	.109	.077	-.317	-.415*	.226	-.152	.978**	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 2.4b: Pearson correlation among different traits measured in 9 double haploid wheat genotypes grown at high (with supplemental) Zn treatment

	Grain yield	Tiller no.	TGW	Spikes wt.	DSM	Biomass	Plant height	No. of spikes	weight/ spike	Harvest Index	Grain Zn conc.	Grain Zn content	Shoot Zn conc.	shoot Zn content
Grain yield	1													
Tiller no.	.448**	1												
TGW	.147	-.275	1											
Spikes wt.	.820**	.346*	.315	1										
DSM	-.562**	-.470**	-.363*	-.577**	1									
Biomass	-.004	-.206	-.171	.192	.632**	1								
Height	-.495**	-.699**	.351*	-.363*	.434**	.117	1							
No. of spikes	.472**	.851**	-.392*	.414*	-.323	.020	-.640**	1						
wt./Spike	-.118	-.752**	.451**	.059	.218	.297	.551**	-.593**	1					
H. Index	.906**	.499**	.187	.641**	-.763**	-.419*	-.513**	.427**	-.244	1				
Grain Zn (mg/kg)	.326	.178	-.065	.225	-.401*	-.316	-.333*	.228	-.121	.438**	1			
Grain Zn content	.135	-.081	.450**	.015	-.053	-.210	.381*	-.213	.149	.218	-.042	1		
Shoot Zn	-.384*	-.349*	.046	-.289	.168	-.001	.249	-.315	.167	-.360*	.162	-.153	1	
Shoot Zn content	-.390*	-.413*	.066	-.316	.213	.011	.303	-.371*	.243	-.369*	.139	-.114	.979**	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed)

2.4 Discussion

The aim of this study was to investigate if any associations exist between phenotypic traits and Zn concentration in a field grown Berkut and Krichauff mapping population, and it was hypothesized that the field results can be reproduced and extended in a detailed study of selected genotypes in the glasshouse.

Grain Zn levels in the DH mapping population

The parents of this double haploid mapping population showed Zn concentrations of 21 and 23 mg/kg when grown in the fields. Given that standard errors seen in ICP analysis are around 2-3 mg/kg (95% confidence interval), the Zn concentrations of parents are not to be different. Across the whole DH population, Zn concentration ranged from 17 to 46 mg kg^{-1}, suggesting that transgressive segregation did occur with this population. This range of Zn concentrations is similar to that seen other published studies with wheat. Zn concentrations ranged from 26 to 40 mg kg^{-1} in one study with 27 modern wheat cultivars (Peterson et al. 1986); in another study with 132 modern genotypes the range was from 25 to 53 mg kg^{-1}(Graham et al., 1999); and in another study with 57 modern genotypes of durum wheat, the range of Zn concentrations was from 29 to 46 mg kg^{-1} (Ficco et al., 2009). However, the range was much lower than that seen by Cakmak et al (2004), who studied 825 wild emmer accessions and obtained grain Zn concentrations over a range 14–190 mg kg^{-1} in the greenhouse. These wild lines exhibited great variation and much higher Zn compared with cultivated lines.

In addition to errors caused in ICP analysis during the analysis itself, another source of error that could have affected the accuracy of the Zn concentrations in this screening is error cause by soil contamination of grain samples. Soil contamination may have inflated the Zn values for some samples in this screening.

Yield in the DH mapping population

In 2007, Berkut and Krichauff, the parents of this mapping population had grain yield of 1.8 and 2.8 t/ha. In this double haploid mapping population, the range of grain yield was from 0.3 to 3.3 t/ha with average yield of 2.0 t/ha. In 2009, another year in which the population was grown at Roseworthy, the average yield for the population was 3.3 t/ha. Overall, it appears that yields were somewhat lower in 2007 than in typical years and were lower in the 2007 DH plots than is typical for wheat grown at Roseworthy because it was drought year (Hugh Wallwork, unpublished data). An average of 2.7 t/ha occurs for wheat globally (www.PAKISSAN.com) and 3.03 Mt/ha (www.nue.okstate.edu/Crop).

Associations between grain Zn and yield in the field

In the fields, grain Zn concentration was negatively associated with grain yield and there was no significant relationship between Zn concentration and thousand grain weights (TGW) (Fig.2.1). Grain yield was positively correlated with TGW. These finding were consistent with the literature confirming that it is very difficult to breed lines with both high yield and high Zn concentration (Murphy et al., 2008; McDonald et al., 2008; Garvin et al,. 2006). It is likely that a relatively constant amount of Zn is taken up by plants and that in higher yielding plants this is diluted by extra carbohydrates. A significant negative correlation existed between yield and Zn concentration, a positive correlation existed between yield and TGW, but the correlation between TGW and Zn concentration was only weak or non-existent. This means that while low yielding lines generally are high Zn and have lower TGW, low TGW lines are not necessarily high Zn. This is reassuring since it indicates that high Zn can have good grain size, even though it may be difficult to breed high Zn lines with good yield.

Grain Zn and yield in the glasshouse

i) Zn levels were higher in glasshouse than field – why?

Grain Zn levels in glasshouse grown plants were consistently higher than in plants grown in the field, even without Zn supplementation. The increase was slight, but consistent, in the lines selected to be high Zn lines. With lines selected to be low lines, the increase was even greater. Other studies have had similar problem i.e. environmental effect on Zn concentration (Brennan and Byth, 1979; Hintsa et al., 2011; Camak et al., 2010).

This was surprising since the yields of plants grown in the glasshouse were higher than the yields of field grown plants. In addition the glasshouse grown plants only had the chance to explore a small root volume. Hence it could have been expected that Zn concentration would actually have been lower in the high yielding glasshouse plants. One reason for the high Zn concentrations seen in the glasshouse could be that the potting mix used contained high Zn levels, either in the soil mix itself or in slow release fertilizers that are added to the mix. Another possibility is that glasshouse grown plants received higher N treatments than those in the field. When Zn-coated urea fertilizer was applied, significant improvement in grain yield and grain Zn concentrations was observed compared to when only Zn treatments were applied (Shivay et al. 2008; Cakmak, 2010). Nitrogenous fertilizers increase grain proteins which may be the sink for Zn concentration (Morgounov et al., 2007). Thus the applications of urea made in the glasshouse could have had a role in increasing Zn transport to the grain.

ii) Zn treatment did increase Zn level

The application of ZnSO4 solution to pots did increase Zn levels for all genotypes, (Table 2.2) relative to glasshouse grown plants with no $ZnSO_4$ treatment, i.e., the addition of ZnSO4 to potting mix did lead to an increase in plant available Zn, which was transported to grain. It is likely that the fact the pH of potting mix is near about

neutral contributed to this result (in contrast, soil with high pH could be expected to bind Zn and the element would not be available to plants).
Zinc sulphate was applied at a rate of 1 mg/kg of soil, which equated to 3 mg per pot. Considering that the surface area of soil in the pots was 2.8×10^{-6} ha, this equated to a rate of 1 kg/ha of $ZnSO_4$ (similar to the application rates of $ZnSO_4$ in other pot studies, e.g., (Habib, 2009), but less than is often used in agronomic biofortification, because soil used in this study was not Zn deficient.

There are several examples in the literature where increased Zn concentrations of grains have been achieved by increasing Zn fertilizers (Maralian Habib, 2009). Increases in Zn concentration of grain and straw and grain yields were reported by Shaheen et al., 2007: Singh and Singh, 1989; Martens and Westerrmann, 1991; Dwivedi and Tiwari, 1992; Mortvedt and Gilkes, 1993; Yilmaz et al., 1997; Rengel et al., 1999; Bybordi and Malakouti, 2003; Seilsepour, 2007). Zinc concentration of cereals can be improved by adding Zn fertilizer into the soil or directly applying on the plants (Broadley et al., 2007). Mandal and Mandal (1990) also reported that applied zinc increases available zinc concentrations of the soils. Addition of NPK fertilizers blended with Zn was very effective in improvement of Zn concentration and grain yield (Mortvedt, 1991).

iii) What did the Zn treatment do to other traits?

Besides higher grain Zn, the application of Zn to soil led to a range of other morphological changes, even though the soil was not considered Zn deficient and there were no symptoms of Zn deficiency in the glasshouse grown plants. Zn application enhanced the tiller number, number of spikes, dry shoot matter and Zn concentration of grains. This is similar to what has been observed in other studies where Zn fertilization has been made to Zn deficient plants. Zn responses have included an increase in shoot dry matter production (11-109%), grain yield (9-256%)

and grain Zn concentration (9-912%) of wheat with applied Zn (Anonymous, 1998; Yilmaz et al., 1997; Rengel et al., 1999; Rafique et al., 2006; IZA, 2009). Most of these studies by other authors were done on Zn deficient soils.

iii) Did Zn treatment affect the relationship between Zn concentration and other traits?

Given that Zn concentrations were not able to be reproduced between field and glasshouse conditions, it was considered unlikely that the study would reveal much useful information about the relationship between different traits and Zn concentrations. Preliminary efforts were made to look for relationships between grain yield, Zn concentration and TGW in glasshouse grown plants at both Zn treatments. However, no relationships were evident ($p > 0.05$).

iv) DH 57

DH 57 had highest Zn concentration in field as well as the glasshouse. It was exceptionally low yielding in the field, which suggested initially that perhaps the high Zn was a consequence of the 'concentration effect', i.e. that whatever Zn was taken up was transported to just a few grains, in which case the line would be of minimal commercial and practical interest. However, the line continued to be high Zn in the glasshouse, and that in this environment the line was also high yielding. It is not clear whether or not DH57 had high Zn in the vegetative tissues before grain filling, but it did not have exceptional biomass, nor did it have exceptional Zn concentration in the straw after grain filling (Table 2.3). Thus it was not clear from this study if the line took up more Zn, or was just better at relocating that Zn. It was concluded that more work should be conducted on this line.

2.5: Conclusions

This study confirmed the negative association of grain yield with Zn concentration, but showed that lines with high TGW can still be high yielding. Clearly breaking the negative association between yields and grain Zn must be a priority for wheat biofortification breeders. In addition, the line DH 57 produced high Zn in two different environments, suggesting it to be a line worthy of further study.

The experience of this study served to highlight several important lessons. Firstly, it would have been better to make selections of high and low Zn lines based on Zn concentrations in multiple environments – not just on information on Zn from one environment. This would maximise the chance of identifying robust variation for Zn that would hold up in different environments. Secondly, the Berkut x Krichauff population may not have been particularly suitable for this study since variation between parents was small. No purpose built high Zn by low Zn crosses have yet been made in wheat for genetic studies of Zn concentration, but these could be considered in future. Thirdly, conducting screens in the glasshouse in a potting mix cannot necessarily be expected to produce the same results and rankings among lines as were obtained in the field. In future, field soil could be used to get more realistic (field-like) Zn levels. Finally, another factor that may have confounded the results of this chapter could be soil contamination, and whether soil contamination lead to mis-identification of high lines in the initial screen. Thus it was concluded further work is needed to understand the impact of soil contamination on Zn screens.

Chapter 3 Cleaning grains for accurate micronutrient analysis

3.1 Introduction

Inductively Coupled Plasma–Optical Emission Spectrometry (ICP-OES), Atomic Absorption Spectrometry (AAS) and more recently, X-ray Fluorescence spectrometry (XRF) (Paltridge et al., 2012a, 2012b) analysis of plant tissues for nutrient concentration are essential technology underpinning many plant nutrition and biofortification studies. Within the HarvestPlus biofortification network, the main nutrients of interest are Fe and Zn, and the tissues of primary interest are mature grains. Both Fe and Zn are micronutrients, present in grain at very low levels (generally between 10 and 50 mg kg^{-1} dry weight Paltridge et al. (2011, 2012). Major seed crops of interest in biofortification include wheat, rice, maize, pearl millet and beans (Nestel et al., 2006). For wheat grain, Fe and Zn are present at very low levels (generally between 10 and 50 mg kg^{-1}) and a major obstacle in wheat breeding is that analysis of Fe and Zn in grain is often confounded by the presence of soil derived contamination particularly for Fe rather than Zn (Pfeiffer and McClafferty, 2007; Sillanpaa, 1982).

Soil borne micronutrients adhere to the outer epidermal surfaces (Djingov and Kuleff, 1994; Hall, 1995; Jones, 2001) that leads to the contamination. Soil typically contains around 4% Fe (40,000 mg kg^{-1}) (Sparks, 2003), several orders of magnitude more than is found in grain, so traces of soil on grain lead to erroneously high reports of grain Fe concentration. Soil contamination is also mostly considered a problem for the plants grown and collected from windy areas (Cherney and Robinson, 1983; Jones, 1991, 2001).

Other common sources of Fe and Zn contamination are residues from grain handling equipment or threshers, and residues from human hands or even gloves used during the preparation of seed samples (Sager and Mittendorfer, 1997; Jones, 2001). There

are two main methods used to identify Fe and Zn contamination in wheat. Firstly, major sources of contamination may be seen as concentration 'spikes' in the analysis, which are generally not reproduced in replicated analysis. Secondly, since Al is even more abundant in soil than Fe (Ibia, T.O., 2002; Rauch and Pacyna 2009) but is not found in clean plant tissue, and is easily analyzed by ICP, it can be used as an indicator of soil contamination. Typically, soil contamination of grain is considered to be acceptably low in grain analysis reports where Al is present at <5 $mgkg^{-1}$ (Pfeiffer and McClafferty, 2007).

There is very little information available in the literature as to how contaminated wheat grain can be cleaned of contamination prior to analysis. In one unpublished study, attempts were made to remove contamination by manual wiping of the seeds with Chux™ cloth (James Stangoulis, personal communication). However, this method was only partially successful, with Al levels on grain contaminated with soil were only to be reduced to 5-10 $mgkg^{-1}$, even after wiping for 90 sec, and the method was considered too labour intensive to pursue further.

Washing experiments of wheat and maize grains have been conducted to remove the iron contamination and have been successful to some extent although there is concern that washing of samples can leach out the micronutrients and this then distorts results (Djingov and Kuleff, 1994; Hall, 1995; Jones, 2001).

The Kett "Pearlest" Polisher has been shown previously to be suitable for the light milling ('polishing') of small rice samples (e.g., 10 g samples; Bautista and Siebenmorgan 2002). As supplied by the manufacturer, the Kett mill is unsuitable for micronutrient work, since the rubber spinning disk and walls have been shown to introduce Zn contamination to rice samples (James Stangoulis, unpublished data). However, modified Kett mills, in which the rubber disk and gasket in the polishing chamber are replaced with polyurethane PU40 90A, are suitable for rice micronutrient work; i.e., to mill rice without introducing detectable micronutrient or Al contamination (James Stangoulis, unpublished data). The work of this chapter is

divided into three main parts. A hypothesis was proposed that contamination of wheat samples by dust masks genotype variation with respect to Zn. The first experiment investigated what milling time should be used to clean wheat, and whether the process of cleaning itself introduces contamination. The second experiment, a validation, investigated the effect of cleaning on highly contaminated lines from two environments. The third experiment, investigated the effect of cleaning on the precision of QTL analysis.

3.2. Materials and methods

3.2.1 Milling time study

Initial work was conducted using lines from the Berkut x Krichauff DH population described previously (Chapter 2). Three contaminated genotypes from the 2007 growing season at Roseworthy (RAC), DH35, DH57 and DH135, were selected. These showed Al concentration in the range of 6 to 12 mg kg^{-1}(Table 3.1). Two non-contaminated genotypes, HPAN 1 and HPAN 2, were also selected, which were from HarvestPlus irrigated wheat experiments and showed very low levels of dustiness. It was not possible to select non-contaminated genotypes from RAC, 2007, because all lines from that site were slightly or highly contaminated. HPAN1 and HPAN2 showed Al concentrations of 1.6 and 1.5 mg kg^{-1} respectively.

All samples were dried overnight at 60 °C prior to the milling treatment. Milling times used were 5, 10, 20 and 40 s, in addition to an un-milled control. Approximately 2 g of wheat grain were cleaned for each treatment. The experiment was conducted with four replications, i.e., 4 different subsamples of each genotype were milled at each milling time. It was not necessary to further clean the milled samples with a sieve after each run as the Kett mill is equipped to separate out bran

from the polished rice, but the Kett mill was thoroughly cleaned between samples. Samples were milled in random order and each treatment was replicated four times. Wheat samples were carefully weighed to the nearest 0.001g before and after milling to allow the calculation of dry matter removed and retention percentages for each genotype across the entire range of genotypes. In order to observe the changes brought about by milling, photographs of seed samples were taken before and after milling. After milling, samples were analyzed for Al, Fe and Zn at Waite Analytical Services. To investigate the concentrations of Zn, approximately 0.6-0.8 g of oven dried wheat seeds were weighed into 75 mL Pyrex tubes and digested in 11 ml mixture of $HNO_3/HCLO_4$ (10:1 v/v). This mixture was wrapped with polyethylene and kept overnight in a fume cupboard for pre-digestion. Tubes were then removed from cupboard and digested to reduce the sample to around 1ml $HClO_4$. Then samples were vortexed after adding water and analyzed (Wheal et al. 2011). Quality control and quality assurance was assessed using control sample data generated when performing routine analyses Averaged results were compared to published values from either NIST certified values (mean ± 95%CI) or ASPAC consensus values (median ± MAD) (Wheal et al.2011).

