High zinc wheat for sub-Saharan Africa

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M.Sc.

Thesis submitted to the University of Nottingham for the Degree of Doctor of Philosophy

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February 2023

ABSTRACT

Zinc deficiency affects over 17% of the global population. Risk deficiencies of up to 96% have been reported in Sub Saharan Africa, mostly due to increased poverty levels and high dependence on cereal diets with low bioavailable zinc. In Africa, although wheat provides up to 20% of dietary energy, cultivated wheat is inherently low in essential micronutrients such as grain zinc (Zn) and iron (Fe), and the genetic variability is relatively narrow. Genetic biofortification of food crops is considered a sustainable and cost-effective approach for alleviating mineral nutrient deficiencies. Wheat progenitors and wild relatives are considered as potential sources of genetic variation for crop improvement. Inductively coupled plasma mass spectrometry (ICP-MS) was undertaken to determine the natural variation in grain Zn and selected essential mineral nutrients (Fe, Ca and Se) of wheat wild relative accessions, in order to identify novel sources of genetic variation. Accessions from the genus *Triticum, Aegilops, Thinoprum, Amblyopyru*m and *Secale* were screened. Results showed a wide variation in grain Zn, Fe, Ca and not Se. *Triticum urartu* and *Amblyopyrum muticum* accessions showed the highest grain Zn and Fe, whilst *Thinopyrum* species showed the highest Ca concentration. A preliminary study of 48 pre-breeding introgression lines (doubled haploids) derived from *T. urartu* and *Am. muticum* also showed a wide variation in grain Zn, Fe, Ca and not much in Se.

Selected *T. urartu* and *Am. muticum* doubled haploid (DH) lines were also phenotyped under two contrasting soil types, to investigate the effects of soil type on grain Zn, Fe, Se and Ca concentration. One soil type was characterised by higher Zn, Fe, and lower pH (Chitedze soils), and the other soil type was characterised by lower Zn, Fe and higher pH (Ngabu soils). Analysis of variance (ANOVA) revealed a ~two-fold higher grain Zn concentration in low pH, higher Zn soils compared to high pH, lower Zn soils. Variation in grain Zn concentration was associated with the genotypes, soil type, and the interaction between soil and genotypes. Grain Fe concentration was influenced by genotypes and soil type only, grain Se was highly influenced by soil type whilst grain Ca was independent of soil type but highly influenced by genotypes and partly by the interaction between genotype and soil type.

Two high-Zn DH lines (DH-348 and DH-254) were selected, and crossed with three Malawian wheat varieties (*Kadzibonga*, *Kenya nyati* and *Nduna*), to transfer the *Am.*

muticum and *T. urartu* chromosome segments potentially increasing grain mineral concentration in the DH lines. From the crossing program, 41 Malawian wheat/*Am. muticum* and 11 Malawian wheat/ *T. urartu* BC_1F_3 introgression lines were generated. A field based phenotyping study of the 11 Malawian wheat/*T. urartu* and the 37 Malawian wheat/ *Am. muticum* alongside three Malawian wheat, DH-348, DH-254, Paragon, Pavon 76 and Chinese Spring showed high yields and 11-30 mg kg^{-1} improvement in grain Zn in 11 introgression lines, above the three Malawian wheat varieties and Chinese Spring and Paragon. These lines also showed 8-12 mg kg^{-1} improvement in grain Fe than *Nduna* and *Kenya nyati*, whilst four lines showed a 6-10 µg kg^{-1} Se concentration improvement above paragon and the three Malawian checks. Across the four experiments, grain Zn showed strong and significant positive correlations with grain Fe concentration. Grain Ca moderately and significantly correlated with grain Zn and Fe, whilst grain and straw Zn, Fe, Ca and Se showed positive and low significance or positive but insignificant associations. Grain Zn and Fe also showed significant negative correlations with TKW/yield.

Acknowledgements

First and above all, I would like to thank God for making it possible for me to go through my PhD studies amidst different challenges, including the Covid-19 pandemic. It was not an easy journey, but definitely, the most fulfilling.

My sincere gratitude goes to my supervisors, Professor Julie King, Professor Moses Maliro, Professor Martin Broadley, Professor Malcolm Hawkesford and Professor James Bokosi for the mentorship, encouragements and technical inputs over the years of practical work and writing. Their commitment to the success of this work is beyond words. I am also extremely grateful for the immense support they rendered me, to ensure the work is not affected much during the Covid-19 lockdown.

My profound gratitude to The Future Food Beacon (FFB) of the University of Nottingham for funding my PhD studies. Special thanks to Joanna Smuga-Lumatz for all the logistical support during the entire study period. I also thank the GeoNutrition project funded by the Bill & Melinda Gates Foundation (INV-009129) for partially funding my PhD.

I must thank the Nottingham BBSRC Wheat Research Centre (WRC) staff and PhD students for all the support rendered me throughout my study period. Special thanks to Professor Ian King, Dr Surbhi Grewal, Dr Stella Hubbart-Edwards and Dr Manel Othmeni for all the encouragements and technical inputs during my PhD studies. I am also thankful to the WRC technical team, Dr Caiyun Yang, Stephen Ashling and Duncan Scholefield for showing me all the laboratory techniques and their continuous help through the years of practical work.

I must thank Dr Jaswant Khokar for initiating the work leading to this PhD, and for all the valuable inputs in developing the concept note. Many thanks to Dr Liz Bailey, Dr Saul Vazquez Reina, Lolita Wilson, Kenneth Davis and Dr Mark Wilkinson for all the assistance provided during digestion, multi-elemental, and seed size analysis of my samples.

My sincere gratitude to the Department of Crop and Soil Sciences, Lilongwe University of Agriculture and Natural Resources (LUANAR). Special thanks to Mike Banda, Emmanuel Mbewe and Ladson Chirwa for all the assistance rendered in managing the field trials and laboratory activities.

Finally, I would like thank my dear parents Adrian and Olive Guwela, my siblings, and all my friends in Malawi and UK for all the love and moral support throughout my studies. Special thanks to Valerie and John Owens, Dr Olanrewaju Ogunyinka and Dr Abdul Moussa.

I dedicate this work to the late Professor J.H.A Maida and late Mr Mafeni Soko, for believing in me, and giving me the necessary support in my earlier studies. May your souls rest in peace.

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List of abbreviations

AFLP: Amplified fragment length polymorphism

AEDO: Agriculture extension development officer

AME: Apparent metabolisable energy

ANOVA: Analysis of variance

Am.: *Amblyopyrum*

Ae: *Aegilops*

bp: Basepairs

BBSRC: Biotechnology and Biological Studies Research Council

CINaO: Sodium hypochlorite

CIMMYT: International Maize and Wheat Improvement Centre

CRM: Certified reference material

Cv.: Cultivar

DTPA: Diethylene triamine penta-acetic acid

DAPI: 4-6-diamidino-2phenylindole dihydrochloride

dNTPs: Deoxyribo-nucleotide triphosphate

DAES: Department of Agriculture Extension Services

DNA: Deoxyribonucleic acid

DH: Doubled haploid

EDTA: Ethylene-diamine-tetraacetic acid

EAR: Estimated average requirement

EPA: Extension planning area

FAO: Food and Agriculture Organization

FISH: Fluorescence *in situ* hybridisation

FISP: Farm input subsidy program

GWAS- Genome wide association study

GISH: Genomic *in situ* hybridisation

GDP: Growth domestic product

GAP: Guide in agriculture production

HMW-GSs: High molecular weight glutenin subunits

HPYT: HarvestPlus yield trials

Ha: Hactare

IWGSC: The International Wheat Genome Sequencing Consortium

ICP-MS: Inductively coupled plasma-mass spectrometry

ITC: International Trade Centre

IHS: Integrated household survey

IFC: International Finance Cooperation

JIC: John Innes Centre

KASP: Competitive Allele-Specific PCR

LMW-GSs: Low molecular weight glutenin subunits

LRM: Laboratory reference material

LSD: Least significant difference

LUANAR: Lilongwe University of Agriculture and Natural Resources

LOD: Limit of detection

MIT: Ministry of Industry and Trade

MGDS: The Malawi growth and development strategy

MNDs: Mineral nutrient deficiencies

Mbps: Megabase pairs

mg/kg: Miligrams per kilogram

mcGISH: multi-colour GISH

ml: mililitre

mM: millimolar

NGS: Next-generation sequencing

NAP: The National Agriculture Policy

NSO: National Statistical Office

PCR: Polymerase chain reaction

PFA- Perfluorolkoxy

PEEK: Polyethylethylketone

Pina: Puroindoline a

Pinb: Puroindoline b

Ph I: Pairing homoelogous 1

pm: powdery mildew

QTL: Quantitative trait loci

qPCR: Quantitative polymerase chain reaction

RAPD: Random Amplified Polymorphic DNA

RNA: Ribonucleic acid

RNase: *Ribonuclease*

RCBD: Randomised complete block design

SSC: Saline-sodium citrate

SNP: Single nucleotide polymorphism

TPS: Template preparation solution

TEA: Triethanolamine – $(HOCH_2CH_2)3N$

Th: *Thinopyrum*

TKW: Thousand kernel weight

Tris-HCl: Tris hydrochloride

T: Triticum

USDA-FAS: United States Department of Agriculture Foreign Agricultural Service

USAID: The United States Agency for International Development

UV: Ultraviolet

VAT: Value added tax

WHO: World Health Organisation

WRC: Wheat Research Centre

WGS: Whole genome sequencing

µg/kg: Microgram per kilogram

µl: Microlitre

$C_2H_3NaO_2$: Sodium acetate

CH_3COOH: Acetic acid

$FeNH_4SO_4$: Ammonium ferrous sulfate

HNO_3: Nitric acid

HCl: Hydrochloric acid

$K_2Cr_2O_7$: Potassium dichromate

$SrCl_2$: Strontium chloride

CHAPTER 1

1 Background and introduction

This chapter is comprised of some background information of the research topic and two introductory review articles. The first review article was published in CAB Reviews as:

Wheat value chains in Malawi: trends, gaps, challenges and opportunities

Veronica F. Guwela, Moses F. A. Maliro, Edward J.M. Joy, Kevin Tang, James Bokosi, Malcolm J. Hawkesford, Martin R. Broadley and Julie King (2021) Wheat value chains in Malawi: trends, gaps, challenges and opportunities. *CABI Reviews. CABI International*. 046: 1-16. https://doi.org/10.1079/PAVSNNR202116046

The second review article has been formatted according to guidelines for publication in the Journal of Trends in Plant Science. The review will be submitted as:

Veronica F. Guwela, Moses F. A. Maliro, Surbhi Grewal, Martin R. Broadley, Malcolm J. Hawkesford, James Bokosi, and Julie King

1.1 Background

Over 50-96% of the population in in sub-Saharan Africa (SSA) are at risk of zinc (Zn) deficiencies (Kumssa et al., 2015). Wheat is one of the major cereals consumed by a majority of people in sub-Saharan Africa. Improving wheat for a particular trait largely depends on availability of variation within its gene pool. A gene pool is a set of all genes or genetic information of a particular species that can be tapped into for plant breeding and crop improvement (Ayala, 2019). Exploitation of the wheat gene pool is based on the genetic distance of the wild species to the cultivated wheat genomes (Mujeeb-Kazi et al., 2013). Harlan and de Wet 1971 divided the genepool into 3 groups based on the degree of relationship to the cultivated species.

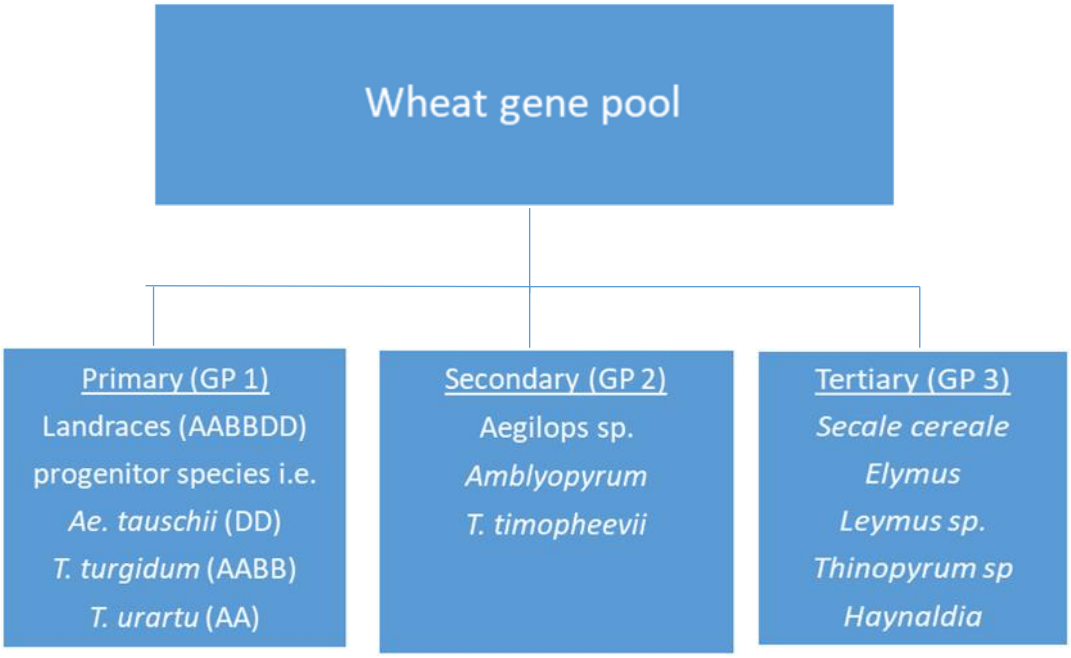

Figure 2.1: Wheat genepool (adapted from Chaudhary et al., 2014)

The wheat primary gene pool consists of species that can inter-mate freely to produce fertile hybrids (Chaudhary et al., 2014). It includes; hexaploid landraces, cultivated tetraploids, wild emmer (*T. dicoccoides*) and the A and D genome progenitors of hexaploid wheat (Harlan and de Wet, 1971). Transfer of genetic material can be achieved through normal hybridization processes (Chaudhary et al., 2014). F_1 hybrids have normal chromosome pairing and gene transfer is usually easy. The wheat primary gene pool has been extensively exploited for useful traits such as disease tolerance (Olson et al., 2013, Yaniv et al., 2015, Wiersma et al., 2017) and high mineral content (Velu et al., 2014, Singh et al., 2017).

The secondary gene pool consists of the polyploid *Triticum* and *Aegilops* species which share one genome among the three genomes of wheat (Chaudhary et al., 2014). *Amblyopyrum and T. timopheevii* also belong to the secondary gene pool. The species in the secondary gene pool usually results in partial sterile/weak F_1 hybrids with poor chromosome pairing (Cox, 1997, Chaudhary et al., 2014). Species in the secondary gene pool have previously been exploited for grain mineral concentration (Rawat et al., 2008, Wang et al., 2011b, Neelam et al., 2010, Neelam et al., 2012, Farkas et al., 2014) and disease and pests tolerance (Hsam et al., 1998, Marais et al., 2005, Martin-Sanchez et al., 2003, Liu et al., 2015).

The tertiary gene pool consists of species that are distantly related to the species in the primary genepool. It includes diploid and polyploidy species of *Triticeae*-carrying genomes other than A, B and D (Chaudhary et al., 2014). The tertiary gene pool includes species such as *Secale cereal, Thinopyrum, Leymus, Haynaldia and Elymus.* F_1 hybrids are usually sterile and gene transfer requires special techniques such as embryo rescue and induced polyploidy.

1.1.1 Approaches to increase mineral concentration in wheat

Food fortification, agronomic biofortification and genetic biofortification have been outlined as the food-based approaches for increasing micronutrients in staple crops (Gibson and Ferguson, 1998, Graham et al., 1999, Ruel and Bouis, 1998, Bouis, 2003, Welch, 2002, Velu et al., 2014). Previous studies have shown that these approaches can significantly increase the concentration and bioavailability of micronutrients such as Fe and Zn (Horton, 2006, Bouis and Saltzman, 2017, Osendarp et al., 2018, Mejia et al., 2019), although many efficacy and effectiveness studies have to be conducted to validate these approaches.

1.1.1.1 Food fortification

Food fortification involves the addition of a mineral fortificant at processing level. Food vehicles for mineral fortification include; sugar, rice, wheat flour, maize flour, margarine, salt and oil, and target minerals include; folic acid, iodine, Se and Zn (WHO, 2006). Country mandatory and voluntary legislations for food fortification helps to increase availability of fortified foods with essential mineral nutrients. For example, the United Kingdom and Northern Ireland have a mandatory fortification legislation of domestically produced and imported wheat since 1940. Currently 88%

of wheat flour produced in the UK and Northern Ireland is fortified. Wheat fortification with folate, riboflavin, thiamine, iron and calcium is also mandatory in America and Canada with 92% of wheat fortified in America and 100% in Canada (Dwyer et al., 2014). Mandatory fortification of wheat in Africa was issued after the year 2000 with South Africa being the first country to have legislation in 2003. Zn fortified wheat flour offers bioavailable Zn (Bouis and Saltzman, 2017) and significantly increase plasma and serum Zn in adult women (Huo et al., 2011, Engle-Stone et al., 2017), whilst Fe fortification increases haemoglobin levels, serum ferritin and reduces anaemia and iron deficiency (Dwyer et al., 2014, Abdollahi et al., 2011, Bouis and Saltzman, 2017). In low to middle-income countries, food fortification is challenging because it is normally done at an industrial level, resulting in an increase in the price of the products (Gomez-Galera et al., 2010, Horton, 2006). In some countries in the developing world, wheat, milling is also done locally, which makes it difficult to add the required fortificants (GFDx, 2019).

1.1.1.2 Agronomic biofortification

Agronomic biofortification aims at increasing concentration of minerals in edible portions of crops through application of micronutrient rich fertilisers (Velu et al., 2014, Cakmak and Kutman, 2018). The effectiveness of agronomic biofortification depends on the type of fertiliser, application method and rate, time of application (Rengel et al., 1999, Hussain et al., 2012) and understanding of both plant and soil factors (Prasad et al., 2014, White and Broadley, 2009). Combining both soil and foliar application has been reported to significantly increase grain zinc concentration (Cakmak et al., 2010, Zou et al., 2012, Dhaliwal et al., 2019) and yield (Dhaliwal et al., 2019). However, agronomic biofortification of wheat with Zn has also shown to decrease the concentration of other micronutrients such as calcium (Ca) and Manganese (Mn) (Zhang et al., 2019). Although successful, agronomic biofortification programs may be challenging to implement at full scale in developing countries due to accessibility and cost of micronutrient rich fertilisers (Cakmak, 2008).

1.1.1.3 Genetic biofortification

Genetic biofortification aims at enhancing grain micronutrient concentration and substances that promote nutrient bioavailability through plant breeding (Velu et al., 2014, Bouis and Saltzman, 2017). Breeding as a food-based mechanism for

biofortification was first discussed in 1992 (Graham et al., 1999). Initial work involved screening of cultivated varieties, landraces, progenitors and wild relatives to identify sources of variation (Graham et al., 1997, Graham et al., 1999, Gregorio, 2002, Cakmak et al., 2004, Calderini and Ortiz-Monasterio, 2003). Several screening studies have identified variation among cultivated wheat (Welch and Graham, 2004, Zhao et al., 2009), spelt wheat (Gomez-Becerra et al., 2010a), durum wheat and wheat progenitors and wild relatives (Neelam et al., 2011, Rawat et al., 2011a, Tiwari et al., 2015, Rawat et al., 2009, Chhuneja et al., 2006).

Table 3.1: Variation in grain zinc concentration of wheat and wheat wild relatives

Species	**No. of entries**	**Range (mg kg^{-1})**	**Reference**
Morden bread wheat	132	25.2-53.3	Graham *et al.,* 1999
T. monococcum ssp boeoticum	2	121-154	Cakmak *et al.,* 1999
T. monococcum ssp monococcum	4	29.1-48.2	
T. turgidum ssp diccocoides	6	113-122	
T. turgidum ssp polonicum	3	11-37	
T. aestivum ssp spelta	3	31-34	
T. aestivum ssp aestivum	2	11-14	
Hexaploid wheat genotypes	170	25-65	Monasterio *et al.,* 2000
Hexaploid wheat cultivars	2	22.2-22.4	Calderim and Monasterio, 2003
T. turgidum ssp. *dicoccoides*	825	30-118	Cakmak *et al*., 2004
Morden bread wheat	25	25-53	Genc *et al.,* 2005
Durum wheat (*T.diccocon*)	24	39-62	
Durum landrace	7	28-46	
Ae. tauschii	30	36.9-90.4	Chhuneja *et al.,* 2005
Ae. kotschyii	8	55.8-90.4	
Bread wheat accessions	175	16.1-39.5	Oury *et al.,* 2006
Spelts, einkorn, emmer, durum and bread wheat	150	13.5 – 34.5	Zhao *et al.,* 2008
Wild emmer	22	69-140	Peleg *et al.,* 2008
T.boeticum	19	22.12-39.06	Rawat *et al.,* 2008
T. turgidum ssp. *dicoccoides*	17	22.50-66.51	
Ae. kotschiyi	14	22.29-58.61	
Ae. peregrina	10	33.13-49.14	
Ae. longissima	5	24.99-50.52	
Ae. cyrindrica	3	32.38-52.18	
Spelt	714	19-145	Gomez-Becerra *et al.,* 2010
Wild emmer	19	39-115	
Ae. caudata		71.3	Wang *et al.,* 2011
Ae. geniculate		42.5	
Ae. searsii		65.5	

Breeding strategies to improve wheat for high micronutrient density include; increasing the concentration, reducing the amount of inhibitors i.e. phytate and increasing the concentration of promoters such as sulphur containing amino acids (Ruel and Bouis, 1998, Bouis and Saltzman, 2017, Velu et al., 2014). Reducing the concentration of inhibitors affects seedling vigour in infertile soils because some inhibitors are a source of P, energy and minerals (Frossard et al., 2000). Breeding for increased promoters may have significant impact on the bioavailability of the nutrients, as most promoters are normal plant metabolites (Welch, 2002, Welch and Graham, 2004).

Development of synthetic hexaploid wheat (SHW) is one approach to increasing wheat grain mineral concentration through breeding. Synthetic hexaploid wheats (SHW) are developed by artificially hybridizing improved durum wheat (AABB) or wild tetraploid *T. dicoccon* (AABB) and diploid *Aegilops tauschii* (D'D') (Singh et al., 2017, Rosyara et al., 2019). SHW introduces a genetic base from diploid *Ae. tauschii*, which may not be present in the D genome of cultivated hexaploid wheat (Li et al., 2018). Studies have shown high Zn concentration and high micronutrient uptake in some synthetic hexaploid wheats compared to their recurrent wheat parents. Highest concentration of up to 115.4 mg/kg has been reported in synthetic hexaploid durum/ *Ae. tauschii* (Calderini and Ortiz-Monasterio, 2003, Singh et al., 2017, Velu et al., 2019).

Mineral nutrients can also be improved genetically through hybridization of cultivated wheat with other closely or distantly related species in the secondary and tertiary gene pools. Previous studies have shown increased Zn concentration in wheat/wild relative introgressions compared to their cultivated wheat parents (Rawat et al., 2009, Tiwari et al., 2010b, Neelam et al., 2011, Wang et al., 2011a, Farkas et al., 2014). For example, *T. turgidum* ssp. durum/*Ae. longissima* amphiploids developed for iron and zinc biofortification resulted in more than 25 mg kg^{-1} Zn above durum wheat parent. In *Ae. kotschyii*/wheat derivatives, the 7U and 2S chromosomes were suggested to increase zinc concentration by 146% (Tiwari et al., 2010b). Wheat-*Ae. kotschyii* addition and substitution lines developed for high grain protein, Fe and Zn also showed increased grain Zn concentration at a range of 43% to 195% above the recurrent parent (Rawat et al., 2011b). Neelam et al., 2010 showed a 3-4 fold increase in zinc concentration of

Ae. peregrina addition lines associated with introgression of group $4S^p$ and $7U^p$ and a $5U^p$ translocation.