3.2.2 Validation of 5 sec milling method on contaminated lines from two environments

To validate the cleaning method on highly contaminated lines, a set of thirty highly contaminated wheat lines from the RAC site were selected and analyzed both as 'dirty' samples and as 'cleaned'. This enabled the calculation of the amount of Fe and Zn removed per unit of Al removed, in a larger group of lines. Finally, validation was conducted on a larger set of 240 lines from Pakistan. This population of 240 lines had been grown in the field at Islamabad in Pakistan during 2010 as part of HarvestPlus trials. From initial analysis by ICP-OES, it was clear that all samples contained Al > 5 mg kg^{-1}, with many others showing Al in the range of 5-20 $mgkg^{-1}$.

These lines were milled for 5 seconds and then re-analyzed by ICP-OES, again allowing investigation of the ratios of elements removed and the efficacy of the cleaning method.

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3.2.3 Precision of QTL analysis

To investigate whether cleaning of wheat grains leads to greater precision of QTL analysis, lines from the Berkut x Krichauff DH population grown in 2009 at Roseworthy Agricultural College (RAC) were analyzed for the identification of QTLs. In first step dust contaminated samples were analyzed for identification of QTLs. In second step the same samples were cleaned by using Kett mills and again analyzed for identification of QTLs by using the newest version of ICIM software.

3.3 Results

3.3.1 Milling time study

The first parameter to be examined at the different milling times was dry matter retention (Table 3.1). Data indicated a steady reduction in dry matter retention as milling time increased, with approximately 0.45% dry matter reduction after 5 sec milling, 1% reduction after 10 sec milling, 2% reduction after 20 s milling and 3% reduction after 40 s milling.

The second set of parameters to be examined was concentrations of Al, Fe and Zn at each milling time, for each genotype, as well as pooled concentration data across each contamination class (i.e., across the two non-contaminated lines and three contaminated lines). Both the amount of element removed and % removal are presented in Table 3.2.

For three contaminated lines (average Al 9 mg kg^{-1}), Al concentration was reduced, on average by 5.6 mg kg^{-1} by the first 5 s milling, then a further 0.5 – 1 mg kg^{-1} was removed at each subsequent time-point. In percentage terms this translated to 60% Al removal in the first 5 s then removal of an additional 3-7% at each time point. In absolute terms, Al was reduced to < 5 mg kg^{-1} in all contaminated genotypes after just 5 s of milling but did not ever reduce to below 1 mg kg^{-1}, even after 40 s of milling. The detection limits of the ICP-OES are around 3 mg kg^{-1} so it was considered that very low levels of Al to be ambiguous and mostly base-line noise.

The Fe concentration in non-contaminated lines (average Fe around 31 mg kg^{-1}) was decreased by 1.5 mg kg^{-1} after 5 s of milling, then decreased by an additional 0.2-2.5 mg kg^{-1} at each subsequent time-point. In percentage terms 5 s removed 5% of Fe, then subsequent milling periods of 5-20 s removed an additional 0.7-6.5 % per time-point. For contaminated lines (average Fe level 48.8 mg kg^{-1}), Fe concentration was reduced, on average, by around 4 mg kg^{-1} by the first 5s milling, then a further 0.7-2.3 mg kg^{-1} at each subsequent time-point. In percentage terms this translated to 8% Fe removal in the first 5 sec then removal of an additional 1.5-4.6 % after 10-20s, then 8% after 40 s.

Zn concentration in non-contaminated lines (average Zn 36.5 mg kg^{-1}) was decreased by around 0.3 mg kg^{-1} after 5 s of milling and either stayed similar or decreased slightly up to 20 s, then decreased by about 3 mg kg^{-1} at 40 s milling. With the contaminated lines (average Zn concentration of 30 mg kg^{-1}), Zn concentration decreased by about 1-1.5 mg kg^{-1}, except after 40 s milling where the decrease was around 2 mg kg^{-1}. In percentage terms, a similar trend was observed as with Fe, except that less Zn was removed than Fe.

Overall, the generalization can be made that most of the Al that can be removed by milling was removed after the first 5 s, and that additional removal of Al after the first 5 s was slight but increased progressively with time. Dirty lines tended to retain more Al than clean lines. For Fe, a quick reduction of 2 mg kg^{-1} (clean lines) or 4 mg kg^{-1} (contaminated lines) was observed after the first 5 s of milling, and subsequent removal was again slight but steady. For Zn, there was no sudden decrease of Zn after the first 5 s; instead the decrease was minor and steady across all time-points. Another generalization is that Al and Fe were removed in similar amounts over the first 20 s of milling (at approximately a 1:1 ratio). With 40 s of milling, however in clean lines, more Fe was removed than Al. Over the first 20 s milling much less Zn was removed than Al and Fe, with Zn typically removed at about 0.2 mg kg^{-1} for every 1 mg kg^{-1} of the other elements.

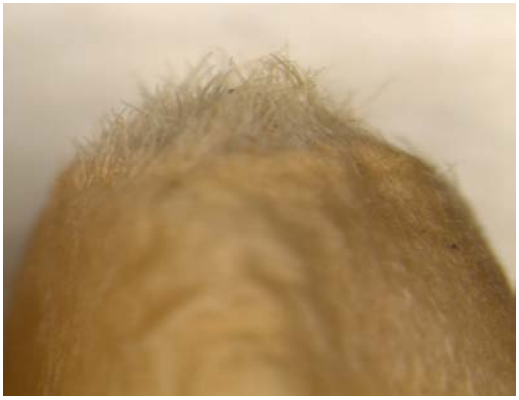
Visual inspection of the 5 genotypes revealed the milling action to remove a majority of seed hairs, even after 5 s; (for examples, see Berkut and DH 135 before and after milling (Figure 3.1)). Milling for longer times (Fig 3.2) led to a more severe erosion of the seed coat.

Table 3.1 Dry matter retention (%) among 2 non-contaminated and 3 contaminated genotypes after milling for different times

Variable	Genotype	Conc. Al	Contamination class	Milling time													
				0		5			10			20			40		
				Original	% orig	DM	Milled	% orig	DM	Milled	% orig	DM	Milled	% orig	DM	Milled	% orig
Dry Matter	HPAN1	1.6	Clean	2.18	100	2.21	2.2	99.55	2.12	2.1	98.11	2.21	2.2	97.29	2.2	2.1	94.55
	HPAN2	1.5	Clean	1.97	100	1.98	1.97	99.49	2.03	2.0	98.03	2.12	2.1	97.17	2.08	2.0	94.23
	DH35	12.1	Contaminated	2.02	100	2.07	2.06	99.52	1.97	1.94	98.48	2.07	2.02	97.58	1.86	1.8	96.77
	DH57	9.1	Contaminated	2.02	100	2.39	2.38	99.58	2.3	2.28	99.13	2.23	2.19	98.21	2.24	2.18	97.32
	DH135	6	Contaminated	2.01	100	2.28	2.27	99.56	2.21	2.2	99.55	2.35	2.31	98.30	2.18	2.12	97.25
	Mean				100			99.55			99.05			98.03			97.11

Table 3.2: Concentration of Al, Fe and Zn in 2 clean and 3 contaminated genotypes after milling for different times

Element	Genotype	Cont class	Milling time													
			0		5			10			20			40		
			Original conc.	% orig	Conc.	Decrease	% orig	Conc.	Decrease	% orig	Conc.	Decrease	% orig	Conc.	Decrease	% orig
Al	**HPAN 1**	**Clean**	**1.58**	100	0.59	0.99	37	0.44	1.14	28	0.44	1.14	28	0.34	1.24	21
Al	**HPAN 2**	**Clean**	**1.52**	100	0.50	1.0	33	0.42	1.10	28	0.30	1.22	20	0.23	1.29	15
Al	**Mean**	**Clean**	**1.55**	**100**	**0.54**	**1.01**	**34.9**	**0.43**	**1.12**	**27.7**	**0.37**	**1.18**	**23.7**	**0.29**	**1.27**	**18.3**
Al	**DH35**	**Contaminated**	**12.1**	100	3.9	8.2	32	3.8	8.3	31	3.6	8.5	29.8	2.5	9.6	20.7
Al	**DH57**	**Contaminated**	**9.1**	100	3.7	5.4	41	2.5	6.6	27	2.5	6.6	27.5	2.3	6.8	25.3
Al	**DH135**	**Contaminated**	**6**	100	2.8	3.2	47	2.4	3.6	40	2.1	3.9	35.0	1.4	4.6	23.3
Al	**Mean**	**Contaminated**	**9.1**	**100**	**3.5**	**5.6**	**39.9**	**2.9**	**6.2**	**33.0**	**2.7**	**6.3**	**30.7**	**2.1**	**7.0**	**23.1**
Fe	**HPAN1**	**Clean**	30.32	100	29.50	0.8	97.3	29.20	1.1	96.3	28.66	1.7	94.5	26.90	3.43	89
Fe	**HPAN2**	**Clean**	32.02	100	29.89	2.1	93.4	29.72	2.3	92.8	29.68	2.3	92.7	27.40	4.62	85.6
Fe	**Mean**	**Clean**	**31.17**	**100**	**29.69**	**1.5**	**95.3**	**29.46**	**1.71**	**94.6**	**29.17**	**2.0**	**93.6**	**27.15**	**4.02**	**87.1**
Fe	**DH35**	**Contaminated**	50.7	100	43.5	7.2	85.8	44.9	5.8	88.6	44.4	6.3	87.6	42.2	8.5	83.2
Fe	**DH57**	**Contaminated**	58.4	100	54.5	3.9	93.3	52.6	5.8	90.1	54.1	4.3	92.6	51.4	7	88.0
Fe	**DH135**	**Contaminated**	37.2	100	36.1	1.1	97.0	34.5	2.7	92.7	34.2	3.0	91.9	32.4	4.8	87.1
Fe	**Mean**	**Contaminated**	**48.8**	**100**	**44.7**	**4.1**	**92.1**	**44.0**	**4.8**	**90.5**	**44.2**	**4.5**	**90.7**	**42.0**	**6.8**	**86.1**
Zn	**HPAN1**	**Clean**	33.52	100	33.46	0.1	99.8	33.24	0.3	99.2	33.21	0.3	99.1	31.01	2.5	92.5
Zn	**HPAN2**	**Clean**	39.54	100	39.05	0.5	98.8	38.77	0.8	98.1	39.36	0.2	99.5	36.24	3.3	91.7
Zn	**Mean**	**Clean**	**36.53**	**100**	**36.26**	**0.27**	**99.3**	**36.00**	**0.53**	**98.6**	**36.28**	**0.25**	**99.3**	**33.62**	**2.9**	**92.1**
Zn	**DH35**	**Contaminated**	29	100	26.1	2.9	90	26.8	2.2	92.4	27.1	1.9	93.4	26.1	2.9	90
Zn	**DH57**	**Contaminated**	38.7	100	37.8	0.9	97.7	38	0.7	98.2	38.6	0.1	99.7	36.6	2.1	94.6
Zn	**DH135**	**Contaminated**	21.6	100	21	0.6	97.2	20.6	1.0	95.4	20.4	1.2	94.4	19.5	2.1	90.3
Zn	**Mean**	**Contaminated**	**29.8**	**100.0**	**28.3**	**1.5**	**95.0**	**28.5**	**1.3**	**95.3**	**28.7**	**1.1**	**95.9**	**27.4**	**2.4**	**91.6**



DH135 before milling

DH 135 after milling

DH57 before milling

DH57 after milling

DH35 before milling

DH 35 after milling

Figure 3.1 Photographs of seeds in DH135, DH57 and DH35, before and after milling for 5 sec showing removal of hair

Figure 3.2 Photograph of seed coat after cleaning for 40 s, with erosion of the seed coat clearly visible.

3.3.2 Validation of 5 sec milling method on contaminated lines

To confirm typical removal rates of Al, Fe and Zn from contaminated wheat samples, 30 genotypes of the Berkut x Krichauff Double Haploid population that was grown in the field at Roseworthy in 2007, were selected and divided into three categories of contamination: Category (Cat) 1, samples with 4-6 mg Al kg^{-1}; Cat 2, samples with 6-8 mg Al kg^{-1}; Cat 3, samples with 8-10 mg Al kg^{-1}. These samples were all subjected to 5 s milling. Removal of Al was greatest from the most contaminated lines (Table 3.3): 6 mg kg^{-1} was removed from lines which originally contained 9 mg kg^{-1}, 5 mg kg^{-1} from lines that contained 7, and 3.6 mg kg^{-1} from lines that contained 5.4. Similar concentration of Fe was removed as Al. For Zn, however, much less was removed and the amount of Zn removed was not highest in the most contaminated lines, consistent with the idea that Zn levels are not proportional to soil contamination level. Thus the generalization may be made that, in these highly contaminated lines, Fe was removed by cleaning at approximately a 1:1 ratio with Al, but that Zn removal only occurred at about 1.4 mg kg^{-1} per 5 mg kg^{-1} (equivalent to 0.2 mg kg^{-1} per 1 mg kg^{-1} of Al).

Table 3.3: Concentration (mg kg^{-1}) of Al, Fe, and Zn in 3 categories of contaminated lines from Australia (Berkut x Krichauff population) prior to and after analysis.

Number of samples	**[Al] contaminated**	**Al**			**Fe**			**Zn**		
	B x K 07	**Orig**	**Cl**	**Red**	**Orig**	**Cl**	**Red**	**Orig**	**Cl**	**Red**
10	mean4-6	5.37	1.8	3.6	39	35	4.0	23	21	1.6
10	mean6-8	6.97	2.3	4.7	39	35	4.4	24	22	2.0
10	mean8-10	8.92	3.0	6.0	41	37	4.1	23	23	0.4
	mean	7	2	4.7	40	35	4.2	23	22	1.4

Orig.=original; Cl= cleaned; Red= change in concentration

For final validation of the 5 s milling method, a further 240 highly contaminated lines were cleaned by milling (average Al before cleaning was 10 mg kg^{-1}). This study was conducted firstly as a service to HarvestPlus, who did not consider data obtained from the original ICP-OES analysis to be reliable, and wanted the analysis repeated after cleaning of samples, and secondly because it provided a good opportunity to see how effective cleaning was on a large collection of lines, and to investigate ratios of Al, Zn and Fe removal, in another environment.

Before cleaning, average Al concentrations were 10 mg kg^{-1}, and only 9 out of 240 or 4% of wheat lines showed Al concentrations <5.0 mg kg^{-1} (i.e. were clean enough for results to be considered by Harvest Plus to be reliable). After cleaning, 183 out of 240 or 76% had Al <5.0 mg kg^{-1}, and average Al levels were 4.2 mg kg^{-1} (Table 3.4). Thus the cleaning procedure was highly successful in reducing Al levels in contaminated samples, but only partially successful at reducing Al to acceptable levels (<5 mg kg^{-1}). Fe levels were reduced from an average of 49.4 mg kg^{-1} to an average of 42.8 mg kg^{-1} in the cleaned set, and Zn levels were reduced from an average of 38.1 mg kg^{-1} to 35.8 mg kg^{-1} in the cleaned set.

Regarding the ratios of elements removed, a ratio of 1:1 for Al: Fe was evident, with slightly more Fe removed than Al and with much less Zn removed than Fe (approximately 0.4 concentration units of Zn removed per unit of Al removed).

Table 3.4: Average concentrations of Fe, Zn and Al among 240 wheat lines, before and after cleaning. Units are $mgkg^{-1}$ unless otherwise indicated

Element	Before cleaning	After cleaning	Reduction	Retention
Al	10.0	4.2	5.8	42%
Fe	49.4	42.8	7.2	87%
Zn	38.1	35.8	2.3	94%

Finally, a highly important attribute of genotypes which can be of interest in micronutrient screening programs is ‘rank ’of grain concentration for the element of interest. To investigate if cleaning affects the ranking of lines, 21 lines were chosen from the 240 line population and ranked for Fe and Zn concentration (Table 3.5).