Table 1.4: Zn concentration of wheat/wild-relative introgression lines and wild relative based-synthetics

Species	No. of entries	Range (mg/kg)	Reference
Synthetic hexaploid wheat	36	28-66	Genc *et al*., 2005
Rye translocation lines	62	38.6-57.6	Velu *et al.*, 2019
Sythetic hexaploids and landraces (Year 2)	354	38-72	
Sythetic hexaploids and landraces (Year 1)	416	35-69	
Aegilops kotschyi derivatives	24	39.90-65.44	Verma *et al*., 2016
Ae. kotschyii derivatives	13	22.3-63.9	Rawat *et al.*, 2011
Ae. biuncialis substitution and translocation	3	21.7-23.2	Farkas *et al*., 2014
Ae. peregrina	10	23.8-66.7	Neelam *et al*., 2010
Ae. longissima amphiploids	4	45.6-50.4	Tiwari *et al*., 2010
Ae. longissimi F2 population	4	45.8-56.2	
Ae. Kotschyii Substitution lines	13	34.6-48.6	Tiwari *et al*., 2010
RILs (*T. boeticum/T.Monococcum)*	93	17.8-69.7	Tiwari *et al*., 2009
Ae. caudata additional lines	3	71.3	Wang *et al*., 2011
Ae. geniculate additional lines	10	42.5	
Ae. longissima additional lines	12	42.0	
Ae. peregrina additional lines	13	37.5	
Ae. umbellulata additional lines	4	30.2	
Ae. searsii additional lines	5	65.5	

1.1.2 Genetic basis of grain mineral concentration in wheat

In a plant breeding program, understanding the genetic basis of micronutrient accumulation is the prerequisite for their manipulation (Tiwari et al., 2009a). Several studies have identified a number of quantitative trait loci (QTLs) that are associated with grain Zn concentration in wheat and its progenitors. QTLs on chromosome 6B have been reported across several studies (Cakmak et al., 2004, Distelfeld et al., 2007, Srinivasa et al., 2014b, Genc et al., 2009, Peleg et al., 2009, Velu et al., 2017b, Crespo-

Herrera et al., 2016). For example, in *T. turgidum* substitution lines, a QTL on chromosome 6B increased zinc concentration by a 3 factor (Cakmak et al., 2004) and in a wheat/wild emmer RIL population, a QTL on chromosome 6B showed significant G*E interaction (Peleg et al., 2009). Velu et al. (2017) showed that QTLs on chromosome 6B can be stably detected. Interestingly, a QTL on the short arm of chromosome 6B has also shown to carry the *Gpc-B1* locus which is responsible for increasing grain protein (Joppa et al., 1997). Distelfeld et al. (1997) suggested that the locus is involved in efficient remobilisation of proteins, Zn, Fe and Mn from leaves. The *Gpc-B1* locus was shown to encode a NAC transcription factor (*NAM-B1*), which is associated with earlier senescence and increased grain protein, Fe and Zn content to developing seeds (Uauy et al., 2006). *NAM-B1* homologues, *NAM-A1* and *NAM-D1* are also associated with senescence and grain nutrient accumulation (Cormier et al., 2015, Harrington et al., 2019, Andleeb et al., 2022).

QTLs mapped on chromosome 7B have also been reported to explain significant variation in grain zinc concentration (Crespo-Herrera et al., 2016, Peleg et al., 2009, Velu et al., 2018b) and associated with the Zn finger motif candidate gene (Velu et al., 2018a). QTLs associated with grain Zn accumulation have also been detected on chromosomes 1A, 1B, 2A, 2B, 3A, 3D, 4A, 4B, 5A, 5B, 6A and 7A (Cakmak et al., 2004, Genc et al., 2005, Peleg et al., 2009, Srinivasa et al., 2014b, Velu et al., 2017b). Studies have shown that grain Zn is controlled by polygenes each with minor effects (Shi et al., 2008, Adonina et al., 2015). Holasoua et al. (2021) suggested that additive gene action is significant for grain Zn and Fe concentration.

Identification of genomic regions/QTLS underlying mineral accumulation in wheat and wheat wild relatives is significant in the application of new and targeted approaches in genomics and transformation for improving grain mineral concentration (Borrill et al., 2014, Borrill et al., 2019). Recently, CRISPR/Cas9 genome editing has been exploited for vitamin A and E enrichment, targeted increases in grain Zn and Fe, and reduction of grain anti-nutritional factors in wheat and Barley (Kumar et al., 2022). In wheat, CRISPR/Cas9 mediated disruption of *Inositol Pentakisphosphate 2-Kinase 1* (*TaIPK1*) was shown to reduce phytic acid and improve Fe and Zn accumulation (Ibrahim et al., 2022).

1.2 A review of the wheat value chains in Malawi: trends, gaps, challenges and opportunities

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Abstract

Wheat (*Tritcum aestivum* L.) is an important cereal crop, consumed by over 2.5 billion people globally. The current demand for wheat in Malawi is estimated to be 200,000 tonnes/year with a projected growth in consumption of 3–6% annually. We conducted interviews and reviewed literature and databases on wheat production, imports, processing, marketing and consumption to describe current wheat value chains in Malawi, and to identify possible future economic and food security opportunities. The current gap between the supply and demand of wheat in Malawi is large with 99% imported due to low domestic production. The main actors in the value chain include importers, millers, commercial and small bakeries, biscuit manufacturers, wholesalers and retailers. In total, 45% of milled flour is utilised by commercial bakeries, 46% is distributed to rural and urban outlets through primary and secondary distributors, and biscuit manufacturers utilise 9%. Although there is no information on wheat exports between 2016 and 2019, the Food and Agriculture Organization of the United Nations (FAO) statistical database (FAOSTAT) and the International Trade Centre (ITC) Trade Map databases show small quantities of wheat flour exports prior to 2016. Production constraints include the lack of a national wheat development strategy, lack of stable markets, unavailability of improved varieties, low input use, limited knowledge among technical staff in the management of wheat crops, and a lack of funds for research and development. Currency devaluation, transport and other logistical costs, and limited forex reserves further affect the annual volume of wheat

imported and prices of wheat flour on the domestic market. We conclude that domestic production and wider value chain opportunities could be increased through policy support, including research for development, expansion of production into non-traditional wheat growing areas, development of improved and adaptable varieties, investing in irrigation, farmer incentives, and developing market systems and good road networks.

Key words: Wheat (*Triticum aestivum* L), value chains, production, imports, consumption

1.2.1 Background

Wheat (*Tritcum aestivum* L.) is a strategically important crop across Africa (Negassa et al., 2013) where it accounts for over 20% of total calorie intake (FAO, 2019). Over the last decade, annual wheat consumption in Africa has increased from approximately 59 million tonnes (MT) in 2009 to 79 MT in 2018 (USDA-FAS, 2010, USDA-FAS, 2018). Mason et al. (Mason et al., 2015) identified a number of key drivers for rising wheat consumption in Africa which include, increase in GDP, population growth, wheat supplied through food aid and increased participation of women in the wider labour force, which makes women opt for wheat-based foods with short cooking time. Improved income at an individual level and the related shift in food consumption habits are also potential drivers to increased wheat consumption in African countries.

Growing demand for wheat in Africa is constrained by low domestic production. For example, in 2018 wheat consumption in Africa reached ~79 MT (USDA-FAS, 2018) but only 37% of this was produced within the continent (FAOSTAT). To reduce the supply gap, the majority of the wheat consumed is imported, and over the last decade, wheat imports have risen from ~35.7 MT in 2009 to 44.7 MT in 2018 (FAOSTAT). Imports are projected to rise to 63 MT by 2028 (USDA-FAS, 2018). Although total production increased from 19.6 MT in 2008 to 29.2 MT in 2019, and the total area under wheat increased from 8.5 to 10.2 million hectares (FAOSTAT), domestic supply is still much lower than demand. A 2018 USDA report on global wheat imports shows that the sub-Saharan Africa (SSA) region has been a major driver of rising global wheat trade over the last decade. The year-over-year growth in wheat imports for SSA

is greater than any region across the globe. Current annual production in SSA is ~7 MT (FAOSTAT) which accounts for only 28% of total annual demand.

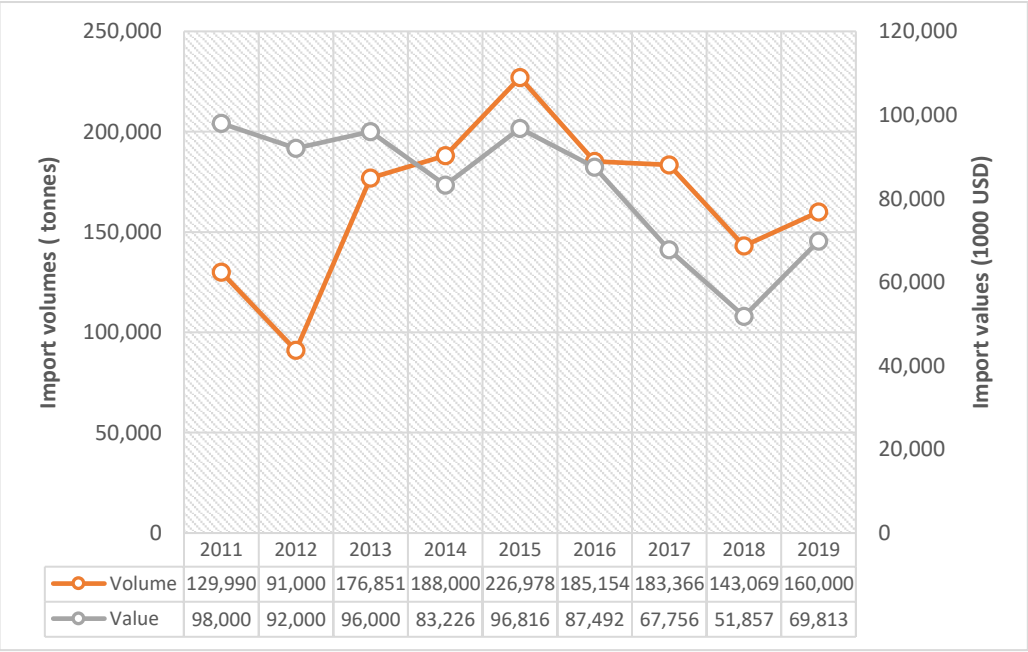
Agricultural systems of Malawi are dominated by maize (*Zea mays* L.) and the wheat value chain is driven almost entirely by imports, which currently represent >99% of demand. In Malawi, wheat is used for making bread and scones, mostly consumed by people in urban areas, and *mandazi* which are consumed as snacks across rural and urban areas. Chapattis are also consumed in restaurants and among Asian communities. Imported products include wheat grain, flour, and wheat-based products such as breakfast cereals and pasta. In 2013, wheat demand in Malawi was projected to increase by 6% annually (IFC, 2012), however current projections using FAOSTAT and ICT Trade Map import data for the past 10 years show an annual growth rate of 3%. Wheat production in Malawi has fluctuated over the past three decades, with a general increase from 1995 to 2007. However, average production has declined annually since 2008 to less than 2000 tonnes in 2017 (FAOSTAT). Wheat research has received little or no support for most of this period. A small National Research Station for wheat was established in Ntcheu district (fig. 3) in 1968 and remained active until 1980 (Mnyenyembe, 1983). Although the Department of Agricultural Research Services have evaluated a number CIMMYT wheat varieties on trial nurseries since the 1960s, little progress has been made to promote these varieties among smallholder farmers.

This review focuses on wheat value chains in Malawi, drawing on information from various databases, interviews, published papers, conference papers, unpublished research reports, unpublished thesis reports accessible online and short interviews with some value chain players. Import and export data were obtained from the Malawi Ministry of Industry and Trade (MIT), the Food and Agriculture Organization of the United Nations (FAO) statistical database (FAOSTAT) and the International Trade Centre (ITC) Trade Map database. Production data were sourced from the FAOSTAT databases, profitability analysis data was collected from various value chain actors using a short semi structured questionnaire and consumption data were taken from the Malawian National Statistical Office (NSO)/World Bank database.

1.2.2 Wheat imports

Wheat imports to Malawi from 2011–2019 are shown in Fig. 1.2 Wheat imports were largest in 2015 (226,978 tonnes), which was likely associated with food shortages in the country. Zant (Zant, 2005) suggested that increase in cereal imports in specific years in Malawi arose due to food shortages associated with natural disasters; this was the case in 2014/15 and 2015/16 growing seasons (GoM, 2016). A sharp decrease observed in the volume of wheat imported in 2012 was associated with a 34% devaluation of the Malawi kwacha by the Government of Malawi. (Naziri et al., 2013). A decrease in imports of ~18% was seen from 2015 to 2016, 1% in 2017 and 21% in 2018 while in 2019 imports rose by 11% from 143,069 tonnes to 160,000 tonnes.

Current annual wheat imports are valued at ~USD70 million. This value includes shipping to the nearest port but excludes port charges, freight within Malawi and import duties and taxes. According to the Malawi Revenue Authority Customs and Excise Act, Customs and Excise (tariffs) Order 2018, there is an exemption of import duties and taxes on all whole grain wheat imported into Malawi and resold, however a 20% import duty is applied for wheat flour and 16.5% value added tax (VAT) for reselling (GoM, 2019). Previously, a tax exemption was also made for wheat flour imported for use in the food manufacturing industry. Naziri et al., (Naziri et al., 2013) suggested that this was one way of encouraging companies to manufacture biscuits and other confectionaries locally.



	2011	2012	2013	2014	2015	2016	2017	2018	2019
Volume	129,990	91,000	176,851	188,000	226,978	185,154	183,366	143,069	160,000
Value	98,000	92,000	96,000	83,226	96,816	87,492	67,756	51,857	69,813

Figure 1.2: Annual import volumes and values of wheat in Malawi.

Source: Ministry of Trade and Industry, ITC Trade Map database and FAOSTAT (2020).

The volume of imported wheat (Figure 1.2) represents different categories of wheat and wheat products. Millers usually import hard red winter wheat, soft red winter wheat and hard red spring wheat grain (Sergeant, 2009). Hard wheat has a high protein (12–15%) and gluten content (11–12%) mostly used in breads and all-purpose flour as it develops strong elastic dough. Soft wheats have low protein (8–12%) and gluten content (7–8%) (Kasarda, 2013, Shewry and Hey, 2015) and they are used for cake, pastries and self-raising flour. Soft wheat can also be used as a blend for all-purpose flour. From 2008 to 2014, millers imported only the cheaper soft wheat, while the hard winter wheat was purchased in country through the USAID funded "Food for Peace" (PL 480) programme, a US Government programme providing donations of agricultural commodities to International Organizations (IOs) and Non-Governmental Organizations (NGOs), to support specific emergency and non-emergency food needs, either by monetization or for direct food distribution (Sergeant, 2009, Naziri et al., 2013, Schnepf, 2016). Wheat flour and wheat products made from both durum and common wheat (Figure 1.3), i.e. pasta and breakfast cereals, are also imported by over 50 companies including wholesalers, tea estates, sugar manufacturers, beverage companies, supermarkets and bakeries (ITC, 2020). Sergeant (Sergeant, 2009) reported that biscuit companies imported 15,000–20,000 tonnes of wheat flour annually, although this may not be the case currently due to the duties levied on imported of flour.

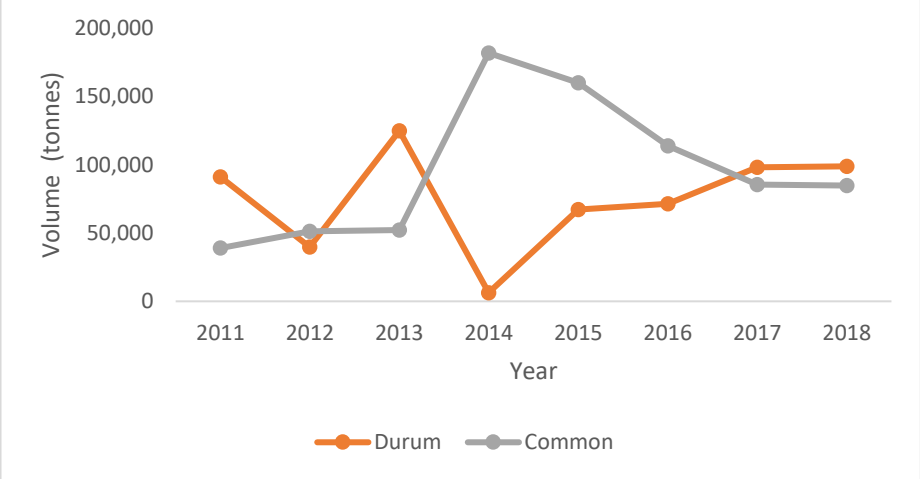


Figure 1.3: Annual imports by wheat type data in Malawi

Source: Ministry of Industry and Trade, ITC Trade Map (2020)

Malawi imports wheat from different countries including Russia, Australia, Germany, Argentina, Turkey, Canada, Latvia, the United Arab Emirates (UAE), USA and Mozambique (ITC, 2020). From 2015–2019, 34% of wheat was imported from the Russian Federation, 21% from Canada, 13% from Switzerland, 12% from Australia, 5% from UAE and 15% from 14 other countries contributing between 0.4–4%. Low prices of wheat in Russia compared to most EU countries has increased its competitiveness on the global market, pushing low to middle income countries to import most of their wheat from Russia (USDA-FAS, 2018).

1.2.3 Transportation

Malawi is a landlocked country and most imported goods come through the Nacala or Beira ports in Mozambique, the Dar es Salaam port in Tanzania and some through the Namibian ports. Wheat often comes from Nacala port through the Nacala corridor; a 912 km railway line that comes through Liwonde in southern Malawi, to Mchinji in the Central Region. Transporting goods from Nacala to Malawi is cheaper compared to the Beira port (Sergeant, 2009). From Nacala, millers use both road (trucks) and rail to transport their consignments (Nagasaki, 2013, Naziri et al., 2013). Although rail transport is considered cheaper and helps to increase the profit margins on wheat flour, it is not always available for all consignments (Sergeant, 2009).

1.2.4 Wheat production in Malawi

Wheat was introduced in Malawi in the 1870s (Mnyenyembe, 1983, Mkamanga et al., 1985), and it is mostly grown by smallholder farmers in the high-altitude areas (>1500 masl). The crop is mainly cultivated under residual moisture and rain showers during the cool months of April to August.. Land under cultivation of wheat is estimated to be less than 3000 ha (FAOSTAT, 2020), however, an estimated 30,000 ha land is suitable for wheat production in Malawi (Ganunga, 1999, Kamalongo, 2012). Three wheat varieties (*Kenya nyati, Kadzibonga* and *Nduna*) are the most widely cultivated across the wheat production areas. *Kenya nyati* and *Kadzibonga* were released in the early 1980s and lost resistance to leaf and stem rust(Ganunga, 1999, Kamanga, 2013), while *Nduna* was introduced in 2007/08 by SeedCo, one of Malawi's largest seed companies (Kamalongo D., personal communication, 5 June 2020). Primary crops such as maize, rice (*Oryza sativa* L.), groundnut (*Arachis hypogaea* L.) and common bean (*Phaseolus vulgaris* L.) are cultivated under rainfed conditions in the months of

November to April. Maize forms the bulk of the countries' cereal output and it is cultivated on an estimated 1.7 million ha. Use of improved maize varieties and fertilisers has increased since 2005/06 when the Farm Input (seed and fertiliser) Subsidy Program (FISP) was introduced. This has resulted in increased production and productivity of maize (Dorward and Chirwa, 2011).

Wheat is grown in the high altitude areas of Ntcheu, Neno, Dedza, Chitipa, Rumphi, and Zomba (Figure 1.4). This represents only 20% of the total districts in Malawi. National wide wheat production is limited by climatic conditions. Wheat is a cool season crop which requires an optimum temperature range of 17- 23°C over the entire growing season(Porter and Gawith, 1999, Pirttioja et al., 2015). Temperatures outside the optimum usually results in a short grain filling period and grain size, low dry matter accumulation and total yield (Thaler et al., 2012).Existing weather patterns in the traditional growing areas makes it suitable for wheat production. For example Dedza to Ntcheu usually has minimum temperatures of 9–14°C and maximum temperatures of between 20–22°C within the growing months of May–August. Daily rainfall in the form of light showers usually ranges from 1.5–6.2 mm within the growing season. Zomba has minimum temperatures of 11–16°C and maximum temperatures of 23–27°C, with 0.3–1.9 mm rainfall across the growing season Across the country minimum temperatures vary between 17 and 27°C in the winter months of May to August (DCCMS, 2021).

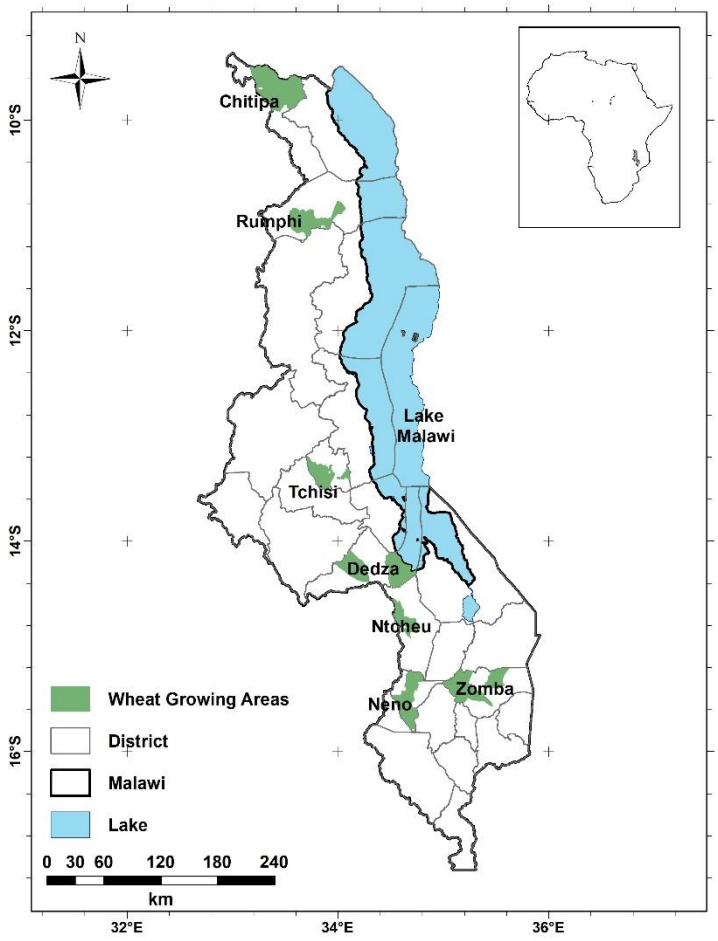


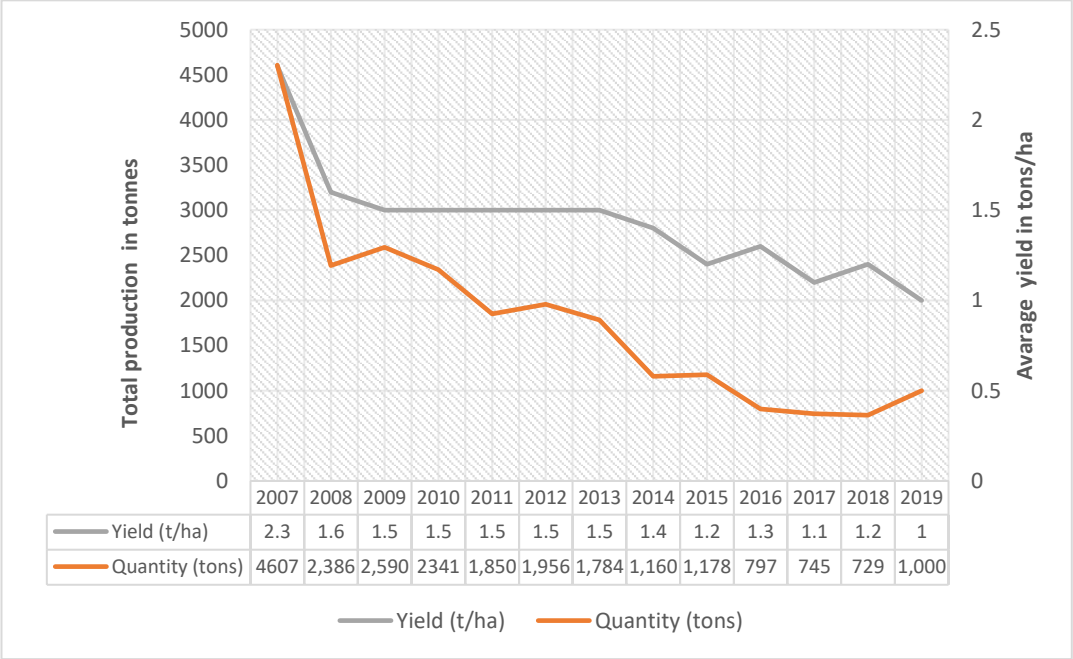
Figure 1.4: Wheat production areas of Malawi by districts