Table 3.5 Ranking of 20 genotypes with reference of Fe and Zn concentrations before and after cleaning

Genotypes	Fe (mg/kg) Before	Fe (mg/kg) After	Zn (mg/kg) Before	Zn (mg/kg) After	Al (mg/kg) Before	Al (mg/kg) After	Fe Ranking Before	Fe Ranking After	Zn Ranking Before	Zn Ranking After
H+AN #13	51	42	34	32	13.7	5.4	**1**	**2**	**1**	**1**
H+AN #100	50	48	43	40	5.7	2.5	**2**	**1**	**2**	**2**
H+AN #2	48	32	30	27	21.5	4.4	**3**	**21**	**3**	**3**
H+AN #4	46	36	28	27	15.4	4.6	**4**	**10**	**4**	**4**
H+AN #5	46	33	28	27	15	3.4	**5**	**20**	**5**	**5**
H+AN #15	45	38	31	30	13.3	5.3	**6**	**3**	**6**	**6**
H+AN #10	44	38	31	30	10.1	4.4	**7**	**5**	**7**	**7**
H+AN #14	44	37	29	27	8.1	3	**8**	**7**	**8**	**8**
H+AN #6	43	35	30	28	11.7	3.6	**9**	**15**	**9**	**9**
H+AN #18	42	37	31	30	10.3	3.5	**10**	**8**	**10**	**10**
H+AN #9	42	38	30	29	9.4	4.9	**11**	**4**	**11**	**19**
H+AN #1	42	36	34	31	11.6	4.8	**12**	**9**	**12**	**12**
H+AN #12	42	35	33	31	11.5	5.6	**13**	**12**	**13**	**11**
H+AN #20	42	36	28	25	9.3	4.2	**14**	**11**	**14**	**13**
H+AN #8	42	38	28	27	10.2	5.7	**15**	**6**	**15**	**15**
H+AN #19	41	33	27	25	10.3	3.6	**16**	**19**	**16**	**16**
H+AN #11	41	35	32	30	11.4	5	**17**	**13**	**17**	**17**
H+AN #16	40	34	36	34	8.8	3.5	**18**	**17**	**18**	**20**
H+AN #17	40	34	32	30	8.6	4	**19**	**18**	**19**	**18**
H+AN #3	40	35	28	29	9.5	4.1	**20**	**14**	**20**	**14**
H+AN #7	39	34	30	28	9.9	4.6	**21**	**16**	**21**	**21**

Data showed that after cleaning, the ranking of genotypes for Fe concentration changed markedly (Table 3.5) e.g., genotype H+ AN # 2 changed from 3 to 21, H+ AN # 4 from 4 to 10 and H+ AN # 5 from 5 to 20. For Zn, rankings only changed slightly after cleaning: the top 10 lines retained the same rank after cleaning, but there were some changes in rank after cleaning in lines ranked 11-21.

Finally, the 240 wheat lines were divided into different categories depending on the concentration of Al: namely; Cat 1, Al 4-6 mg kg^{-1}; Cat 2, Al 6-8 mg kg^{-1}; Cat 3, Al 8-10 mg kg^{-1}; Cat 4, Al 10-12 mg kg^{-1}; Cat 5 Al 12-14 mg kg^{-1}; Cat 6 Al 14-16 mg kg^{-1}; Cat 7 Al 16-20 mg kg^{-1}. Data showed that the higher the contamination class of Al, the more Al was removed, and the higher the proportion of Al removed (Fig 3.3). Similarly, the higher the contamination class, the more Fe was removed. However, removal of Zn was not highest in the most highly contaminated classes.

It was also apparent that milling can only be expected to reduce samples with 'medium' levels of contamination (around10 mg kg^{-1}) to below 5 mg kg^{-1} (the acceptable limit for contamination with HarvestPlus samples). Above that level of Al contamination, the samples could not be cleaned to < 5 mg kg^{-1} within the 5 s milling period.

3.3.3Precision of QTL analysis

In contaminated lines of Berkut x Krichauff (2009), two QTLs for Fe were found, one QTL on 2B with a LOD score of 3.6, with flanking markers cfa2278 - gwm55 and a second on 3B with a LOD score of 1.1 and flanking markers wPt-4209 - wPt-5390 (Table 3.6; Fig. 3.4). These contaminated lines were cleaned by polishing method and analyzed again for QTLs. After cleaning the same two QTLs on 2B and 3B chromosomes were found with the same flanking markers but with greater precision after cleaning. The QTL on chromosome 2B had a LOD score of 3.4 after cleaning, while the QTL on 3B had a LOD score of 3.4, which identified it as a was

significant QTL (Table 3.6, Fig. 3.4) which means cleaning had icreased its precision. Seed cleaning either narrowed the QTL peak (2B QTL) or bring up the significance (3B QTL). Both are better for QTL detection (Fig.3.4).

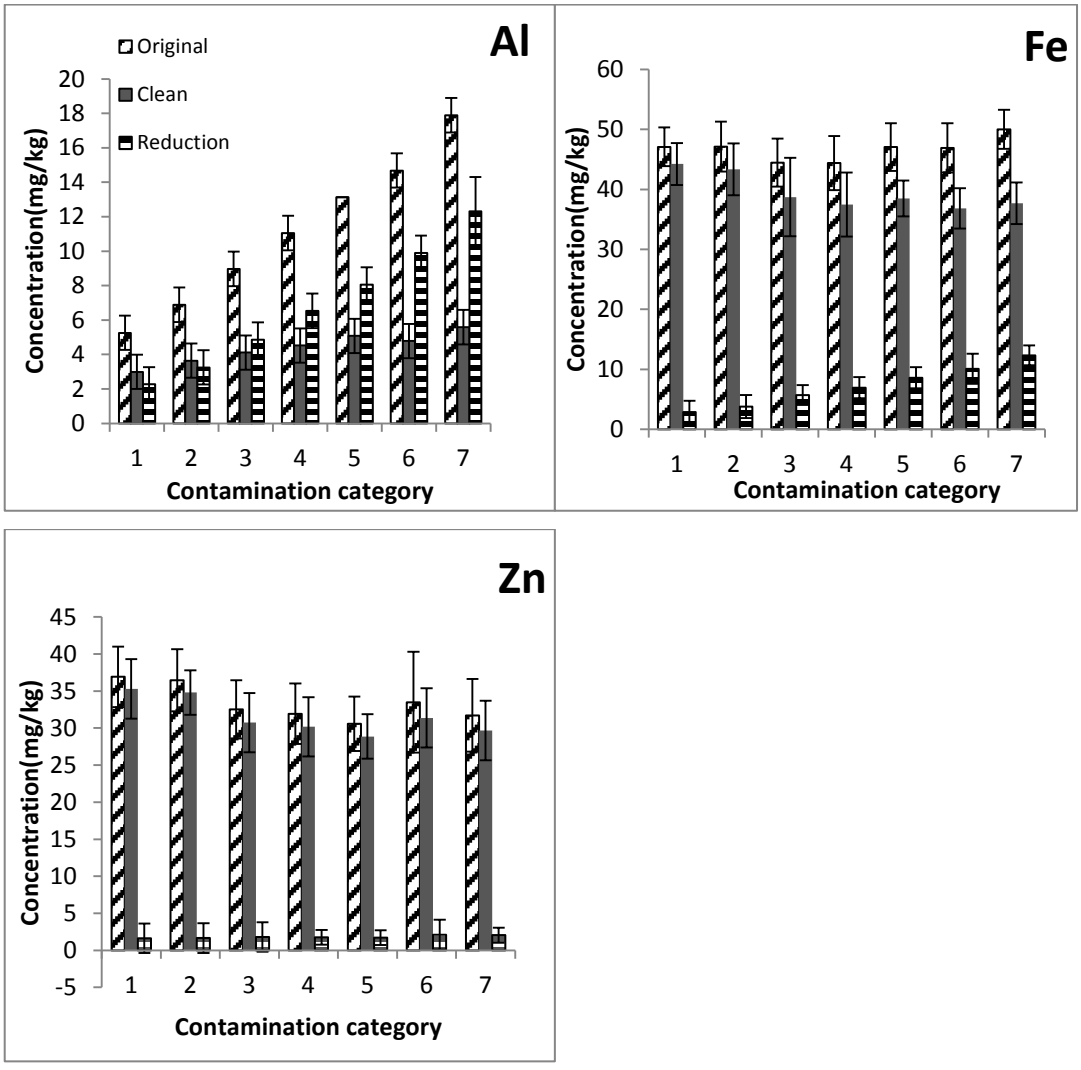


Fig 3.3: Average changes in concentration of Al, Fe, and Zn in different categories of 240 contaminated wheat lines before and after cleaning.

Table 3.6 QTLs for Fe concentration (% of dry weight) measured on the dirty *versus* clean grain of the Berkut/Krichauff doubled-haploid population grown at Roseworthy in 2009.

QTL	Chromosome	Position (cM)	Flanking markers	LOD score	Percentage of variance (%)	Additive effect* (ppm)
QGFe.ta-2B (dirty)	2B	116	cfa2278 - gwm55	3.6	10.6	-1.1
QGFe.ta-2B (clean)	2B	112	cfa2278 - gwm55	3.4	8.2	-1.0
QGFe.ta-3B (clean)	3B	63	wPt-4209 - wPt-5390	3.4	7.9	0.9
QGFe.ta-3B (dirty)	3B	63	wPt-4209 - wPt-5390	1.1	2.7	0.6

*A positive effect indicates that the allele from Berkut contributes to higher grain Fe levels, while a negative effect indicates that the allele from Krichauff contributes to higher grain Fe levels

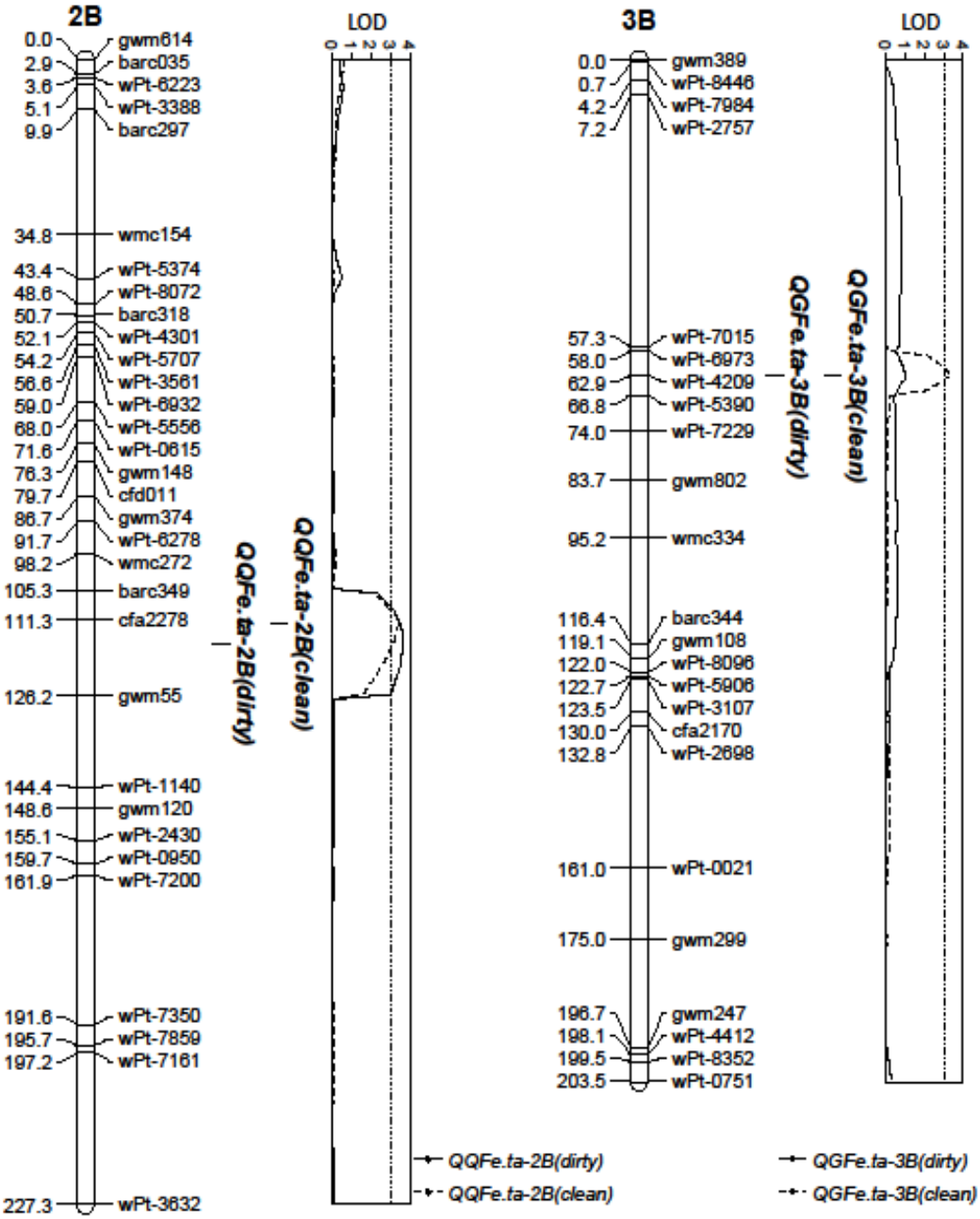


Fig. 3.4 Chromosomal locations and LOD scores of Fe QTLs in Berkut x Krichauff (2009) before and after cleaning.

3.4: Discussion

This chapter has provided information about how to clean grain to ensure that soil contamination does not confound Zn and Fe analysis, and provided information about the levels of Zn and Fe contamination that can be expected at given levels of Al contamination.

Results from the milling experiment indicated that 5 s milling removed a large proportion of the Al and Fe that can be removed by milling, with Zn levels only slightly affected (Table 3.2). These results are consistent with Al existing mostly on the outside of the grain, probably in dust stuck to grain hairs, with very low levels of Al in the seed coat or endosperm. Fe, in contrast, exists on both the outside of the grain in contamination and in the seed coat. Thus there is a sudden drop in both Al and Fe as soon as seed hairs and associated superficial contamination are removed by milling. After this initial effect, not much more Al is removed – remaining Al is probably located in cracks in the testa and the grain crease. With Fe, there is an initial drop in Fe values as contaminant Fe is removed, then steady removal of Fe with longer milling time as the seed coat, known to be relatively rich in Fe (Moore et al.,2012: Singh et al., 2012), is eroded. Photographs of seeds milled for 5 sec confirmed that most hairs are removed from seeds by the 5 s milling treatment. With Zn, results were quite different in that there was only a slight drop in Zn with 5 s milling, and then steady slight decreases with later time-points. This is consistent with Zn being present at low levels in soil (the global average is only 48 mg kg^{-1}, compared to 33,000 mg kg^{-1} Fe and 62,000 mg kg^{-1} Al (Rauch and Pacyna 2009). Furthermore, this finding is consistent with the fact that there is no particular localization of Zn to the seed coat, so erosion of the seed coat did not particularly decrease Zn levels.

It was important to include ‘clean’ samples in this study, as a way of checking how much ‘plant’ micronutrient may be removed by cleaning. Thus two ‘clean’ genotypes were also included in the milling time study. Data indicated that while milling of ‘clean’ seeds did remove some Al, Fe and Zn, the amount removed was only around 1 mg kg^{-1} for Al and Fe, and 0.3 mg kg^{-1} for Zn. It is likely that the slight decrease in elemental concentrations was caused in part by the removal of low

level contamination from the ‘clean’ lines: the lines probably did still have some dust on them which was removed by milling. It is also possible that the slight decrease was caused by removal of hairs and the slight erosion of the seed coat. It is significant, however, that the removal occurred at < 1 mg kg^{-1} or < 1%. Thus it appears that removal of actual ‘seed’ micronutrient (e.g., in seed hairs) only occurs at a maximum of 1 $mgkg^{-1}$ for Fe and Al, and at a maximum of 0.3 mg kg^{-1} for Zn.

It is also noteworthy that, among all the lines milled (clean and contaminated), elemental concentrations never increased with milling treatment; therefore it does appear that the modified, by contrast with original mill, can complete the milling process without introducing any contamination itself.

Having established that 5 sec milling was the most suitable milling time, the next step in this study was to apply the milling method to contaminated lines – firstly on 30 highly contaminated Australian lines, then on 240 highly contaminated lines from Pakistan.

The cleaning method was highly successful in reducing contamination, with Al in the range 5-10 mg kg^{-1} generally cleaned to the point where Al was 2-3 mg kg^{-1} (Australian lines), and among Pakistani lines average Al reduced from around 10 $mgkg^{-1}$ to around 4 $mgkg^{-1}$. However, it is noted that the most highly contaminated lines (24% of the Pakistani collection) were not able to be brought back to the <5 mg kg^{-1} Al level used by Harvest Plus for acceptably clean. Thus the cleaning method could be considered suitable for bringing contaminated lines to a sufficiently clean state for analysis, but is not suitable for use on highly contaminated lines.

Given the likelihood that some of the contamination that remains on grain after milling for 5 sec could be present as fine dust on the surface of grain, one possibility to improve the cleaning method would be to briefly wipe seed with a damp cloth after milling. However, removal of contamination from the crease would be more difficult due to inaccessibility to cleaning methods, in which case it may not be practically possible to ever bring highly contaminated lines to the 5 mg kg^{-1} level.

One particular result was that the ratio between Al concentration removed and Fe concentration removed was fairly consistent at approximately 1:1, across two environments (Pakistan and Australia); that is similar concentration of Fe and Al were removed by cleaning. This confirms that, at levels of contamination that are often seen in the field (of, say, 5-10 mg kg^{-1} Al), practically significant concentration of Fe will also be present, inflating Fe values by a similar amount. This result was consistent with the finding that the ranking of Fe in lines changed markedly after cleaning; i.e., because levels of soil contamination vary in a set of field samples, then levels of contamination vary, and ranking of Fe concentrations in dirty samples becomes confounded. Thus, it is clear that cleaning is important for accurate Fe analysis.