1.2.5 Seed systems

There are no formal seed production and distribution systems for wheat in Malawi. Farmers usually save seeds and perform farmer-to-farmer exchange every season. Currently, private seed companies are not involved in marketing of wheat seed. Previous reports and an interview with a SeedCo sales and marketing manager show that SeedCo Malawi head office in Lilongwe was importing the varieties SC Select and SC Nduna from SeedCo Zimbabwe for evaluation and promotion between 2006 and 2009, however the lack of a stable market forced the company to stop importing and stocking seed (Kamalongo D., personal communication, 5 June, 2020; W. Lipenga, personal communication, 2 June 2020). A focus group discussion with farmers in Tsangano and Mwera Hills area in Ntcheu district and showed that some farmers buy preferred wheat seed varieties from Mozambique, which is few kilometres away from some parts of Ntcheu district (Kamanga, 2013). In most of the wheat

traditional growing areas, farmer access to certified seed and associated farm inputs is also hampered by poor road networks that becomes inaccessible during rainy season.

1.2.6 Production Levels



	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Yield (t/ha)	2.3	1.6	1.5	1.5	1.5	1.5	1.5	1.4	1.2	1.3	1.1	1.2	1
Quantity (tons)	4607	2,386	2,590	2341	1,850	1,956	1,784	1,160	1,178	797	745	729	1,000

Figure 1.5: Total annual production and average yield of wheat in Malawi from 2007 to 2019

Data source: FAOSTAT, 2020

There has been a general decline in wheat production since 2007 (Figure 1.5), with total annual production below 2000 tonnes since 2011. The much larger production in 2007, was attributed to the Clinton Hunter Development Initiative project, which focused on increasing wheat productivity through improved varieties, subsidized fertiliser, capacity building in best agronomic practices and linking farmers to millers offering premium prices (A. Ngwira, personal communication, 10 June 2020). Yields have also been declining from 2.3 t/ha in 2007 to 1 t/ha in 2019 (Fig. 4). The yield gap is very wide compared to some countries in the southern Africa (Table 1.3), for example, Zambia with a national average of 6.6 tonnes/ha, Namibia 5.6 tonnes/ha, South Africa 3.4 tonnes/ha, and Zimbabwe 2 tonnes/ha (FAOSTAT, Dube E and 2020). Differences in yields could be attributed to less use of improved varieties, low input use, heavy reliance on rainfed production, poor agronomic practices, lack of

extension support (Kamanga, 2013, Ganunga, 1999, Kamalongo, 2012) and climate and soil factors(DCCMS, 2021).

Table 1.3: Average yield, area harvested and production of wheat in southern African countries (2014-2018)

Country	**Yield (tonnes/ha)**	**Area harvested (ha)**	**Production (tonnes)**	**Potential yield (tons/ha)**
Zambia	6.6	26,376	176,688	11
Namibia	5.6	1,527	8,433	
South Africa	3.4	492,407	1,700,600	7.6-11.5
Zimbabwe	2.0	19,219	37,517	10-12
Eswatini	1.8	397	715	
Malawi	1.2	757	922	7-9
Mozambique	1.1	17,092	19,048	7.5

Data source: FAOSTAT, 2020, Tadesse et al., 2018, Dube et al., 2020

1.2.7 Marketing

In 2020/21 season wheat farmers were selling wheat grain to vendors at USD 947 per/tonne. The vendors in turn sold the wheat to milling companies. Some of the wheat is sold and milled locally for household consumption and for making wheat flour products (Figure 1.6) such as *mandazi* (a deep fried sweet and fluffy snack made from wheat, yeast/baking powder and sugar), *kanyenya* (a deep-fried fish snack made of small *cichlid* fish dipped in a mixture of wheat flour, salt and curry powder) and *madonasi* (doughnuts), often by women operating small scale businesses (Sergeant, 2009, Naziri et al., 2013). Farming households also mix wheat flour with maize flour for cooking *nsima*, Malawi's staple food, which is made from a mixture of water and milled whole kernel maize/corn known as *mgaiwa* or maize milled with refined flour where the outer kernel shell and seed germ have been removed, locally known as *ufa oyera.*

Figure 1.6: *Madonasi (A, B), kanyenya (C)* and *mandazi (D)* made by women living in rural areas.

1.2.8 Challenges in wheat production

The challenges for wheat production among smallholder farmers in the Tsangano and Mwera Hills in Ntcheu district were studied using focus group discussions (Kamanga, 2013). Farmers reported unavailability of improved varieties, low input use, insufficient extension services, lack of stable markets for their grain, post-harvest losses due to weevils and mice, shattering of some varieties and birds eating the grains and especially the awnless varieties. We used a short semi-structured questionnaire to get an overview of the current production challenges in and Dzinjiriza village in Tsangano, Ntcheu district. We randomly selected 10 smallholder farmers who have been cultivating wheat for over five years. Challenges were ranked according to their importance. Lack of reliable markets was cited as the major challenge followed by wheat rust diseases, limited access to improved seeds, unaffordable inputs especially fertiliser, low selling price, low soil fertility and effects of climate change. Millers have attributed unavailability of stable markets to low quantities produced by the local farmer and lack of proper aggregation coupled by poor road networks (Sergeant, 2009, Masauli, 2019). According to the Department of Agriculture Extension Services and

(DAES) and Department of Agriculture Research Services (DARS), the lack of a national crop development strategy, limited knowledge and skills by technical staff in production and management of wheat crop, lack of funds for research and development, lack of recommended improved varieties for local and export market production, low investments in irrigation facilities, poor road infrastructure networks and lack of a proper seed system affects wheat production in the country. Poor road networks affects both access to farm inputs and transportation of products to markets especially during rainy season. (S. Magomero and Kamalongo D., personal communication, 5 June, 2020)

1.2.9 The wheat value chain

Importers control the wheat value chain in Malawi. Wheat millers, wholesalers, retailers, supermarkets, and grocery stores, commercial and rural/small bakeries, wheat-based product manufacturers and consumers are other key actors in the chain. The value chain has been summarised in Figure 1.7 and it represents all the possible market channels from production, milling to end use. Information from multiple reports, online articles and personal communication have been used to make the figure.

1.2.9.1 Millers/processors

The milling industry is comprised of three main companies: Capital Foods Limited (http://www.capitalfoodsmw.com/) in Lilongwe, in the Central Region, and Bakhresa Grain and Milling (http://bakhresa.com) and HMS Foods Grain Limited (http://hmsmalawi.net/) in Blantyre, in the southern region. Bakhresa Grain and Milling (BGM) also has branches in Lilongwe and Mzuzu in the northern region. Bakhresa began with a 250 tonnes per day capacity mill in 2003 but doubled its capacity to 500 tonnes/day with another 250 tonnes/day capacity mill in 2011 (Nagasaki, 2013). At full operation capacity, BGM mills 400 tonnes/day (Naziri et al., 2013). Capital Foods started with a 200 tonnes/day mill and doubled its capacity to 400 tonnes in 2010 (Sergeant, 2009) while HMS has a capacity of 200 tonnes. In 2013, BGM had a national market share of 80% (IFC, 2012). Across the regions, BGM had 90% market share in the south, 50% in the central region and 75% in the north (Nagasaki, 2013). The types of flour that the three mills package include brown bread, all-purpose bread, biscuit, cake flour and special *mandazi* flour by HMS. Flour package sizes from all companies range from 2, 5, 10, 25 and 50 kg depending on the

target market. Millers use different primary and secondary market channels via wholesalers and retailers to reach consumers

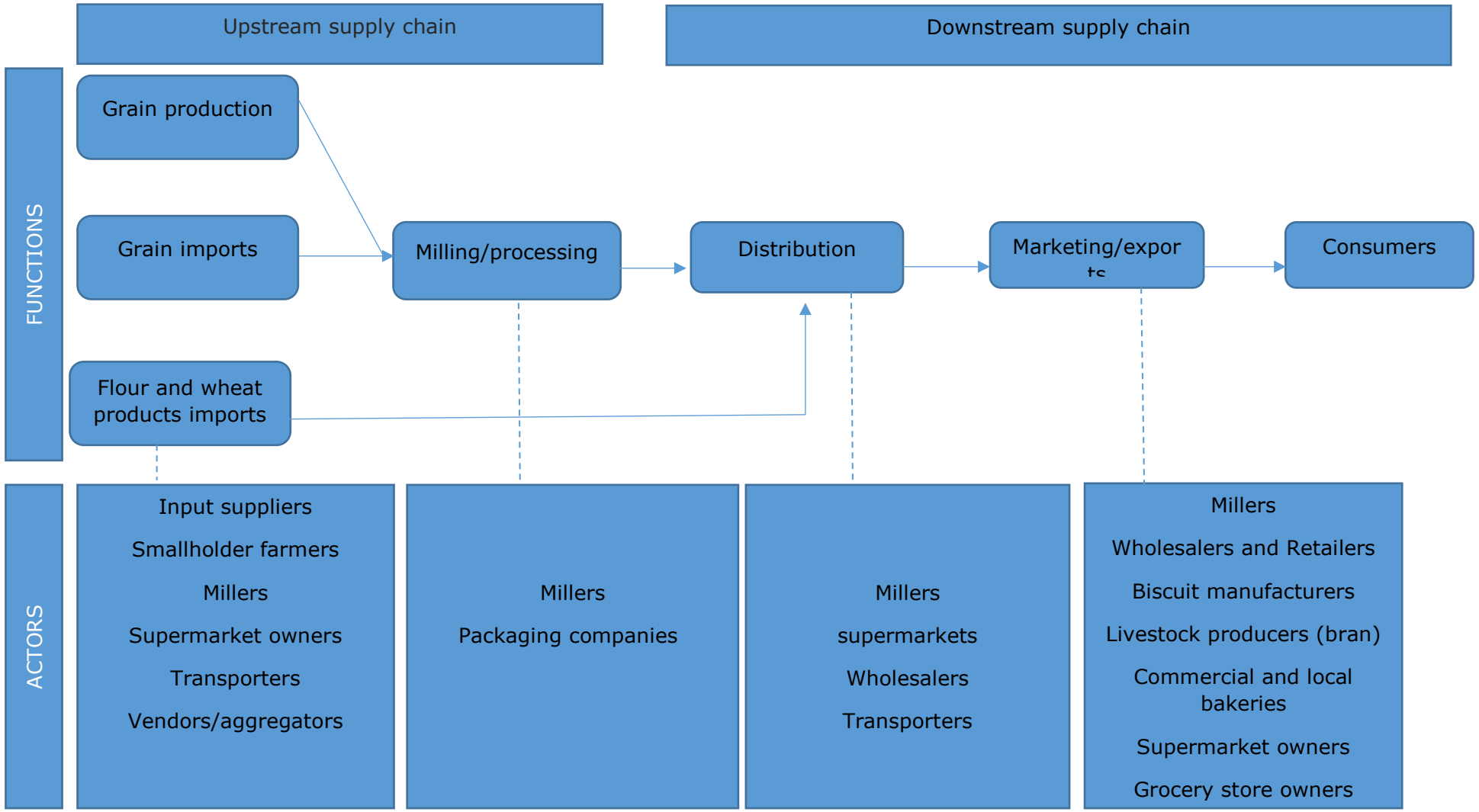


Figure 1.7: Value chain mapping of wheat in Malawi

1.2.9.2 Wholesalers

About 50% of the processed wheat is distributed through wholesale channels as the primary distributors. For example, BGM distributes through four major wholesalers: Rab Processors Limited, Right Price and Woollies in Blantyre, and Farmers World in Lilongwe, together have a total of over 110 retail outlets across the country in both rural and urban areas (Nagasaki, 2013). The wholesalers target markets are small/rural bakeries, retailers and individuals who make products such as *mandazi* for selling on.

1.2.9.3 Retailers

Retail shops are the secondary distributors of wheat in Malawi. They are supplied by wholesale outlets and sometimes directly by millers from their headquarters. Local grocery stores and roadside vendors are the major retailers of wheat flour. Their target markets are small to medium sized businesses, typically run by women entrepreneurs selling *mandazi* for home consumption. According to Naziri et al. (Naziri et al., 2013), 40% of the wheat flour milled in Malawi is used for making mandazi. In the BGM business model, retailers take up 90% of the BGM wheat flour distributed by wholesalers and about 20% of the flour directly sold by BGM at its headquarters in Blantyre (Nagasaki, 2013). Apart from wheat flour, local grocery stores and roadside vendors also sell imported wheat products, especially pasta.

Supermarkets sell wheat flour, pasta and breakfast cereals. The main supermarkets, which are mostly located in main cities (Blantyre, Lilongwe, Zomba and Mzuzu) of Malawi, include Shoprite, Sana, Chipiku and Peoples. Wheat millers distribute flour directly to supermarkets in 2, 5 and 10 kg packages (Bakhresa 2020; HMS, 2018; Capital Foods, 2020). Supermarkets also stock other imported cake flour brands, pasta and breakfast cereals.

1.2.9.4 Biscuit and confectionary manufacturers

Wheat millers distribute wheat flour directly to biscuits and confectionary companies. Four manufacturers, Universal Industry, Bakeman's Limited, Cresta and Bakelines Limited, absorb 9-10% of total wheat flour from millers (Sergeant, 2009, Naziri et al., 2013). Universal Industries has a bigger market share and fulfils over 60% of the total wheat flour demand from the biscuit manufacturers. Bakeman's absorb 25% and the rest is shared between Cresta and Bakelines (Sergeant, 2009, Naziri et al., 2013).

Previous reports show that some biscuit manufactures directly imported flour from Turkey (J. Pankuku, personal communication, 31 May 2020) and Tanzania (Sergeant, 2009). Currently biscuit companies depend on local production although prices on the local market are considerably higher compared to the international market (K. Mittal, personal communication, 9 June 2020).

1.2.9.5 Commercial bakeries

Millers supply wheat flour directly to commercial bakeries. In Malawi, most commercial bakeries operate in groups of several affiliated individual bakeries. Mother's Pride and Royal products in the southern region and Baker's Pride in the Central and Northern Regions are the main commercial bakery groups. From 2009 to 2013, commercial bakeries were using about 45% of the wheat flour from millers (Sergeant, 2009, Naziri et al., 2013). BGM alone supplied 90% of the total volume (Nagasaki, 2013). Bakeries use wheat flour to make bread, scones, cakes and pastries which are sold to supermarkets and retail outlets in rural and urban areas.

1.2.10 Market segmentation for wheat flour

According to estimates made using the 2012 wheat flour balance sheet (Naziri et al., 2013), commercial bakeries accounted for 45% of the total wheat flour, rural outlets 32%, while urban outlets take up 14% and the rest is used by the biscuit companies. Wholesalers and retailers account for over 90% of rural and urban outlet distribution and the rest is directly distributed by millers. Although there has been a growth of wheat imports and consumption since 2012, the market segmentation may still reflect the current situation.

1.2.11 Domestic wheat flour prices

Prices for wheat flour tend to fluctuate depending on the cost of production and currency exchange rate. Final wholesale price is determined by the cost of freight from the country of importation to the mill, port charges, custom clearance charges, administrative costs and cost of processing and packaging(Sergeant, 2009, Naziri et al., 2013). At the beginning of 2020 the cost of wheat flour was USD745 (2020, exchange rate of 1USD:MK751).

1.2.12 Exports

Small quantities of milled (from imported grain) and packaged wheat flour are exported from Malawi to Asia and other African countries (Table 1.4). Top export market destinations include Mozambique, Zimbabwe, Zambia and South Africa. According to the ITC Trade Map and FAOSTAT databases, the highest volume exported was 11,213 tonnes valued at US$183 million in 2011 and 2012. In 2009, Capital Foods Limited and Bakhresa estimated an export quantity of ~5,000 tonnes/year of wheat flour each to Zambia and Zimbabwe (Sergeant, 2009). Export volumes for wheat flour have likely reduced over the years due to the establishment of BMG Mozambique (Naziri et al., 2013) which exports wheat flour to neighbouring Zimbabwe and South Africa.

Table 1.4: Total annual wheat exports from 2007 to 2015

Year	Quantity (tonnes)	Value ($ '000)	Countries importing
2007	5786	436	South Africa, Japan
2008	5679	749	South Africa, Japan, Germany
2009	3809	272	South Africa, Zimbabwe, Japan
2010	7337	659	South Africa, Zimbabwe, Zambia
2011	11213	738	Zimbabwe, India, South Africa
2012	11213	738	Zimbabwe
2013	1515	311	Zimbabwe
2014	123	No data	Zimbabwe
2015	170	75	South Africa

Source: ITC Trade Map database, FAOSTAT (2020)

FAOSTAT and ICT Trade Map data (Table 1.5) also show fluctuating wheat bran exports valued at less than $4 million annually from the period of 2010–2018. Interestingly, the volume and value of wheat bran exported annually surpasses that of wheat flour. In Malawi, wheat millers sell some of the wheat bran locally to livestock feed manufacturers and the bulk of it is exported to feed industries in other countries (E. Nyirongo, personal communication, August 15, 2020). Top export destinations for wheat bran include South Africa, Zimbabwe, and lately Botswana, and these countries also rank highly in maize bran imports from Malawi.

Table 1.5: Total annual wheat bran exports from 2010 to 2018

Year	Quantity (tonnes)	Value ($ '000)	Countries importing
2010	6990	No data	Zimbabwe, Zambia, Kenya,
2011	12986	No data	South Africa, Zimbabwe, Kenya
2012	14155	1560	South Africa, Zambia, Zimbabwe
2013	21248	3878	South Africa, , UAE, Switzerland
2014	23793	3149	South Africa, Zimbabwe, India
2015	30401	2886	Zimbabwe, Kenya, South Africa
2016	23789	2760	Zimbabwe, Botswana, South Africa
2017	22986	1383	Zimbabwe, Botswana, South Africa
2018	21030	1075	Zimbabwe, Botswana, South Africa

Source: ITC Trade Map database and FAOSTAT (2020)

1.2.13 Profitability analysis of wheat production and processing

Profitability analysis of wheat production was conducted by interviewing 10 farmers in Dzinjiriza village in Tsangano, Ntcheu (Table 1.6). The analysis assumed that land used for production is rented and that farmers use hired labour, although, six out of 10 farmers interviewed used their own land and family labour. Another assumption is that all farmers are applying fertilisers, which is not the case for most farmers. The largest cost is land rent followed by fertiliser inputs, land preparation and then harvesting. Based on the production and yield data, average yield was estimated at 1.2 t/ha, the total costs of production were 597.12 USD/ha and at a selling price of 1 USD/kg, a margin of 542.88 USD can be achieved (2021, exchange rate of USD1:MK790).

Table 1.6: Estimated margins of wheat production in Malawi

Activity	Cost (MK)	Cost (USD)
Land rent	148,000	187.34
Land preparation	74,000	93.67
Seed purchase	29,650	37.53
Fertiliser	83,075	105.15
Hand weeding	37,000	46.83
Harvesting	60,000	75.94
Other costs	40,000	50.63
Total cost of production	**471,725**	**597.12**
Yield in tonnes/ha	1.2	1.2
Cost per kg	750	0.95
Revenue	**90,0000**	**1,140.00**
Margin	**428,275**	**542.88**

Data source: Interviews with farmers

(Sergeant, 2009) Conducted a profitability analysis of wheat processing/milling in Malawi and concluded that higher profit margins are realised when wheat grain is transported by rail through the Nacala Corridor. He reported a margin ratio of 5:3:1.5 for Nacala rail, Nacala road and Beira road respectively. The analysis factored in transportation costs from the port, port charges, administration costs and processing costs at 75% processing efficiency.

Retailers buy flour from local millers or wholesale distributors at 32 USD/ 50kg bag of flour and re-sell at 36 USD to make a profit margin of 4 USD. Retailers also purchase flour from Mozambique at a wholesale price of 28 USD/50kg bag and re-sell at 33 USD to make a profit of 5USD.

Gross margin analysis of *mandazi* and scones was done based on interviews with five *mandazi* sellers and five local bakery owners from Kuchipata, Chinseu, Motolosi and Takumana villages in Mitundu, Lilongwe district (Table 1.7). Estimates were calculated based on a 50kg bag of flour. *Mandazi* had a high cost of production (63.7 USD/50kg) as compared to scones (54 USD/50 kg). The production cost difference is attributed to the cost of cooking oil, which is among the main ingredients in *mandazi* compared to scones. At a retail price of 0.19 per scone, a margin of USD 45.23/50kg bag of flour can be achieved and 33.43 USD for *mandazi* sold at 0.06 USD each.

Table 1.7: Estimated margins of *mandazi* and rural bakery made scones

Expenses	**Scones**	**Mandazi**
	Value (USD)	**Value (USD)**
Wheat Flour	34.18	34.18
Transport	2.53	2.53
Yeast	0.63	1.71
Sugar	6.96	6.96
Cooking oil	1.77	8.86
Firewood	6.33	6.33
Products/ 50kg bag of flour	500	1500
Selling price/product	0.19	0.06
Total cost of production	**51.77**	**60.57**
Revenue	**95.00**	**94.00**
Margin	**43.23**	**33.43**

Data source: Interviews with *mandazi* sellers and rural bakery owners

An interview with two supermarkets with their own commercial bakeries across the country showed that bread and biscuits are made using wheat flour premixes (Table 1.8). The premixes only require water or milk to make the dough. Total cost of production for one loaf of bread and a single packet of biscuits factored in operational costs, packaging, costs of wheat flour and transportation. A margin of 120.27 USD/50kg bag of wheat flour is achieved for high value biscuits and 25.00 USD/50kg for bread.

Across the value chain, wheat production has the highest margins and at processing level, biscuit manufacturing has the highest margins followed by scones and *mandazi*. There is also a possibility of high margins from millers; however, there was no available information for current analysis.

Table 1.8: Estimated margins for bread and biscuits

Expenses	**Bread** **Value (USD)**	**High value biscuits** **Value (USD)**
Total cost of production	38.00	69.62
Products/ 50kg bag of flour	100	100
Selling price/product	**0.63**	**1.89**
Revenue	**63.00**	**189.87**
Margin	**25.00**	**120.27**

Data source: Interviews with a commercial bakery and a biscuit manufacturing company

1.2.14 Consumption

In Malawi, the contribution of wheat to total dietary energy is less than 10% and fluctuates because of low domestic production and high costs of imports (Zant, 2005). This is low compared to Africa more broadly, where wheat provides about 20% of total calorie intake(Mason et al., 2015).