A second very interesting result of this chapter was that the ratio between decreased Zn concentration and decreased Al concentration was fairly consistent at approx. 0.2:1, across two environments; i.e., that only about $1/5^{th}$ as much Zn was removed as Al (and Fe). This indicates that soil contamination actually does much affect grain Zn levels – for example a line with soil contamination at 10 mg/kg probably only has Zn levels increased by the soil at around 2 mg kg^{-1}. Similarly, the ranking of lines did not change much after cleaning – those which were highly ranked in the contaminated set were also highly ranked in the cleaned set.

The finding that Fe values were markedly affected by soil contamination clearly indicates that any program targeting Fe should aim to have clean seed. However, the finding that the relationship between Fe levels removed by cleaning, and Al levels removed by cleaning, seem to be similar (approximate 1:1 ratio) raises the possibility that breeders could still conduct screens on soil contaminated material, and simply adjust Fe concentrations down by the approximate concentration of Al in the sample; e.g., if ICP-OES results indicate Al to be present at 8 mg kg^{-1}, breeders would know that Fe values are probably inflated by a similar amount. Thus line rankings could be made on this corrected amount and the screen may still be worthwhile. However, it would still be recommended that breeders aim to keep lines as clean as possible and also validate that Al and Fe concentrations from their particular environment to show the same relationship.

The findings of this chapter for Zn are different from for Fe in the sense that breeders probably do not need to be too concerned that low level soil contamination is altering Zn values. Instead of insisting that all samples in Zn biofortificatiion programs contain < 5 mg kg^{-1} Al, breeders may instead tolerate higher levels of Al, and know that the Zn concentration rankings of their lines are not greatly affected by the soil, and that absolute Zn levels are probably only inflated by approximately a few mg kg^{-1}g (e.g., 1-3 mgkg^{-1}) by the soil contamination. Alternatively, breeders may attempt to correct for Zn contamination by adjusting the values down by $1/5^{th}$ the value of Al concentrations. This study showed that while Fe concentration and ranking is greatly affected by soil contamination, Zn concentration and ranking is not.

The findings of this study also provided evidence to show that cleaning seed prior to analysis improves the precision of Fe QTL. Seed cleaning either narrowed the QTL peak (2B in QTL, Fig.3.4) or increased the probability of a QTL (i.e. the Chromosome 3B QTL in Fig 3.4). The method of cleaning seed is both cheap and easy to run and is worthwhile as it improves analytical outcomes.

Conclusion

The work of this chapter we showed that while Fe concentration and ranking is greatly affected by soil contamination, Zn concentration and ranking is not. One implication of this work is that the results of Chapter 2, in which high Zn genotypes from the field were not able to be reproduced in the greenhouse, were probably not a consequence of soil contamination. It is more likely that the inconsistent Zn results were a consequence of variable Zn levels in the grain itself.

Chapter 4: XRF analysis of grain – further validation and use in genetic mapping of grain Zn concentration

4.1: Introduction

For several decades now, the main method used for micronutrient analysis in biofortification programs, and studies aiming to map grain micronutrient content (for example, Genc et al.,2009), has been inductively coupled plasma-optical emission spectrometry (ICP-OES; Zarcinas et al., 1987). Highly trained analysts, expensive equipment, sample grinding and costly, contamination free reagents are required for this method of analysis (Paltridge et al., 2012). In biofortification programs, most breeders cannot perform analysis 'in house', especially where research is being conducted in less-developed countries. As a result, they have to send their samples overseas for analysis (Velu et al., 2011). The need to send samples abroad, and the cost of analysis, has been a major constraint to progress in biofortification and genetic mapping studies.

X-ray fluorescence analysis (XRF) is a technique which has been used for elemental analysis for several decades. The basis of X-ray fluorescence (EDXRF) technique is that all elements emit secondary 'fluorescent' X-rays of characteristic energy, upon exposure to X-rays of higher energy, which provide information about both elemental composition and concentration. Initially, the technique was mostly applied in geochemistry and manufacturing (Arai 2006; West et al., 2009; West et al.2010). Generally, higher energy is required to obtain fluorescence with higher energy and easier to detect the fluorescence. XRF has been shown to be a reliable method for the analysis of Fe, Zn and Cu (Paltridge et al., 2012a, b)

The use of energy dispersive X-ray fluorescence (EDXRF) for macro and micronutrient analysis of plant tissue has increased in recent years (Kaymak et al. 2010. XRF has been used for elemental analysis of different ground food and drink materials such as beverages (Nielson et al., 1988), spices (Al- Bataina et al., 2003), milk based products (Perring et al., 2005; Andrey, 2003), tea (Salvador et al., 2002),

potato starch (Noda et al., 2006), medicinal plants (Queralt et al., 2005) and bread improvers (Ekinci et al., 2002). Samples are typically dried, and are analyzed without digestion in corrosive and toxic acids (Noda et al., 2006).

EDXRF can also be used on whole grain samples of rice and pearl millet (Paltridge et al. 2012a) and wheat (Paltridge et al. 2012b) and it was concluded the technology to be both convenient and economical for screening Fe and Zn concentration in rice, pearl millet and wheat biofortification programs. However, in the study by Paltridge et al. (2012), the validation on wheat was only conducted on 50 wheat lines, all from the same environment.

The first aim of this chapter was to extend the validation of Paltridge et al. (2012) to include many more lines, from different environments, to better validate the method. Secondly, given that the expense of micronutrient analysis is a major constraint to mapping micronutrient density traits, this chapter explores the suitability of XRF data for use in mapping, comparing mapping results obtained with both XRF and ICP-based data.

4.2: Materials and Methods

The wheat lines used in this chapter included the same 156 Berkut x Krichauff doubled haploid lines used in chapter 2. These lines had been grown in the field of Roseworthy Agricultural College in year 2007. Additional lines used in the study included 200 ‘high performance advanced nursery’ (HPAN) wheat lines from the wheat biofortification program from CIMMYT, Mexico.

All seed samples were analyzed for Zn by ICP-OES using nitric acid /perchloric digestion method, at Waite Analytical Services, Adelaide, Australia (Wheal et al., 2011).

To check how EDXRF data compare to ICP data, all lines were also analyzed by XRF, on an Oxford Instrument X-Supreme 8000 fitted with a 10 place auto-sampler using the same measurement conditions (Table 4.1) used by Paltridge et al. (2012b). Total analysis time was 186 s, including 60 s acquisition times as well as 66 s ‘dead time’. Scans conducted in sample cups assembled from 21mm diameter Al cups combined with polypropylene inner cups sealed at one end with 4µm Poly-4 XRF

sample film. Cups containing samples were gently shaken to distribute grains evenly.

Table 4.1 EDXRF conditions used for the analysis of Zn (Paltridge et al., 2012 b)

Conditions	Zn
Atmosphere	Air
X-ray tube	Tungsten
Voltage	26 kV
Current	115 μA
Peak detected	Kα
Acquisition time	60 s
Tube filter	W5
Detector	Silicon Drift Detector
Sample mass	4 g

To investigate whether similar results are obtained when mapping using ICP and XRF data, respective ICP and XRF data sets were submitted to Window QTL Cartograph V2.5 (Wang et al., 2005), revealing whether QTLs identified by the two approaches were similar. QTL mapping was performed as described by Nguyen et al (2011). For QTL mapping, the linkage map comprised 528 markers, covering 21 wheat chromosomes (Genc et al., 2010; Huynh et al., 2008b). Mapping was conducted using QTLNetwork (Yang et al., 2008) developed, which was based on the mixed linear composite interval mapping method (Yang et al., 2007).

4.3: Results

Validation

Validation data for XRF versus ICP was obtained from two separate, large scale populations: the 156 DH lines grown at Roseworthy in 2007 Fig (4.1) and 200 high Zn breeding program lines from CIMMYT, Mexico. The range of ICP-Zn

concentrations in Berkut x Krichauff was from 16.7 to 46.2mgkg^{-1}, with a mean value of 25mg kg^{-1}(Table 4.2). Correlations were observed between ICP- and XRF-determined Zn concentrations (Fig 4.1), with R^2 = 0.67. Standard errors of prediction (SEP) were 3.3 mg kg^{-1}. Ninety-five percent confidence intervals, calculated from SEP, indicated that wheat XRF results can be expected to be within 6.5 mg/kg of ICP-OES-Zn. Bias was calculated by subtracting each XRF value from corresponding ICP-OES values and averaging these differences (Table 4.2), and indicated XRF values were, on average, 1.9 mg/kg low.

Table 4.2: Data for Berkut x Krichauff validation population. Values are in mgkg^{-1}, except for R^2, the coefficient of determination.

Method	Statistics	Zn
ICP-OES	Range	16.7-46.2
	Mean	25
EDXRF	R^2	0.67
	SEP	3.3
	Bias	-1.9

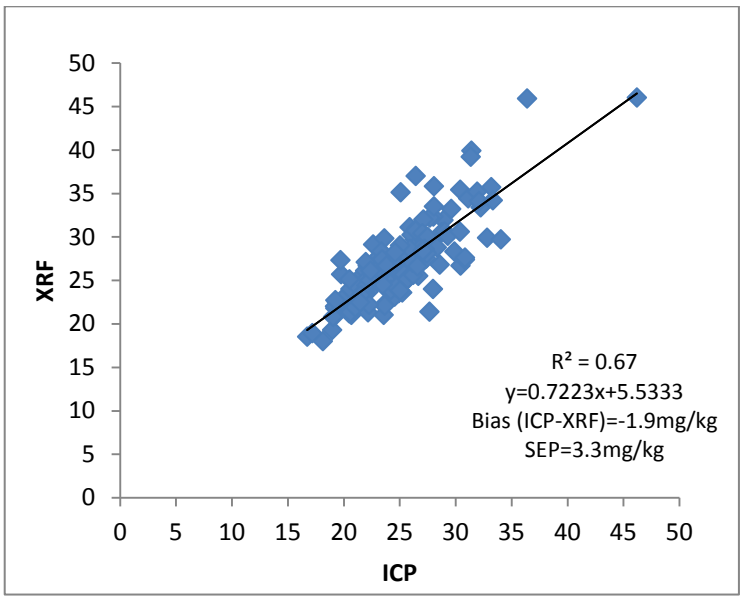


Fig 4.1 Correlation between ICP- and XRF-determined Zn concentration in wheat population of B x K 2007

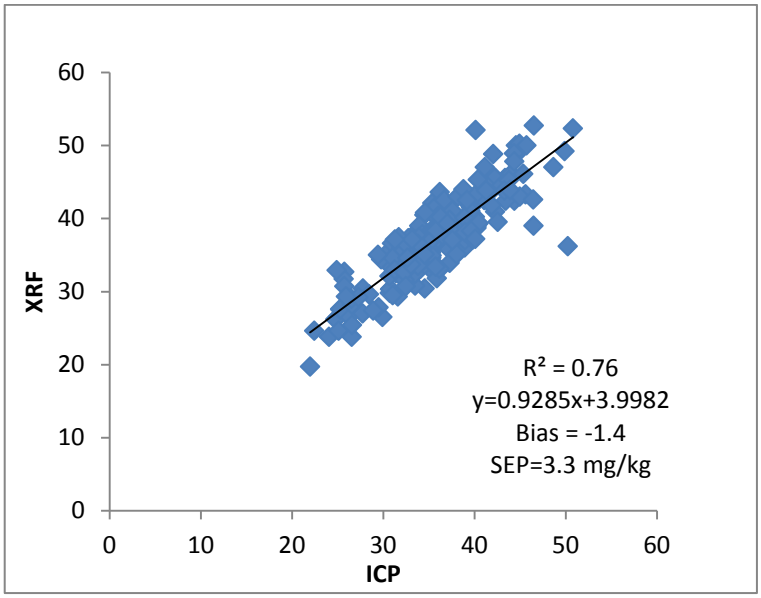


Fig 4.2 Correlation between ICP- and XRF-determined Zn concentration in HPAN wheat population

Table 4.3: Validation data of 200 HPAN lines of CIMMYT populations of year 2010. Values are in $mgkg^{-1}$, except r^2 which is percentage

Method	Statistics	
ICP-OES	Range	21.9.-50.8
	Mean	35.9
	SD	5.6
EDXRF	R^2	0.76
	SEP	3.3
	Bias	-1.43

In the second validation set, HPAN population of CIMMYT Mexico, grown in the year 2010, the range of Zn concentrations were from 21.9 to 50.8.3 in HPAN with mean value of 35.9$mgkg^{-1}$ (Table 4.3). Similarly, correlations were observed

between ICP- and XRF-determined Zn concentrations (Fig.4.2), with R^2 0.76 in HPAN. Bias values for HPAN population was -1.43 (Table 4.3).

Mapping

The second aim of this study was to search for QTLs in a population using both ICP and XRF –i.e. can XRF detect QTLs as efficiently as ICP? Data showed that when the mapping population, Berkut x Krichauff (2007) was analyzed with ICP, two Zn QTLs were found on chromosomes 5B and 5D (Fig 4.3). First QTL identified on chromosome 5B had LOD score of 5.1, flanked by closely linked markers (wmc289 – Pt-3030) with 13.1 % of variance explained(Table 4.4) and second QTL on 5D chromosome had LOD score of 4.8 flanked by closely linked markers (wmc215 - cfd007) with 12.4% of variance explained, (Table 4.4, Fig.4.3). Later on, when

the same mapping population was analyzed with XRF for QTLs, again the same two Zn QTLs were identified on the same chromosomes 5B with LOD score of 3.9, flanked by closely linked markers (wmc289 - Pt-3030) with 11.6 % of variance explained(Fig.4.4) and 5D with LOD score of 3.2 flanked by closely linked markers (wmc215 - cfd007) with 9.5% of variance explained(Fig.4.5). Results showed that both analytical methods, ICP-OES and XRF identified same QTLs on the same chromosomes 5B and 5D but became weaker in XRF (Table 4.5). Although, XRF produced weaker results than ICP, but is still highly suitable for mapping because it is so cheap and easy. If we want to have better precision then a second replication of XRF will be helpful.

Table 4.4 QTL detected in the Berkut x Krichauff population (grain samples from the 2007 growing season ICP screening)

QTL	Chromosome	Position (cM)	Flanking markers	LOD	% of variance explained	Additive effect (ppm)	Favorable allele
ICP-OES-Zn	5B	85	wmc289 – Pt-3030	5.1	13.1	-1.51	Krichauff
ICP-OES-Zn	5D	76	wmc215 - cfd007	4.8	12.4	-1.48	Krichauff

Table 4.5: QTL detected in the Berkut x Krichauff population (grain samples from the 2007 growing season, EDX screening)

QTL	Chromosome	Position (cM)	Flanking markers	LOD	% of variance explained	Additive effect (ppm)	Favorable allele
EDX-Zn-Dirty	5B	84	wmc289 – Pt-3030	3.9	11.6	-1.60	Krichauff
EDX-Zn-Dirty	5D	77	wmc215- cfd007	3.2	9.5	-1.46	Krichauff

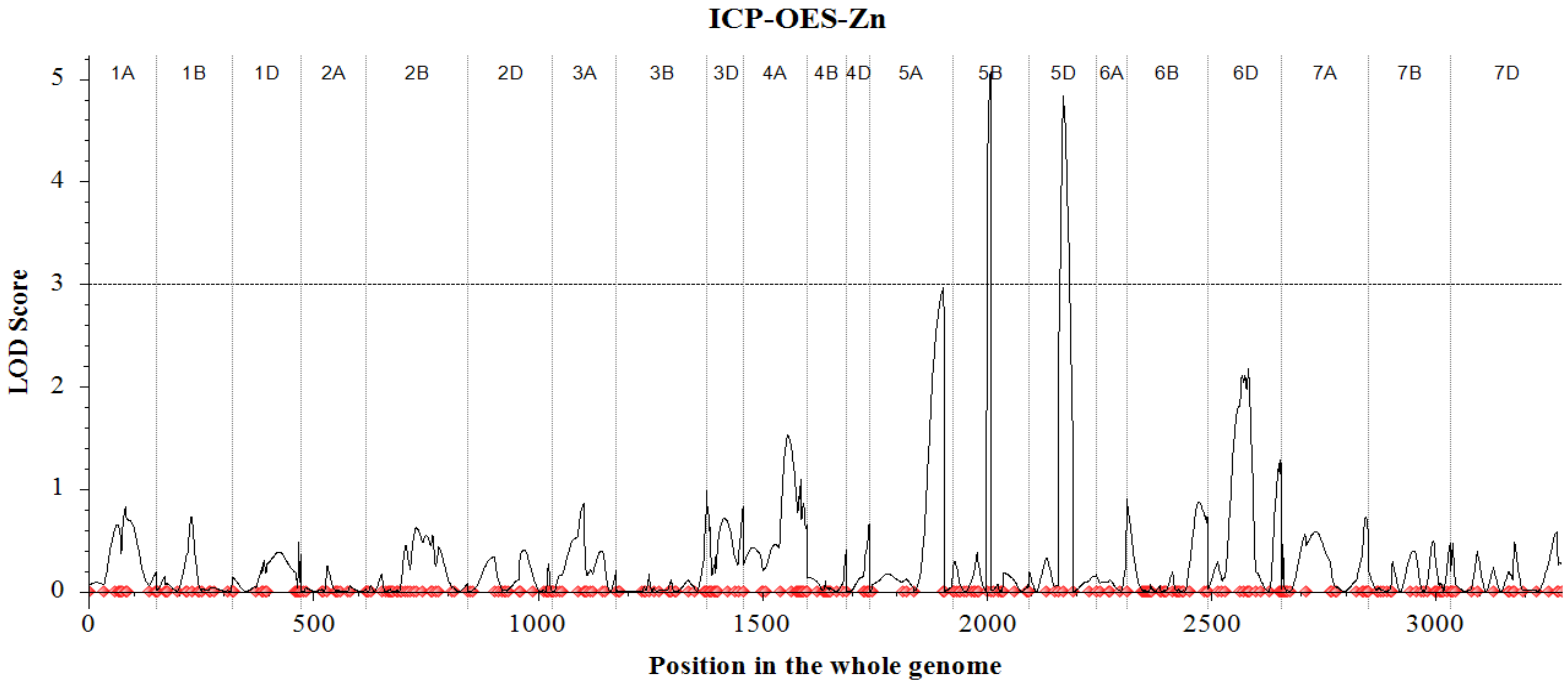


Fig. 4.3.:Chromosomal location and LOD scores of Zn QTLs in Berkut x Krichauff (2007) in ICP screening

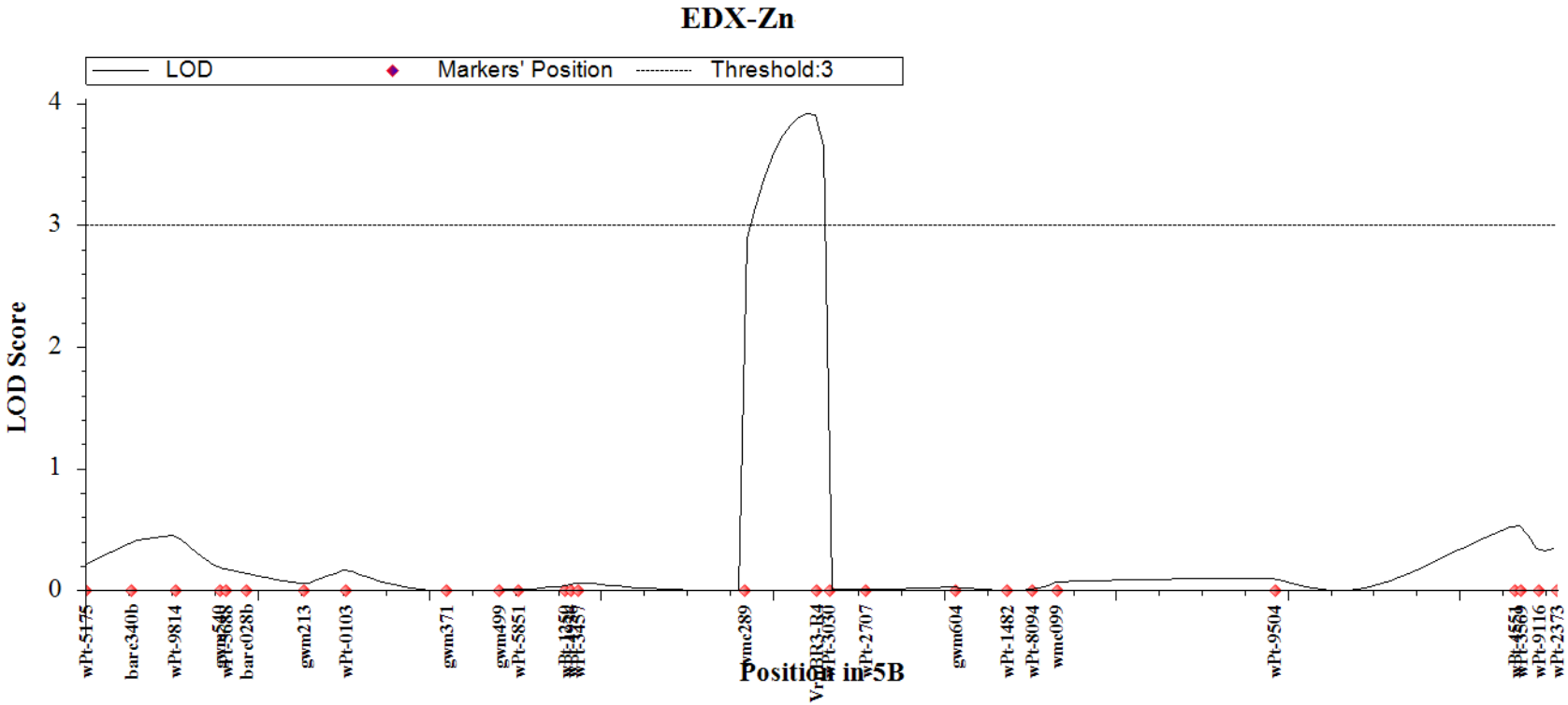


Fig.4.4Chromosomal location of QTL on 5B chromosome for Zn in Bx K 2007 in XRF screening

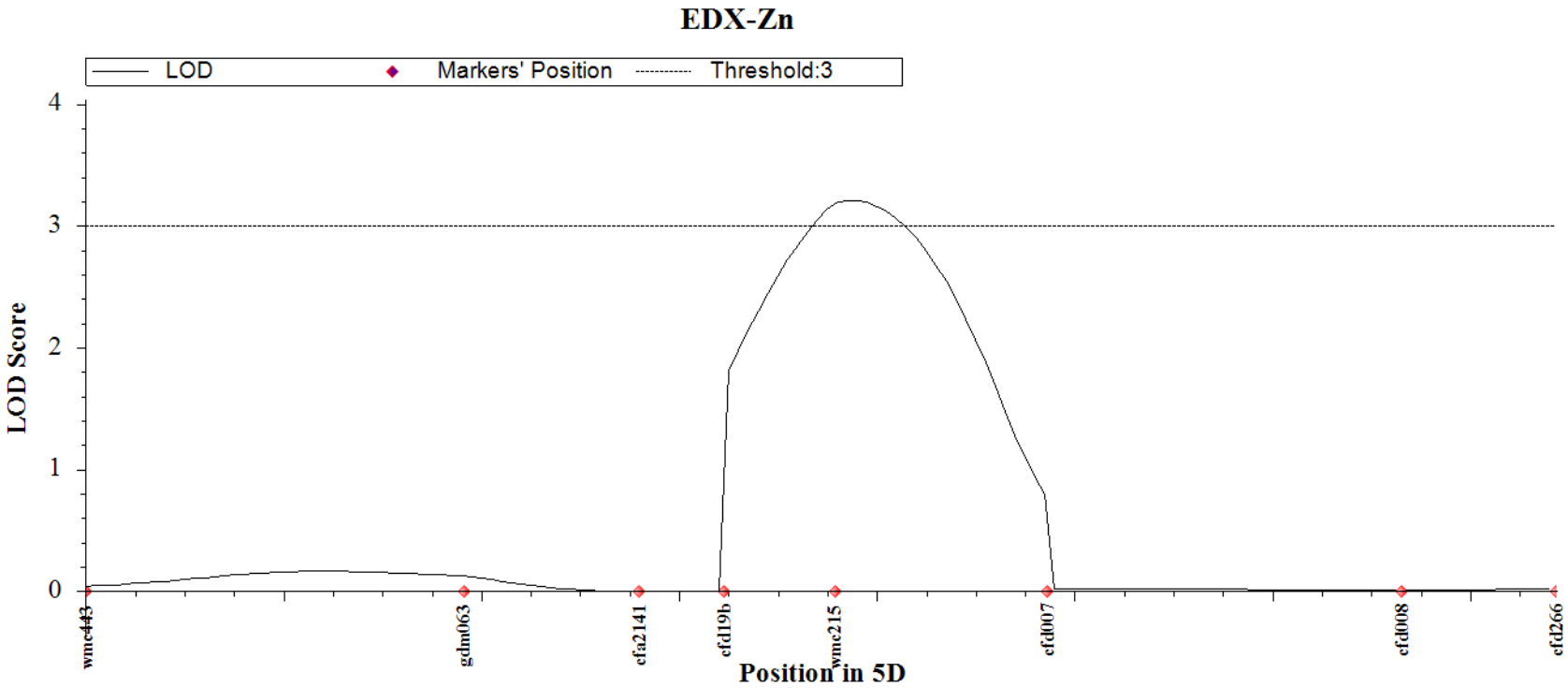


Fig.4.5 Chromosomal location of QTL on chromosome 5D for Zn in Bx K 2007 in XRF screening

4.4: Discussion

This chapter provides important new information on the performance of XRF in additional populations of wheat grain, and on the utility of XRF for mapping Zn density loci in wheat.

In the validation sets of this study, correlations were observed between ICP and XRF concentration for Zn in wheat, with R2 values in all populations in the range 0.65-0.75. Standard errors were 3.3 mg/kg in both validation sets, equating to 95% CI for predictions of 6.5 mg/kg, and bias was minimal (<2 mg/kg). These performance statistics were not as good as were seen by Paltridge et al (2012b), in which SEP was 2.2 mg/kg for Zn. There could be several reasons for the difference. Firstly, Paltridge et al 2012b used duplicate ICP values as reference values, whereas in this study, due to the funding constraints, only single ICP analyses were performed. Thus some of the error contributing to the higher SEP was probably ICP error. [Indeed, Paltridge et al. (2012b) did show that the standard deviations of ICP Zn values are about 1.3 mg/kg.] Secondly, in this study scanning time for XRF was 30 sec. In contrast, Paltridge et al (2012b) used 1 min scanning time. Paltridge et al (2012b) did suggest that reducing scanning time from 60 sec to 30 sec would increase error statistics by ~0.4 mg/kg. Thus it was not surprising that the SEP here was ~1 mg/kg higher than in the study by Paltridge et al (2012b). Similarly the r^2 values seen here were not as high as in the study by Paltridge et al. (2012b), likely because of the higher SEP and the fact that the Zn populations here showed a smaller concentration range.

As presented here, XRF can still be recommended for use as a screening tool, particularly in populations where there is a large difference between ‘high’ and ‘low’ Zn lines. For example, with the HPAN population from Mexico, samples range from 25 to 50 mg/kg. In this context the SEP of 3.3 is not great, relative to the range, and the 6.5 mg/kg confidence intervals (CI) for predictions can still allow highs and lows to be separated.

It was not so clear from validation results that XRF would be suitable for mapping, since the variation in Zn concentrations was much lower than is typically seen in biofortification programs as the great majority of lines were between 20 and 34 mg Zn/kg). Thus the concentration range was only about twice the size of the 95% CI for XRF predictions.

With ICP data from the Berkut x Krichauff population, two QTLs were identified on chromosomes 5B and 5D with LOD score 5.1 and 4.8. EDXRF data of same population was found the same QTLS with LOD scores 3.9 and 3.2.The finding that the same QTLs were identified by XRF as by ICP suggested that XRF can be used for mapping. Given the larger errors in XRF data compared to ICP data, it was not surprising that QTLs were weaker with the XRF data set than the ICP data set.

The EDXRF analytical approach has many advantages, which have been discussed (Paltridge et al 2012b). The first major advantage of EDXRF with is its low cost of $US 0.15 per sample as compared to ICP analysis cost, which ~$US 25 per sample. Therefore a lot of money can be saved in by analysing samples on an EDXRF machine instead of analysing by ICP-OES. Secondly, sample preparation and analysis is minimal. Thirdly, to conduct analysis by EDXRF, technicians can be trained in a very short time period because this technology requires less analytical skill. Thus, it is likely that EDXRF will allow increased focus in future on screening for high Zn lines and for mapping Zn density traits.

The present study chapter was focused on validating a high throughput XRF method for use in biofortification and mapping studies – hence the use of the 30 sec scanning time. However, as has been discussed by Paltridge et al. (2012b), more accurate wheat data could likely be obtained by scanning flour rather than grain (reducing the subsampling errors that come from scanning whole grains), and by scanning for longer. If the performance of XRF, as described here, is considered unsuitable for use in a given program, researchers would be recommended to either grind the grain or increase scanning time.

Compared to ICP-OES analysis, EDXRF has the disadvantage that the main element used as an indicator of soil contamination, Al, is too light to be detected by EDXRF.

However, given the finding of chapter 3 that soil contamination does not much influence Zn levels, means that this disadvantage of XRF should not be a major concern.

4.5 Conclusion

This chapter confirmed that EDXRF can provide an inexpensive tool for screening Zn concentration in wheat grains. Errors of analysis with XRF were, however, greater than reported by Paltridge et al. 2012b, probably due to errors in reference values and the shorter scanning time used here. Importantly, despite the greater errors of XRF results than ICP results, XRF results allowed the identification of the same number of QTLs as ICP, albeit with weaker LOD scores. Thus, provided a QTL is strong, it can still be identified with XRF. This may enable increased focus on mapping Zn density traits in future, given the economy and simplicity of XRF analysis.

Chapter 5 Further mapping of Zn density in Berkut x Krichauff, and in two additional mapping populations, Excalibur x Kukri and Kukri x RAC 875

5.1 Introduction

Given the difficulties encountered in chapter 2 in finding consistent variation in Zn phenotype in the Berkut x Krichauff mapping population, and the success of the pilot study investigating the use of XRF for mapping Zn density in Berkut x Krichauff, the priority for this thesis became increasing our understanding of the genetic basis of Zn density in wheat.

As indicated Chapter 1 (see 1.9, and Table 1.2), there have been four main studies that sought to map Zn density in wheat. These studies have been successful in identifying 15 different QTLs for Zn density in grain. However, two of these studies were done with wide crosses between wheat and wild relatives, and QTLs were mostly weak. More, stronger QTLs for high Zn density would be needed to assist breeders in their efforts to breed high Zn lines.

In the past the identification of QTLs has been constrained by the expense of ICP analysis. However, it was shown in chapter 4 that XRF can be used for mapping by a pilot study with Berkut x Krichauff lines from Roseworthy in 2007 showing Zn QTLs on 5B and 5D. It was concluded that future mapping work could be conducted with XRF analysis.

Seed from another year when Berkut x Krichauff DH lines were grown at Roseworthy in 2009 was available to this study, providing an opportunity to validate Berkut x Krichauff QTLs. In addition, two other doubled haploid wheat populations, Excalibur x Kukri and Kukri x RAC875, were available. The Excalibur x Kukri population has been studied by Fleury et al. (2010), who focused on drought tolerance, and by Edwards et al. (2011), who focused on the identification of QTL for sodium exclusion. The Kukri x RAC875 population has been used for genetic studies of heat and drought tolerance (Balouchi 2010, Bennett et al. 2012). However, neither of these populations had been assayed for grain Zn prior this this study.
The aims of this chapter were:

(i) to validate Berkut x Krichauff QTLs in another season

(ii) to screen for Zn QTLs in the additional double haploid mapping populations from crosses between Kukri x RAC 875, and Excalibur x Kukri.

5.2 Materials and Methods

The three mapping populations used to scan for QTLs affecting grain Zn concentration were Berkut x Krichauff RAC (2009), Excalibur x Kukri (2006), Kukri x RAC875 (2006).

Seeds from the Berkut x Krichauff population, grown at Roseworthy agricultural college during 2009, were provided by Hugh Wallwork, SARDI, Adelaide.

The variety Excalibur (RAC177/'Monoculm'//RAC311S) was released in 1991 by the University of Adelaide. It is a drought-adapted cultivar that gives good yields in wheat regions South Australia, but its grains are of low quality and are susceptible to rust (Izanloo et al., 2008). Zubaidi *et al.* (1999) proposed that the high root growth of Excalibur in severe conditions may be a contributing factor to its drought tolerance. Kukri is another variety released by the University of Adelaide in 1999 (pedigree 76ECN44/76ECN36//MADDEN/6*RAC177). It is hard white wheat which has excellent grain quality and is rust resistant, but its yield is low to moderate in low-rainfall environments (Izanloo et al., 2008). RAC875 (pedigree RAC655/3/Sr21/4*LANCE//4*BAYONET) is a breeding line from Roseworthy Agricultural Campus which has high grain quality and high yields in dry environments, but is susceptible to rust . The three cultivars Excalibur, Kukri and RAC875 all contain the *Rht2* semi-dwarfing gene and show similar phenology (3–5 d difference in heading time) under field conditions (Izanloo et al., 2008).

In order to provide new populations for mapping studies, SARDI generated DH mapping populations from Excalibur x Kukri and Kukri x RAC875 crosses, in addition to the Berkut x Krichauff mapping population introduced in Chapter 4. Seed of these populations, grown in a single replication at Roseworthy in 2006, was provided for this study by SARDI. Seed samples were analysed by duplicate XRF scans as described in chapter 4. Mapping was also carried out as described in chapter 4.