Table 1.9: Wheat consumption by products in 2010/11 and 2016/17

Wheat/wheat products	Count consumed		Mean of those consuming*			
			g/household/day		g/AME/day	
	2010/11	**2016/17**	**2010/11**	**2016/17**	**2010/11**	**2016/17**
Wheat flour	68	119	329	244	77	99
Bread	2079	2776	236	183	69	74
Buns, scones	2585	1747	120	80	34	32
Pasta	141	619	80	267	24	108
Mandazi, doughnuts	2801	4294	50	40	15	16
Breakfast cereal	35	141	123	110	36	37

*This is the mean mass of the food item consumed, either at household level or per Adult Male Equivalent (AME), over the past seven days

Source: NSO, 2012; NSO, 2017

Wheat consumption data recorded at household level were extracted from the Third (IHS3) and Fourth (IHS4) Integrated Household Surveys of Malawi (NSO, 2012, NSO, 2017). Data extraction was done using methods reported in Joy et al (2015). A majority of wheat in Malawi is consumed in the form of bread, buns/scones and *mandazi* and scones (Table 1.9). In 2010/11, on average per day, individuals (per Adult Male Equivalent, AME) consumed 69 g of bread, 34 g of buns/scones, 24 g of pasta and 15 g of *mandazi*. In 2017 average consumption increased by 7% for bread (69 to 74 g per AME per day), 350% for pasta (24 to 108 g per AME per day) and 29% for wheat flour for home cooking (77 to 99 g per AME per day). In 2010/11, 21% of the sampled households consumed buns/scones, 23% *mandazi*, 16.9% bread and <2% pasta (Table 5). Bread consumption increased by 5% from 2010/11 to 2016/17 (17 to 22%). *Mandazi* consumption increased by 11% (23 to 34%) while scones/buns consumption decreased by 7% (21 to 14%). Across socioeconomic positions (Table 1.10), bread, buns/scones, breakfast cereals and pasta consumption were higher in wealthier households, i.e. those in the 4th and 5th highest total annual household expenditure quintiles. For example, 42% of households in the wealthiest fifth of the population consumed bread in 2010/11 and 65% in 2016/17, while the equivalent values for pasta were 4% in 2012 and 19% in 2017. *Mandazi* consumption was constant across all socioeconomic positions between the survey years with consumption increasing incrementally from the poorest to wealthiest groups. Between 2010/11 and 2016/17, *mandazi* consumption also increased consistently across all socioeconomic positions. The consumption pattern for *mandazi* is in line with the

findings of Naziri et al.,(Naziri et al., 2013) where 40% of all milled flour in Malawi was reported for use in making *mandazi*.

Data on wheat consumption by urban versus rural residency shows that bread and pasta are mostly consumed by the urban population (Table 1.11). The percentage of households consuming *mandazi* and buns/scones was similar for rural and urban dwellers. Between, 2010/11 and 2016/17, consumption of all the products by urban households increased by over 30% except for buns/scones which decreased from 29% in 2010/11 to 19% in 2016/17.

Table 1.10: Total consumption by products and wealth quintiles in 2010/11 and 2016/17

Social economic position	Proportion of households consuming each food item, n (%)											
	Wheat flour		Bread		Buns, scones		Pasta		Mandazi		Breakfast cereals	
	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17
Poorest	0	0	1	2	4	4	0	0	10	17	0	0
Poor	0	0	3	6	11	8	0	0	16	28	0	0
Middle	8	0	8	13	18	13	0	1	26	37	0	0
Wealthy	1	1	17	26	27	20	0	4	28	47	0	1
Wealthiest	1	4	42	65	35	26	4	19	28	44	1	4
Total	1	1	17	22	21	14	1	5	23	34	0	1

Source: NSO, 2012; NSO, 2017

Table 1.11: proportion of households consuming wheat products in the rural and urban areas

Residency	Proportion of households consuming each food item, n (%)											
	Wheat flour		Bread		Buns, scones		Pasta		Mandazi		Breakfast cereals	
	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17	2010/11	2016/17
Urban	1	4	51	68	29	19	5	21	25	43	1	4
Rural	0	0	9	12	19	13	0	1	22	32	0	1

Source: NSO, 2012; NSO, 2017

1.2.15 Wheat research

Wheat research in Malawi's National Agricultural Research System comes under the Cereals Section (small grains) in the Department of Agriculture Research Services, in the Ministry of Agriculture. Wheat breeding has never been undertaken formally in Malawi since its introduction. Varieties that have been tested and released were from CIMMYT- Mexico yield nurseries, while some were introductions from Kenya, Zimbabwe and South Africa (Ganunga, 1999, Kamalongo, 2012). Seed Co. bred varieties SC Shine, SC Nduna, SC Smart, SC Stallion, SC Shield and SC Shangwa, were also introduced and tested at the research stations (Bisiwasi and Masangwa, 2009).

Previous trials focused on selecting early maturing (less than 150 days) and high yielding varieties that were ecologically suited to conditions in Malawi with high levels of resistance to major wheat diseases and lodging (Mkamanga et al., 1985, Chafika and Kauwa, 1998). Trials to determine fertilizer requirements, time of planting, performance in traditional and non-tradition wheat growing areas and rainfed and irrigated winter conditions were also conducted (Mnyenyembe, 1983, Munthali, 1989, Kamanga, 2013).

Wheat research has been limited in Malawi due to lack of funds and policy support, although in recent years Lilongwe University of Agriculture and Natural Resources (LUANAR) has been collecting and evaluating wheat germplasm from all wheat growing areas. On station performance studies showed yield performance of 1.8 to 2.7 tonnes/ha in early planted winter irrigated trials and 0.75 to 1.0 tonnes/ha in late planting trials (M. Maliro, unpublished observations). Chitedze Agriculture Research Station have started evaluating CIMMYT-Mexico nurseries and varieties for abiotic and biotic stresses under irrigation since May 2018 winter season.

1.2.16 Challenges, opportunities and future prospects

Annual demand for wheat and wheat products in Malawi will keep rising over time. A ten-year projection trend using import data from 2011–2019 shows a 3% annual growth rate in wheat demand. The figures are likely to increase further due to population growth, economic growth and increased urbanization. Consumption data from IHS3 (2010/12) and IHS4 (2016/17) indicates high consumption of wheat in urban areas compared to rural areas and thus increased urbanization will tend to further

increase wheat demand. Although Mason et al. (Mason et al., 2015) did not find a statistical significance between urbanization and increased wheat consumption at country level in SSA, they argued that urbanization could still be a driver to increased wheat consumption. Change in eating habits and increase in household income overtime will also increase consumption of other wheat products for example, IHS3 and IHS4 data also show an increase of up to 375% in some of the wheat products such as pasta.

Population growth in Malawi will likely be another driver to increased demand for wheat. Current population in Malawi is at 18.14 million and is projected to rise to 40% by 2070 (GoM, 2017). Mason et al. (Mason et al., 2015) showed that a 1,000-person increase in total population raises a country's wheat consumption by 30 to 50 MT, other factors being constant. Although the figures are too high for Malawi, a combination of several drivers is likely to increase the demand for wheat and wheat products.

The projected increase in wheat demand in Malawi suggests that the country needs to increase imports or domestic production. Increasing wheat imports will potentially drain the country's foreign exchange reserves, which are already limited. Tadesse et al. (Tadesse et al., 2018) argued that wheat imports by African countries is not always easy and reliable, as it depends on the availability of wheat on the global market, political stability and ability to compete in cases of price shocks. Negassa et al. (Negassa et al., 2013) studied the potential economic profitability of wheat production in African countries and from their findings they concluded that wheat production in African countries could be economically profitable with proper policy support, strengthened wheat seed systems, input supply systems, extension services and improved market structures.

In Malawi, the lack of a wheat crop development strategy, a lack of policy support, over reliance on rainfed production, and under-developed roads and market structures have forced most smallholder farmers to abandon wheat farming. Reports shows that only 10% of land suitable for wheat production is under cultivation (Kamalongo, 2012). However, the area under cultivation has likely decreased in the past 8 years as reflected in the annual production figures. Although there is no policy support for domestic wheat production in Malawi, there is potential to increase wheat production and productivity by taking advantage of existing policies and strategies that focus on

increased agriculture productivity and development of irrigation structures. The Malawi Growth and Development Strategy MDGS III (2017–2022) identifies agriculture, water development and climate change as one of eight key priority areas. Key strategies for achieving these goals include increasing agricultural productivity and increase land under irrigation by developing areas with irrigation potential and promoting infrastructural investment in large-scale irrigation. The National Agriculture Policy (2016–2021) and National Irrigation Policy (2016–2020) both support increasing land under irrigation which is currently at 29% of the 407,862 ha potential land area. Another opportunity to boost wheat production in Malawi is to take advantage of government efforts on shifting from subsistence farming to commercialization. With a well-organised market system, commercial farmers can afford irrigation and all necessary inputs thus increasing wheat production and productivity.

Development of irrigation facilities for wheat production coupled with access to subsidized inputs/input loans and well-developed markets and road infrastructure in wheat growing areas have potential to boost both production and productivity of wheat in Malawi. In Zimbabwe, where wheat is produced under irrigation in the winter months of May to October (Macrobert and Savage, 1998) , 28 to 50% of annual demand is supplied through domestic production (USDA-FAS, 2017) and average yields are higher than other countries in the sub-Saharan Africa region (Negassa et al., 2013). Shiferaw et al., (Shiferaw et al., 2011b) and Tadesse et al., (Tadesse et al., 2018) have shown that Malawi, Zimbabwe and Zambia fall under the same wheat production mega environment suitable for irrigated winter production.

One recommendation for economically profitable wheat production in Africa is to exploit the non-traditional wheat growing areas. The Ecocrop wheat suitability map for Africa (Negassa et al., 2013) shows that most parts of Malawi are suitable for wheat production, thus there is a need to exploit the potential of winter irrigated production in non-traditional growing areas. Winter irrigated trials in non-tradition growing areas such as Bunda in Lilongwe, Kasinthula in Chikwawa and Bvumbwe in Thyolo have shown average yields of 1.5-9.0 tonnes/ha (Maliro unpublished data; Bisiwasi, unpublished data) which is 2 to 7-fold higher compared to yields on farmers' fields. Further evaluation and strengthening of wheat research in Malawi will also play an important role in improving wheat production. To address production challenges cited

in this review, priority areas of research could include breeding or introducing widely adaptable and high yielding varieties with excellent end use quality, drought and heat stress tolerant and diseases and pest resistance. To improve farmer access to knowledge on good agriculture practices for wheat production, wheat research institutions should collaborate with the Department of Agriculture Extension Services to build the capacity of Agriculture Extension Development Officers (AEDO) who are the primary providers of extension services at Extension Planning Area (EPA) level. Wheat farmers can also form cooperatives for easy aggregation, access to inputs, produce transportation and price negotiation.

Funding statement

This work was supported by a studentship funded by the Future Food Beacon of Excellence, University of Nottingham, UK (VFG). Additional support was provided by the Bill & Melinda Gates Foundation Micronutrient Action Policy Support (MAPS) project (INV-002855) (MRB, EJMJ, and KT). Rothamsted Research and the University of Nottingham receive strategic funding from the Biotechnology and Biological Sciences Research Council as part of the Designing Future Wheat project [grant number BB/P016855/1] (MJH, JK).

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1.3 The potential of *Triticum urartu* for wheat improvement

Abstract

The narrow genetic base of cultivated wheat (*Triticum aestivum* L.) is a major limitation to improvement for existing and emerging production challenges. To overcome yield plateaus and to feed the projected increase in global population will require a pool of genetic variability for crop improvement to meet future demand. Wheat progenitors and distant relatives contain substantial genetic variability that remains untapped and potentially useful for wheat improvement. *Triticum urartu*, the A genome donor of wheat, shows potential for both biotic and abiotic traits. Availability of validated SNP datasets for wheat and its relatives on public domains and the development and accessibility of low, medium and high throughput genotyping platforms is significantly contributing to acceleration of wheat/*T. urartu* introgression programs.