5.3 Results

Concentration ranges

The range of Zn concentration in seeds of Excalibur x Kukri was 15-33 mg/kg with a mean value of 23 $mgkg^{-1}$. The range of Zn concentrations in seeds of Kukri x RAC875 was 18-54 mg kg^{-1} with a mean of 25 mg kg^{-1}. In Berkut x Krichauff, Zn concentrations ranged from 18-40 $mgkg^{-1}$, with a mean value of 25$mgkg^{-1}$(Table 5.1). Fig 5.1 shows the frequency distribution of Zn concentrations for the three populations.

Table 5.1 Zn concentrations in grain of three mapping populations of wheat grown in the field grown at Roseworthy Agricultural College.

Population	mean	range
Berkut x Krichauff (2009)	25	18-40
Excalibur x Kukri (2006)	23	15-33
Kukri x RAC875 (2006)	25	18-54

Mapping results

In Berkut x Krichauff 2009, two QTLs were detected for grain Zn concentration (Table 5.2; Fig 5.2). One QTL was located on the long arm of chromosome 5D with LOD 3.9 and the nearest QTL markers cfd19b- cfd007; the second QTL was located on chromosome 7A (LOD 4.9) with the nearest markers (cfa2240) (Fig.5.3). For both QTLs, the favourable alleles were derived from Krichauff. These two QTLs on 5D and 7A had additive effects of 1.49 ppm and 1.61 ppm, explaining 10% and 12% of the variation respectively. Importantly, the two QTLs did not co-locate with grain size or flowering time. The QTL on 5D was the same as was found in 2007, but the QTL on 7A was not significant in 2007.

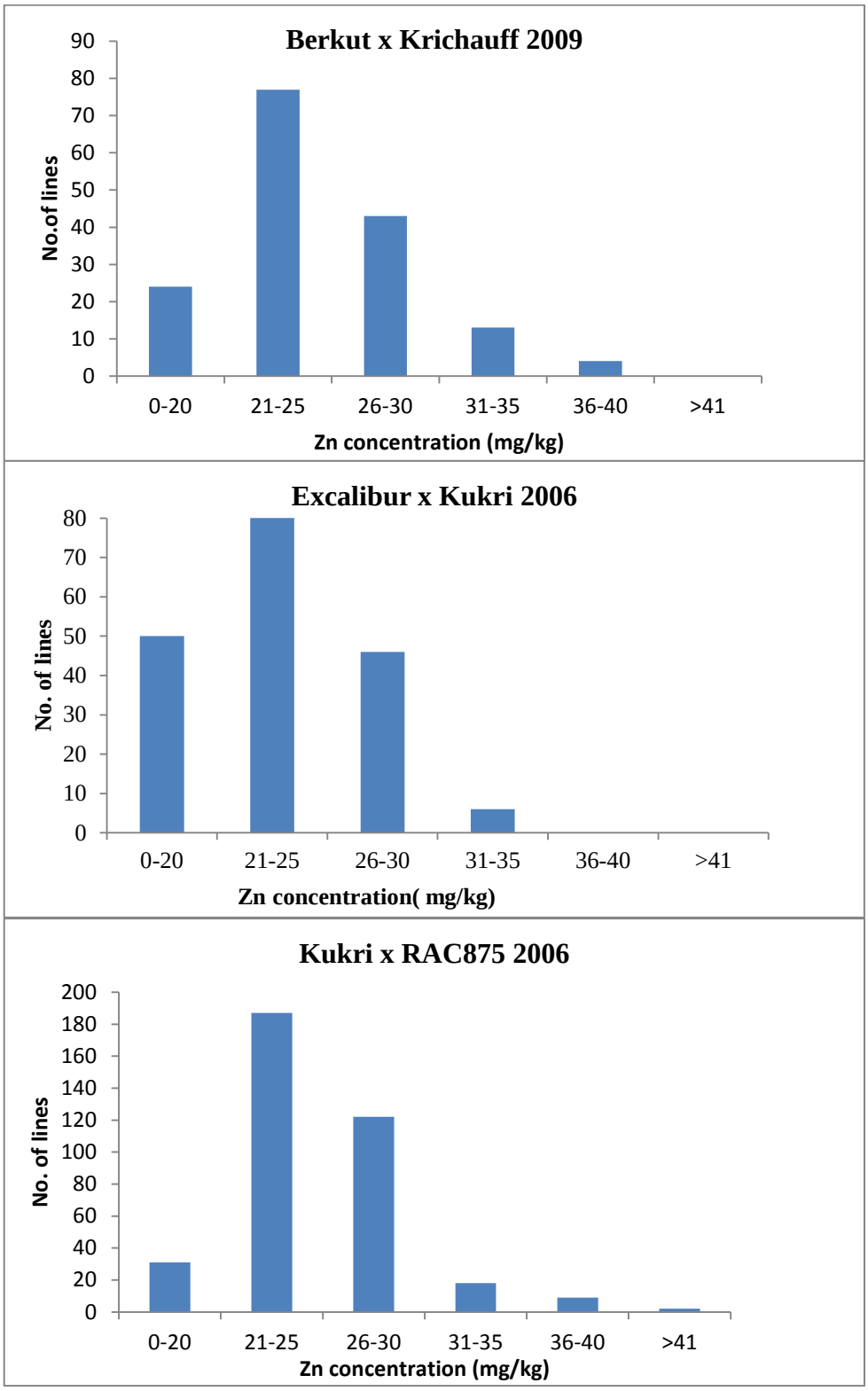


Fig 5.1 Frequency distribution graph for Zn concentration in three double haploid populations

Table 5.2 QTL detected for grain Zn in 3 mapping populations grown at Roseworthy Agricultural College in 2009 and 2006

Population	QTL marker	Chromosome	LOD	Variation explained (%)	Additive effect (ppm)	Favourable allele	Comments
Berkut /Krichauff 2009	cfd19b- cfd007	5D	3.9	10.4	-1.49	Krichauff	
	cfa2240 - gwm344b	7A	4.9	12.2	-1.61	Krichauff	
Excalibur/Kukri2006	wPt-5281	1B	3.1	10	1.24	Kukri	Locus for maturity and grain size
RAC875/Kukri2006	barc13	2B	9.3	11	1.4	RAC875	Locus for maturity and grain size
	barc95	2D	5.2	7	1.05	RAC875	
	cfa2028	7A	3.8	5	0.92	RAC875	

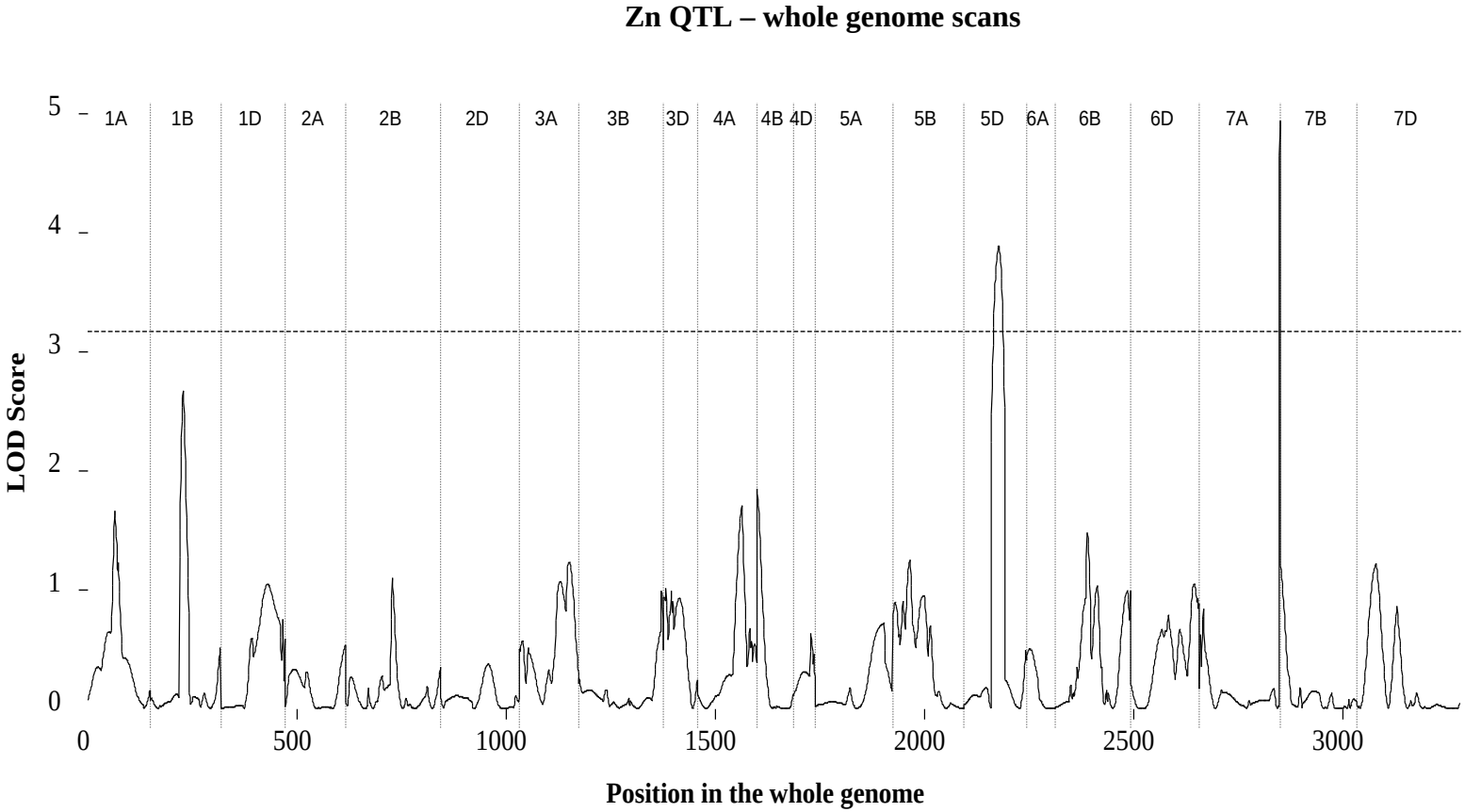


Fig.5.2 Chromosomal locations and LOD scores of Zn QTL in Berkut x Krichauff (2009)

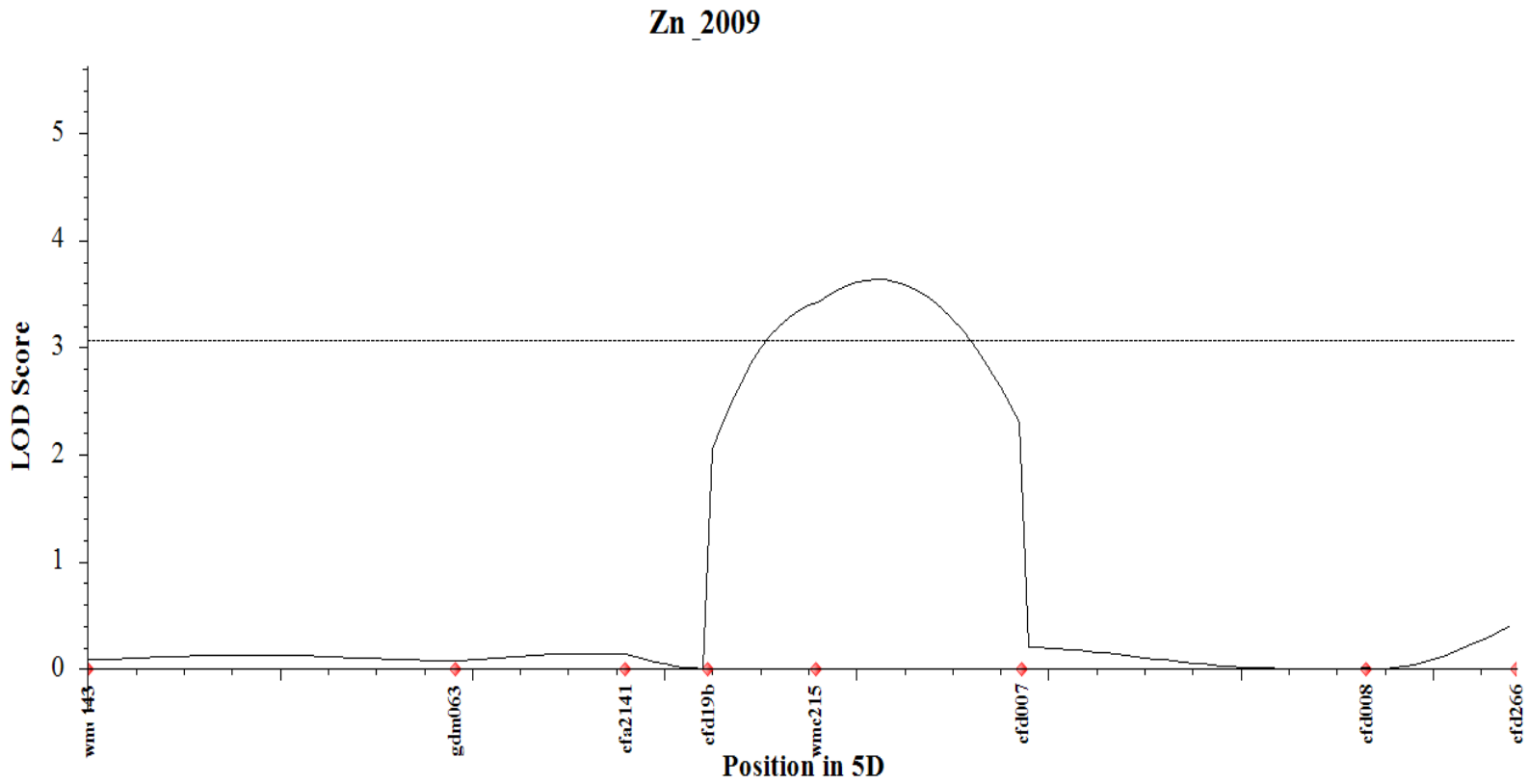


Fig 5.3 Chromosomal location of Zn QTLs on 5D in Berkut x Krichauff (2009)

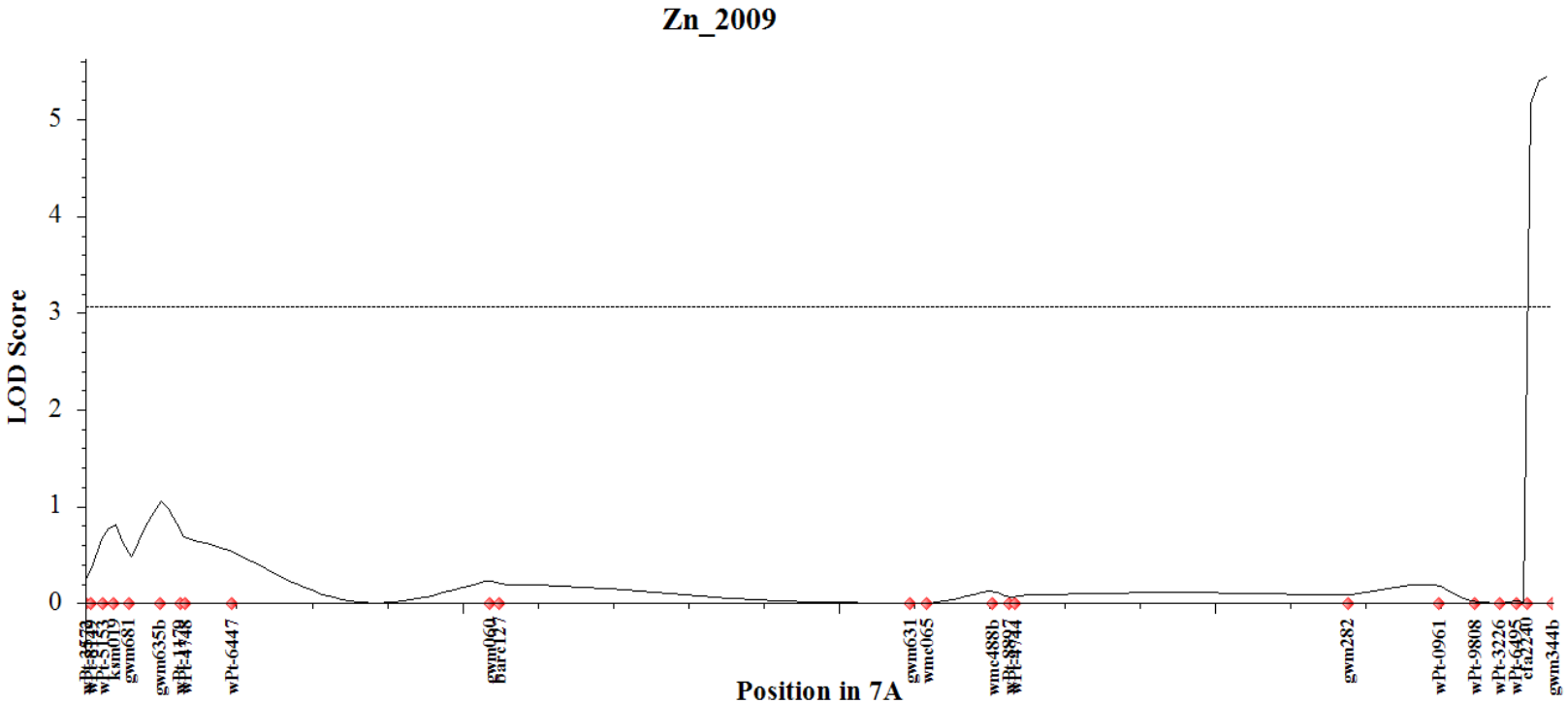


Fig 5.4 Chromosomal location of Zn QTLs on 7A in Berkut x Krichauff (2009)

In the Excalibur x Kukri population grown in Roseworthy in 2006, only 1 QTL was found, on chromosome 1B (LOD 3.1), with nearest marker Wpt-5281(Fig.5.5). The favourable allele came from Kukri. The additive effect of the QTL was 0.89 ppm and it explained 10% of variation. This QTL co-located with maturity and grain size (Table 5.2; Fig.5.5).

In the Kukri x RAC875 population, three QTLs were identified for grain Zn concentration (Table 5.2). The strongest QTL was located on chromosome 2B (LOD 9.3) with nearest QTL marker barc13 and an additive effect of 1.4 ppm, explaining 11% of phenotypic variation (Fig 5.6 and Table 5.2). A second QTL was present on chromosomes 2D (LOD 5.2), with nearest marker barc95 and an additive effect of 1.05 ppm explaining 7% of phenotypic variation (Fig.5.6). A third QTL was on 7A (LOD 3.8), with nearest marker cfa2028 and an additive effect of 0.92 ppm, explaining 5% of phenotypic variation. The favourable alleles all came from RAC 875. These QTLs all co-located with maturity and grain size (Table 5.2; Fig.5.6).

Zn QTL on 1B of the Excalibur x Kukri genetic map

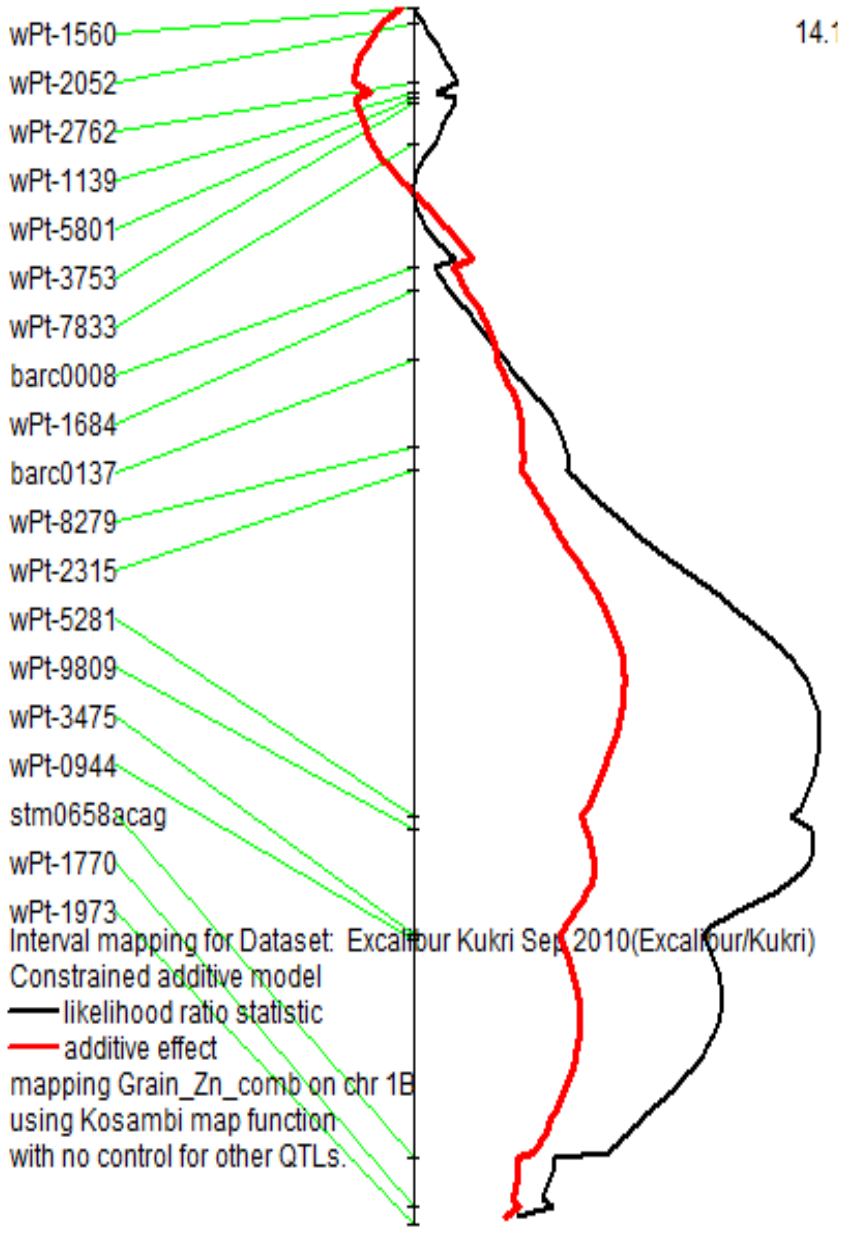


Fig 5.5: Chromosomal location of Zn QTLs in wheat population of Excalibur x Kukri

Zn QTL on 2B of the Kukri x RAC875 genetic map

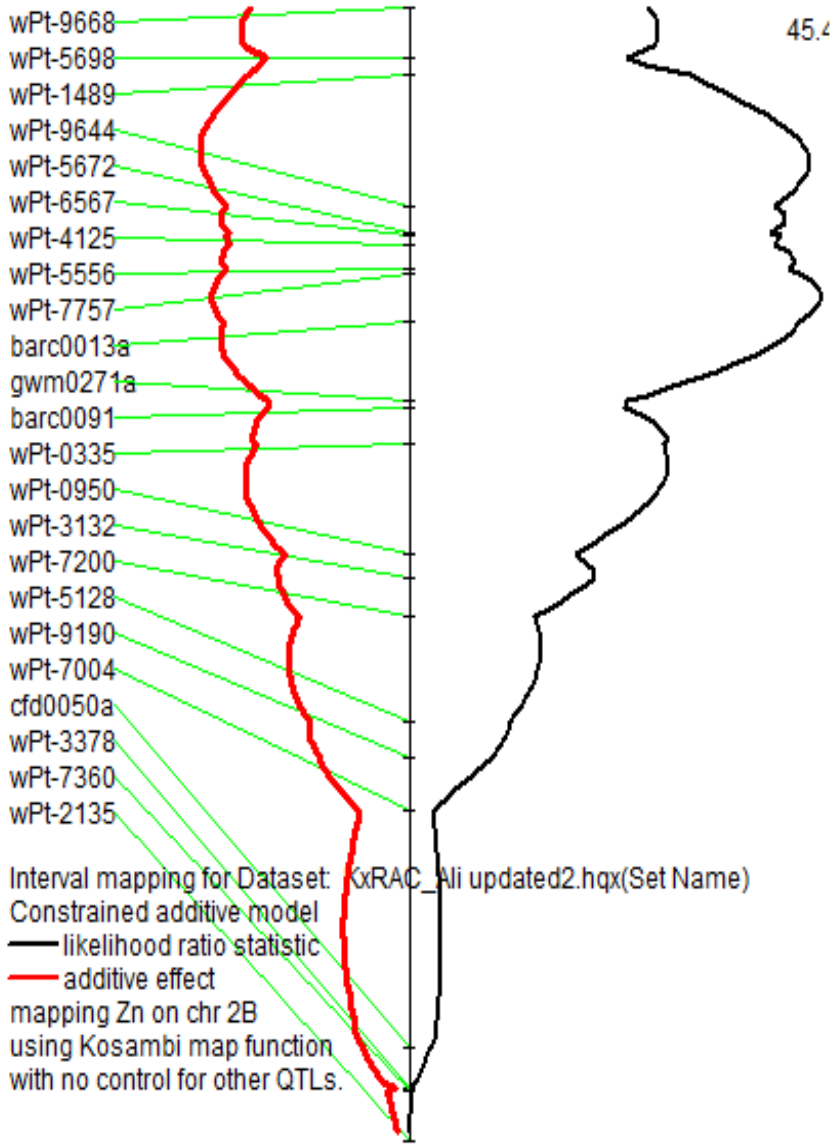


Fig 5.6 Chromosomal location of QTLs in wheat population (Kukri x RAC875)

Zn QTL on 7A of the Kukri x RAC875 genetic map

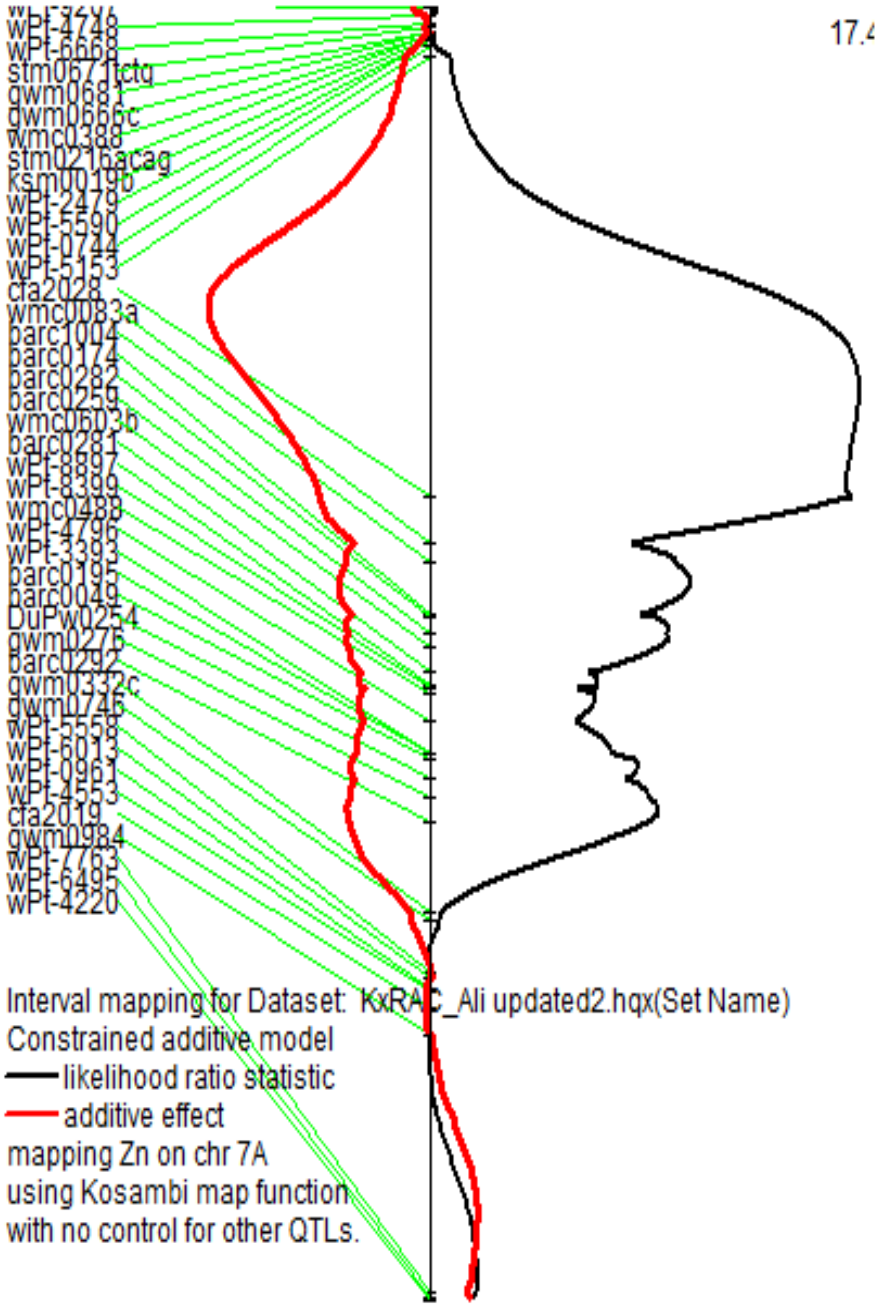


Fig 5.6 continued

Zn QTL on 2D of the Kukri x RAC875 genetic map

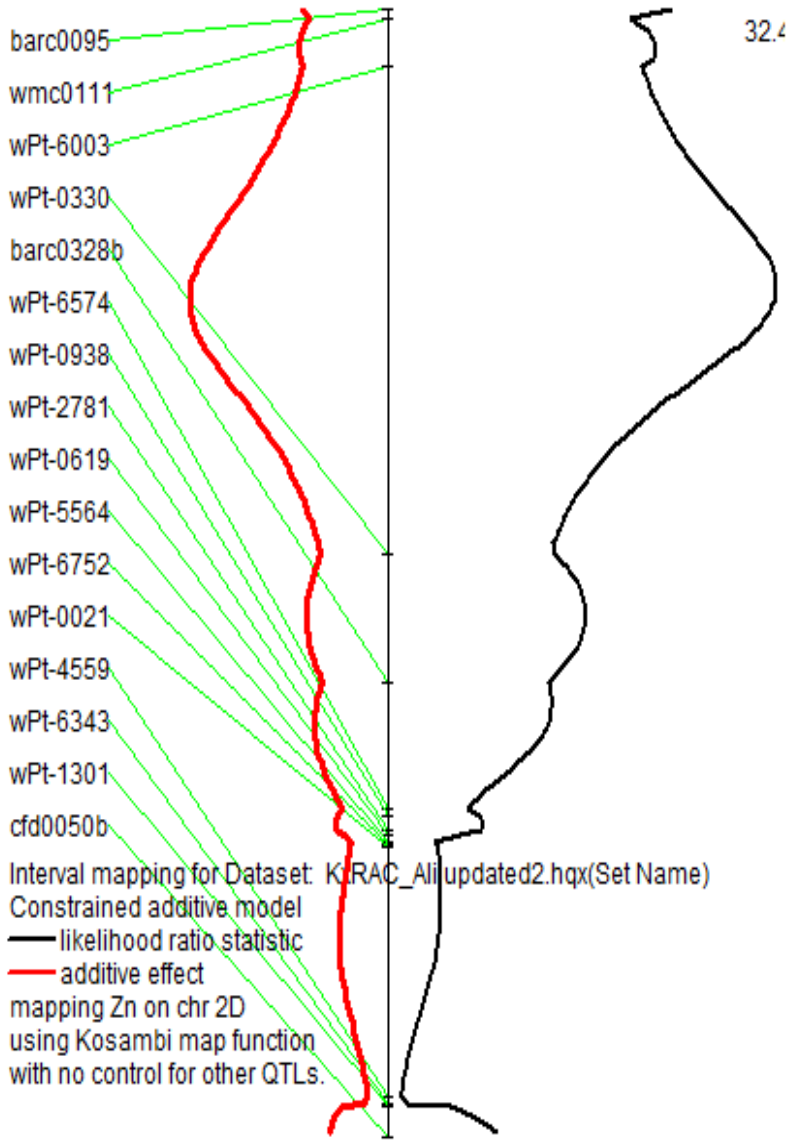


Fig 5.6 continued

5.4 Discussion

Parents

In plant tissues mineral nutrient concentration is dependent on genetic as well as environmental factors (Lickfett et al., 1999; Ernst and Nelissen, 2000; Vreugdenhil et al., 2004). So far a few studies have reported the genetics of Zn accumulation in wheat grains (Shi et al. 2008; Genc. et al. 2009; Tiwari et al.2009; Peleg et al. 2009). The parents of populations used by Shi et al. (2008) had Zn concentration of 34.4 (Hanxyuan 10) and 25.9 (Lumai 14) mg kg^{-1} (a difference of 8.5 mg/kg) and parental lines used by Genc. et al. (2008) had Zn concentration of 30.3 (RAC 875-2) and 33.9 (Cascades) mg kg^{-1} (3.6 mg kg^{-1} difference). The parents of the population used by Tiwari et al. (2009) had Zn of 44.6 (*Tb*5088) and 29.2 (*Tm*14087) mg kg^{-1} (15.4 mg kg^{-1} difference) and the parental lines used by Peleg et al. (2009) had Zn concentrations of 65.7 (Langdon LDN) and 75 (wild emmer wheat, accession # G-16) mg kg^{-1} (with 9.3 mg kg^{-1}). With these latter two studies, by Tiwari et al. (2009) and by Peleg et al. (2009), crosses were deliberately set up to include high Zn lines. All these parental lines had larger differences between parents whereas, parental lines used in this study were as follows: Berkut had 22.7 and Krichauff had 24.4 mg kg^{-1} (1.7 mg kg^{-1} difference); Excalibur had 25 and Kukri 20 mg kg^{-1} (5 mg kg^{-1} difference); and Kukri had 20 and RAC875 30 mg kg^{-1} (10 mg kg^{-1} difference) all in the same soil and season. Therefore, for Berkut x Krichauff and Excalibur x Kukri, the differences between parents in this study were less than is often seen in mapping studies, while the differences between Kukri and RAC875 were larger. It is not surprising that differences were not as large as is often seen as the populations used here were not designed originally for the specific purpose of mapping Zn density.

Segregating populations

The doubled haploid population studied by Shi et al (2008) had grain Zn concentration in the range of 25.9-50.5 mg kg^{-1} (a range of 25 mg kg^{-1}), whereas parents differed by 8.5 mg kg^{-1}; in the study by Genc et al (2009), Zn concentration ranged from 29.0 to 42.7 mg kg^{-1} (a range of 14 mg kg^{-1}), and the parents differed by

3.6 mg kg^{-1}; in the study reported by Tiwari et al (2009), Zn concentration ranged from 19.0-64.2 (a range of 45 mg kg^{-1}) and the parents differed by 15.4 mg/kg; and finally in the study by Peleg et al (2009), Zn concentration ranged from 48.5-114.7 mg kg^{-1} (a range of 66 mg kg^{-1}) even though the parents differed by only 9.3 mg kg^{-1}. In this study, the Berkut and Krichauff DH population showed 18-40 mg Zn/kg (a range of 22 mg kg^{-1}); Excalibur x Kukri showed 15 to 33 mg/kg (a range of 18 mg kg^{-1}) and Kukri x RAC875 showed 18 to 54 mg kg^{-1} (a range of 36 mg kg^{-1}). It is not surprising that the range was greater in the Kukri x RAC 875 population, since the range between parents was greatest with this cross. The range of values seen in Berkut x Krichauff and Excalibur x Kukri was similar to that seen in the studies by Shi et al (2008) and Genc. et al (2009), but less than was seen by Tiwari et al (2009) and Peleg et al (2009). The fact that ranges of Zn concentration in all three populations used in this study were much greater than the ranges between parents was indicative of transgressive segregation which is a major mechanism by which extreme or novel adaptations observed in new hybrid ecotypes or species are thought to arise (Rieseberg et al., 1999). QTL identification is most likely to occur in cases where there is large variation within a population. Thus, based on simple Zn concentration data, it appeared QTLs would be more easily identified in Excalibur x RAC875, and most difficult in Excalibur x Kukri, with Berkut x Krichauff mid-way between the two.

QTL analysis

A most important contribution of this chapter was the identification of a QTL in Berkut x Krichauff on chromosome 5D which (a) has not been described in publications previously (b) did not collocate with any phenotypic traits such as grain size or flowering time and (c) occurred in two different seasonal environments. The QTL explained 10% of the variation seen in Zn concentration in the Berkut x Krichauff cross, and the favourable allele from Krichauff contributed 1.5 mg Zn/kg. It is questionable whether the size of this contribution would be of practical use in breeding programs, and it is also noteworthy that QTLs on 5D were not identified in other populations drawn from other parental lines in this or other studies. Thus is it may be unlikely that the QTL could be expected to exist in the particular genetic background a breeder may want to use. Nonetheless, since the QTL is one of only a

few (15) so far identified in wheat, it could be a target for further study. The identification of gene(s) at the site of the QTL would provide new information on the genetic mechanisms controlling Zn density in wheat.

Another contribution of this chapter was the identification of a QTL on chromosome 7A in two populations (though not in Berkut x Krichauff 2007, see chapter 4), contributing some 5-12% of variation in Zn concentration. The fact the QTL was present in two different genetic backgrounds indicate the QTL to be robust. However, the QTL also collocates with grain size and flowering time in both Excalibur x Kukri and Kukri x RAC875 (Table 5.2). Our explanation for this is that in a dry land situation which is the environment these mapping populations were grown in, end of season drought leads to smaller grain in late flowering varieties, and higher Zn in these grains due to the concentration effect. Such a mechanism is highly unlikely to be of interest to Zn biofortification breeders, since small grains would normally be considered low quality, and lead to low yields. Interestingly, QTLs have also been described for Zn density on chromosome 7A in four published studies ((Shi et al. 2008; Genc. et al. 2009; Tiwari et al.2009; Peleg et al. 2009), but these authors do not identify that the QTLs collocate with grain size and flowering time. Thus an important contribution of this chapter is to identify that the mechanism by which four of the 16 published QTLs (all on 7A, but described separately by Shi et al. 2008; Genc. et al. 2009; Tiwari et al.2009 and Peleg et al. 2000) control Zn density is probably via flowering time and seed size – traits unlikely to be of interest to plant breeders involved in biofortification.

The other two QTLs identified in this chapter were on 2B (LOD score 9.3, explaining 11% of phenotypic variation) and 2D (LOD score 5.2, explaining 7% of phenotypic variation)), both in Kukri x RAC875. These loci have not been reported in the literature before. However, both these QTLs were also associated with unfortunate phenotypic traits (flowering time and grain size), and once adjustments were made for these factors the QTLs disappeared. Thus, similar to the above, they are unlikely to be of interest to breeders.

To improve chances of success in future mapping studies, it may be necessary to study other crosses ideally with greater variation in Zn density. Another possibility

to improve chances of success would be to conduct mapping derived from field trials with greater replication. All XRF scans were conducted in this study in duplicate; however if greater precision is sought, scans could also be conducted with more replication.

5.5 Summary

In this mapping chapter, important contributions were made via the identification of a new QTL for Zn density on chromosome 5D (Berkut x Krichauff only), and the determination that QTLs on 7A in various populations, studied here and in published studies, are associated with late flowering (and in environments with spring drought and smaller grain). Additional QTLs were found on 2B and 2D that were also associated with late flowering time. No QTLs were identified that appear likely to be useful in breeding programs, either because they were not robust across genetic backgrounds, or because of the association with unfortunate morphological traits.

Chapter 6 General Discussion

The main aim of this thesis was to investigate the genetic and agronomic basis of Zn accumulation in wheat. Progress was made in improving our understanding of the genetic basis of variation in Zn concentration but the efforts were only partially successful in identifying the agronomic basis of Zn accumulation. Progress was made in developing methods useful in the wheat biofortification sector.

6.1 Agronomic basis

The thesis began with an investigation into the agronomic basis of Zn accumulation in a population of doubled haploid genotypes from the cross, Berkut x Krichauff grown at Roseworthy in 2007. A hypothesis was proposed that Zn concentration is associated with grain yield and thousand grain weight and could results obtained from the field be reproduced in the greenhouse. A negative association was found between grain yield and Zn concentration which is undesirable but confirms earlier findings (Oury et al. (2006) ;Morgounov et al. (2007); Murphy et al. 2008; McDonald et al. 2008; Garvin et al. 2006; Ficco et al., 2009; Zhao et al. (2009). However, the strength of this association was strongly affected by environment (White and Broadley 2009). On the other hand, there was no relationship between Zn concentration and thousand grains weight (TGW) and this also confirms earlier findings (Morgounov et al., 2007; Velu, 2011; Gomez-Becerra et al. 2010). However, some studies have reported that there was no negative relationship between Zn concentration and grain yield (Graham et al. (1999; Welch and Graham 2004). Clearly breaking the negative association between yield and grain Zn must be a priority for wheat biofortification breeders. It appears that it may be hard to breed wheat with high grain yield and high Zn concentration (Yunfeng et al., 2010). An agronomic approach or use of Zn application is a fast method to increase the grain yield and grain Zn concentration in cultivated genotypes (Hussain et al., 2010). Zn fertilizers may be useful in breaking this negative association between Zn concentration and grain yield.

In an attempt to obtain more detailed information about the agronomic basis of Zn density, genotypes showing a range of grain Zn concentrations were selected and re-grown in the glasshouse and there was no association between Zn concentrations shown in field experiments and the greenhouse experiment (Chapter 2). This lack of correlation between these two very different environments was surprising, and meant that a detailed study of morphological differences between high and low Zn lines could not be conducted, since lines with reproducibly different Zn densities had not been found. The exception to this was DH 57, which did show the highest Zn density in both the field and the glasshouse. However, the basis of the high Zn concentration in DH 57 could not be elucidated, since none of the studied morphological characteristics was exceptional.

The finding that genotypes showing high Zn density in one environment did not show reproducibly high Zn density in a second (albeit very different) environment, does raise questions over how easy it will be to develop wheat genotypes with reliably high Zn density in a range of environments. In the future, breeders certainly need to study the Zn phenotype across a range of different environments, though perhaps not in pot studies. Another general lesson learned in this project was that genotypes under study should have high levels of difference in the Zn phenotype, to maximize the chances of identifying the agronomic basis of differences.
Another hypothesis was made that contamination of wheat samples by dust, masks genotype variation with respect to Zn. Contamination may have led to errors in Zn rankings, leading to a need to better understand the effects of contamination on Zn phenotype and to develop methods to deal with Zn contamination. Finally, the concern was raised that relying on just a single replication of Zn analysis, by the expensive ICP-OES method, may not have been sufficient to obtain good quality Zn concentration data.

6.2 Method development

A hypothesis was that Zn concentration can be measured by a non-destructive, less expensive technique; XRF. This hypothesis was verified and success was had in developing, validating and understanding methods that can be used to clean

contaminated lines, and quickly and cheaply analyze them for Zn phenotype by XRF.

The finding that 5 sec milling can be used to remove 60% of contamination from wheat grains will be of great interest to the biofortification community. Overall, the generalization can be made that most of the Al that can be removed by milling was removed after the first 5 sec, and that removal of Al after the first 5 sec was slight but steady. The cleaning method reduced contamination, with Al in the range 5-10 $mgkg^{-1}$ generally cleaned to the point where Al was 2-3 $mgkg^{-1}$ (Australian lines), and among highly contaminated Pakistani lines average Al reduced from around 10 $mgkg^{-1}$ to around 4 $mgkg^{-1}$.

The first most important finding of this study was that the ratio between Al removed and Fe removed was fairly consistent, approximately 1:1, across two environments; that is, similar amounts of Fe and Al were removed by cleaning, confirming that Al contamination inflates Fe values by the similar amounts. It means, the relationship between Fe levels removed by cleaning, and Al levels removed by cleaning, seems to be similar (approx. 1:1 ratio) which raises the possibility that breeders could still conduct screens on soil contaminated material, and simply adjust Fe concentrations down by the approximate concentration of Al in the sample; e.g., if ICP-OES results indicate Al to be present at 8 mg/kg, breeders would know that Fe values are probably inflated by a similar amount. Fe concentration and ranking is greatly affected by soil contamination (Table 3.5), which indicates that any biofortification program targeting Fe should aim to have clean seeds.
The second most important finding of this study was that cleaning method improves the precision of Fe QTLs and it increases the significance of a QTL.

The ratio between decrease in Zn concentration and decrease in Al concentration was fairly consistent at approx. 0.2:1, across two environments; i.e., that only about $1/5^{th}$ as much Zn was removed as Al (and Fe). This indicates that soil contamination actually does not much affect grain Zn levels and even Zn ranking is not affected by cleaning. The findings for Zn are different from for Fe in the sense that breeders probably do not need to be too concerned that low level soil contamination is

altering Zn values. Instead of insisting that all samples in Zn biofortification programs contain < 5 mg/kg Al, breeders may instead tolerate higher levels of Al, and know that the Zn concentration rankings of their lines are not greatly affected by the soil, and that absolute Zn levels are probably only inflated by approx. a few mg/kg (e.g., 1-3 $mgkg^{-1}$) by the soil contamination. Alternatively, breeders may attempt to correct for Zn contamination by adjusting the values down by $1/5^{th}$ the value of Al concentrations.

Due to lack of any association between field and greenhouse study, the project was focused on understanding of genetic basis of Zn accumulation and the major concern of this study was cost constraints because ICP-OES was the main method of analysis used for micronutrient analysis. Success was obtained in development of an alternate method for micronutrient analysis for screening purposes.

Chapter 4 confirmed that EDXRF can provide an inexpensive tool for screening Zn concentration in wheat grains. Errors of analysis with XRF were, however, greater than reported by Paltridge et al. (2012b) , probably due to errors in reference values and the shorter scanning time used here. Importantly, despite the greater errors in XRF results compared to those in ICP results, XRF results allowed the identification of the same number of QTLs as ICP, albeit with weaker LOD scores. Thus, provided a QTL is strong, it can still be identified with XRF. This may enable increased focus on mapping Zn density traits in future, given the economy and simplicity of XRF analysis.

6.3 Genetic basis

A major outcome of this thesis was the development of a method to interpret the genetics of Zn accumulation in three double haploid populations, and mapping studies were done with this method (Chapter 4). The differences in the Zn concentration of parents, Berkut and Krichauff, were less than is often seen in mapping studies, as indicated in chapter 5 (5.4), while the differences between Kukri and RAC875 were larger (5.4). It is not surprising that differences were not as large as is often seen as the populations used here were not designed originally for the specific purpose of mapping Zn density.

The fact that ranges of Zn concentration in all three populations used in this study were much greater than the ranges between parents was indicative of transgressive segregation. QTL identification is most likely to occur in cases where there is large variation within a population. Thus, based on simple Zn data, it appeared QTLs would be more easily identified in Excalibur x RAC875, and most difficult in Excalibur x Kukri, with Berkut x Krichauff mid-way between the two.

A contribution of this genetic study was the identification of a QTL in Berkut x Krichauff on chromosome 5D which (a) has not been described in publications previously (b) did not collocate with any phenotypic traits such as grain size or flowering time and (c) occurred in two different seasonal environments. The second contribution of this study was the identification of a QTL on chromosome 7A in all three populations (though not in Berkut x Krichauff 2007, see chapter 4). Interestingly, QTLs have also been described for Zn density on chromosome 7A in four published studies ((Shi et al. 2008; Genc. et al. 2009; Tiwari et al.2009; Peleg et al. 2009).

The other two QTLs identified in this study were on 2B and 2D, both in Kukri x RAC875. These loci have not been reported in the literature before. However, both these QTLs were also linked with phenotypic traits (flowering time and grain size), and once adjustments were made for these factors the QTLs disappeared. To improve chances of success in future mapping studies, studies may need to be conducted in other crosses or in crosses with greater variation in Zn density. Another possibility to improve chances of success would be to conduct mapping derived from field trials with greater replication. All XRF scans were conducted in this study in duplicate, however if greater precision is sought, scans could also be conducted with greater replication.

6.4 Recommendations for breeders

a) Harvest Plus/ Zn biofortification

Be very careful about Genotype x Environment interactions, in order to make better selections of high and low Zn lines based on Zn concentrations in multiple environments – rather than rely on information on Zn from one environment. This

would maximize the chance of identifying robust variation for Zn that would hold up in different environments.

In studying Zn concentration, be aware that environment (i.e. absence or presence of water) makes a huge difference. The Berkut x Krichauff population may not have been particularly suitable for this study since variation between parents was small. Therefore, consider making purpose-built high Zn by low Zn crosses in wheat for genetic studies of Zn concentration. Because conducting screens in the glasshouse in a potting mix evidently cannot necessarily be expected to produce the same results and rankings among lines as would be obtained in the field, consider using an agriculturally important field soil to get more realistic (field-like) Zn levels.

b) Recommendation regarding Fe and Zn contamination

For Zn, breeders probably do not need to be too concerned that soil contamination is altering Zn values. Instead of insisting that all samples in Zn biofortification programs contain < 5 mg/kg Al, breeders may instead tolerate higher levels of Al, and know that the Zn concentration rankings of their lines are not greatly affected by the soil, and that absolute Zn levels are probably only inflated by approximately a few mg kg^{-1} (e.g., 1-3 mg kg^{-1}) by soil contamination. Alternatively, breeders may attempt to correct for Zn contamination by adjusting the values downwards by a suitably determined factor (in the case of the present study, by $1/5^{th}$ the value of Zn concentrations). The fact that the relationship between removed Fe and Zn levels in the cleaning process, seemed to be constant in this study (approximately. 1:1 ratio) raises the possibility that breeders could still conduct screens on soil contaminated material, and simply adjust Fe concentrations down by the approximate concentration of Al in the sample.

c) Recommendation regarding XRF

XRF can provide an inexpensive and fast tool for screening Zn concentration in wheat grains. XRF can be used for QTL analysis successfully but the use of duplicates to reduce error must be considered.

d) Recommendation regarding QTLs

For Fe QTLs, breeders probably need to clean the seeds prior to analysis, for precise QTL analysis. QTL analysis in Berkut x Krichauff was not consistent in two environments. QTL analysis showed that in Excalibur x Kukri and Kukri x RAC875 the QTLs were co-located with other phenotypic traits and so were not useful for breeders. In future other QTL analysis programs such as association mapping panel be tried

6.5 Future Work

In the future, low and high Zn lines should be selected based on grain Zn concentration in multiple environments. Populations with larger Zn variation between the parents should be selected. Purpose built high Zn by low Zn crosses should be made in wheat for genetic studies. Field soils should be used to get field like results and DH 57 should be considered for further study because it produced high Zn plants in two different environments.

XRF analysis can also be used for mapping grain Zn density because it’s a cost effective and fast analytical method. Longer scanning time should be used to avoid errors of analysis. Mapping populations with greater variation in Zn concentration should be used for QTL analysis.

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