Key words: Wheat, *Triticum urartu*, introgression, agronomic traits, accessions, molecular markers

1.3.1 Highlights

- Wheat wild relatives and progenitor species have become more important in recent years as a valuable genetic resource for wheat improvement because of their high variability
- *T. urartu* has been successfully introgressed into diploid, tetraploid and hexaploid wheat and in recent years, doubled haploid lines with different *T. urartu* segments have been developed.
- SNP genotyping arrays and KASP markers for detecting *T. urartu* introgressions in a wheat background have been developed and successfully used in pre-breeding programs.
- Disease and stress tolerant traits have been reported in *T. urartu* accessions and some novel resistance genes have been identified.
- Photosynthetic traits and quality traits related to milling, baking and starch synthesis and associated genes have also been identified in *T. urartu* accessions.

1.3.2 Glossary

Doubled haploids: Genotypes formed when cells with one set of chromosomes undergo chromosome doubling to produce fertile homozygous diploids. Chromosome doubling can be achieved by chemical treatment e.g. colchicine, chromosome duplication without nuclear division and fusion of pollen nuclei

Genome assembly: The process of taking a large number of short DNA sequences and putting them back together to create a representation of the original chromosomes from which the DNA originated

Genomic *in-situ* hybridisation: A cytogenetic technique used to distinguish chromosomes from different genitors or different interspecific/intergeneric hybrids through hybridisation of fluorescently labelled total genomic DNA (probes) to chromosome DNA within a cell and assessment through a fluorescent microscope

Introgression breeding: The transfer of alleles from one species into the gene pool of another through hybridisation and repeated backcrossing of the interspecific hybrids with one of the parental species (box 1)

Transcriptome database: An archive of a complete set of RNA transcripts produced by the genome, under specific circumstances or in a specific cell using high-throughput methods, such as microarray analysis

Wheat wild relatives: Wild plant species that are ancestors of cultivated wheat or are closely related

1.3.3 Wheat evolution, the loss of genetic diversity and advances in exploiting *T. urartu*

Grown on an estimated 216 million hectares of land with about ~700 million tonnes of grain harvested annually (FAOSTAT), hexaploid bread wheat (*Triticum aestivum ssp vulgare*) and tetraploid durum (*Triticum turgidum ssp durum*) are the two main species of wheat that are widely cultivated (Dvorak et al., 1993). Hexaploid wheat consists of three sub-genomes: AA, BB, and DD from diploid *T. urartu* (A^uA^u), an unidentified species related to *Aegilops speltoides* (SS) and diploid *Aegilops tauschii* (DD) respectively (Dvorak et al., 1993, Dubcovsky and Dvorak, 2007, Feldman and Levy, 2015). Domestication and polyploidy speciation resulted in loss of genetic diversity of cultivated wheat compared to progenitor species and **wild relatives** (He et al., 2019, Dubcovsky and Dvorak, 2007). Limited variation and full exploitation of

cultivated wheat for breeding over the years, have also posed a challenge to further wheat improvement(Feldman and Sears, 1981) and increased vulnerability of wheat to different biotic and abiotic stresses (Dubcovsky and Dvorak, 2007, Feldman and Sears, 1981). In sub- Saharan Africa for instance, wheat diseases such as yellow rust and stem rust have caused yield losses of up to 100% and lead to the collapse of dominant wheat varieties (Tadesse et al., 2018). Across the globe, an estimated 90% of wheat varieties are reported susceptible to wheat stem rust as new and more virulent races are emerging (Singh et al., 2011, Figueroa et al., 2018). *T. urartu*, though not widely exploited, shows potential for a number of agronomically important traits that could be useful for enriching the genetic base of cultivated wheat. Advances in plant genetics and genomics have accelerated wheat-progenitor species/wild relatives pre-breeding programs and allowed the transfer of alien genomes into cultivated wheat. Here, progress made in the identification and utilisation of *T. urartu* agronomic and quality traits is summarised. In addition, techniques for characterising the introgression lines are discussed.

1.3.4 General description of *Triticum urartu*

T. urartu Thum. ex Gandil. (2n = 2x = 14; genome A^uA^u) is a wild diploid species endemic to the Fertile Crescent (Johnson, 1975, Xiao et al., 2018). Initial studies suggested that *T. urartu* is the B genome donor of cultivated wheat (Johnson, 1975), however, through observing the pairing behaviour of marked A and B telocentric chromosomes of 14 *T. aestivum-T. urartu* hybrids at meiosis, it was discovered that *T. urartu* is the A genome donor of *T. aestivum* (Chapman et al., 1976, Dvorak, 1976). Analysis of the polymorphisms of repeated nucleotide sequences confirmed that *T. urartu* contributed to the A genomes of *T. turgidum*, *T. timopheevii*, and *T. aestivum* (Dvorak et al., 1993). Phylogenetic analysis of some *T. urartu* accessions also provide molecular evidence that *T. urartu* is the A-genome donor of hexaploid wheat (Luo et al., 2015). High quality and draft genome assemblies shows that *T. urartu* has a genome size of about 4.94 GB (Akhunov et al., 2005, Ling et al., 2013, Ling et al., 2018).

1.3.5 Disease resistant traits in *T. urartu* accessions

Disease resistance is a major target trait for any breeding programme, and it is an important trait for sustainability of yield in cultivated crops. Effects of diseases on

wheat crop yields and quality have been widely studied (Laidig et al., 2021, Wellings, 2011). A number of wild relatives and progenitor species have contributed to sources of resistance for some of these diseases and subsequently contributed to food security (Friebe et al., 1996). Several screening studies for *T. urartu* have also revealed some excellent disease resistance traits that could be useful for breeding for resistance to major diseases that are responsible for substantial crop losses in cultivated wheat.

1.3.5.1 Wheat powdery mildew

Powdery mildew is a fungal foliar disease responsible for yield losses of up to 30% (Griffey et al., 1993, Zhao et al., 2020). A more economic and effective way of controlling powdery mildew is through the use of powdery mildew (*Pm*) resistance genes (He et al., 2018, Zhao et al., 2020). *T. urartu* accessions have shown high resistance to wheat powdery mildew (*Blumeria graminis f. sp tritici)* race BgtE09 (Zou et al., 2018). A dominant resistance locus (*Pm60),* which contains two nucleotide-binding domain and leucine-rich repeat immune receptors (NB-LRR) protein encoding-genes, was identified and cloned in *T. urartu* accession P1428309 (Zou et al., 2018). The locus shows hypersensitive reaction (HR) type of resistance that causes programmed cell death. According to the authors, *Pm60* was the first gene to be cloned and characterised in *T. urartu*. Two allelic variants of *Pm60, Pm60a* and *Pm60b* were also identified in resistant accessions. Interestingly, a non-functional *Pm60a*-like allele (*Pm60a*") was mapped at the *Pm60* locus in susceptible *T. urartu* accessions and its sequence is 98.52% identical to the *Pm60a,* with a difference of 58 SNPs and one 3-nucleotide deletion (Zhao et al., 2020). Using the whole genome of *T. urartu*, (Liu et al., 2015) identified and allocated 461 full-length protein sequences containing 4 classes of NBS resistant domain among them NBS-LRR, to seven chromosomes of *T. urartu* with chromosome 7A having the highest number of sequences. Expression analysis of the 461 NBS genes showed that six genes were differentially expressed among the accessions in response to *B. graminis* at the two leaf stage (Liu et al., 2017a). Analysis of resistant gene analogues on chromosome $7A^u$ L of *T. urartu* also revealed 126 Resistant Gene Analogs (RGAs) with 30 of the RGAs in the *PmU* region and 14 with expression data in the *T. urartu* **transcriptome database**. Expression analysis of the 14 *PmU*-RGAs and *Pm60* after inoculation with Bgt race *E09*, showed that *Pm60* was specifically expressed in the *T. urartu* accession carrying *PmU*, but not in a susceptible accession (Zhang et al., 2018). Candidate immune receptor genes

positively associated with powdery mildew resistance were also detected in *T. urartu* accessions through the study of the immune (IM) and hypersensitive reaction (HR) responses of *T. urartu* to powdery mildew infection (Zhang et al., 2016a, Zhang et al., 2018).

1.3.5.2 Wheat stem and stripe rusts

Wheat rusts are one of the major wheat production constraints globally. Together, wheat stem rust (*Puccinia graminis f. sp. tritici*) and stripe rust (*Puccinia striformis f.sp. tritici*) have caused substantial crop losses and led to the collapse of dominant varieties (Tadesse et al., 2018). Identification of novel sources of resistance has the potential to significantly reduce the vulnerability of wheat to rust diseases. In *T. urartu*, resistance to TTKSK (Ug99), a very destructive and relatively new race of stem rust was identified in 95% of 205 accessions (Rouse and Jin, 2011). TTKSK possesses virulence to many resistance genes that have been used in wheat breeding worldwide (Singh et al., 2015). The same study also identified resistance to races QFCSC, MCCFC.

Resistance to stripe rust was first reported in *T. urartu* in the early 80s (Dhaliwal and Gill, 1982). Recently, resistance to two major Chinese stripe rust races CYR33 and CYR32 was assessed in 147 *T. urartu* accessions. Results showed more accessions resistant to CYR33 than CYR32, with some accessions showing resistance to both races. Among the resistant accessions, few exhibited high resistance, while a majority were moderately resistant (Xiao et al., 2018). Intermediate resistance to susceptibility was also reported in 16 *T. urartu* accessions (Ma et al., 1997).

1.3.5.3 Root lesions

T. urartu has shown resistance to root lesions caused by *Platyenchus thorneia*, a migratory parasitic nematode that is associated with yield loss of up to 65% in susceptible varieties (Sheedy et al., 2012). *P. thorneia* feeds and reproduces in the cortex of wheat roots causing lesions and debilitated root systems that are inefficient in nutrient and water uptake from the soil (Mokrini et al., 2019). In *T. urartu*, resistance to *P. thorneia* was reported in five of 21 accessions, with three accessions showing more resistance compared to the partially resistant check (Sheedy et al., 2012). Resistance to *P. thorneia* was previously detected in *Ae. tauschii*; the D genome donor

of hexaploid wheat (Thompson and Haak, 1997) and *Ae. tauschii*-durum synthetic hexaploid wheats (Thompson, 2008).

1.3.6 Quality traits in T. urartu accessions

1.3.6.1 Endosperm storage protein

In wheat, baking properties are controlled by endosperm storage proteins grouped into glutenins and gliadins (Payne, 1987). Dough elasticity properties are determined by glutenins while extensibility and nutritional quality is determined by gliadins (Payne et al., 1984). Glutenins are controlled by high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs) loci (Payne, 1987). A number of glutenins (Hu et al., 2010, Luo et al., 2015, Ahmadi et al., 2018) and gliadin alleles (Zhang et al., 2015) coding for endosperm storage have been identified in *T. urartu* using gene prediction, PCR-based cloning and allele specific markers. Accessions collected from the Fertile Crescent show a high diversity of HMW-GSs in *T. urartu* from Turkey compared to those from Lebanon and Syria (Caballero et al., 2009, Talini et al., 2020). Both tetraploid and hexaploid wheat have a number of known HMW-GSs and LMW-GSs loci located on group one chromosomes (Payne, 1987), however, identification of novel glutenin and gliadin alleles has potential to further improve wheat end-use quality. Hu et al. (2010) [39] identified a novel active gene (FJ404595) coding the y type HMW-GS at the *Glu-A1* locus of *T. urartu*. Expression analysis showed similar electrophoretic mobility with the y-type subunit, *1Dy12*, from the reference variety Chinese spring (Hu et al., 2010). In hexaploid wheat the y gene in the *Glu-A1* loci is completely silent (Halford et al., 1989, Dovidio et al., 1996), and hence not able to express any HMW-GSs to contribute to end-use quality. On the contrary, the gene is active in some *T. urartu* and *T. turgidum* accessions (Waines and Payne, 1987, Bai et al., 2004, Jiang et al., 2009). Bai *et al*.,[47] isolated a complete coding sequence of an expressed active *1Ay* gene and expression analysis produced *1Ay* proteins that were 72% identical to *IDy12* and 90% identical to *1Ay* from *T. timopheevi* (Bai et al., 2004). Recently, *T. urartu* has shown up to 18 different patterns of HMW-GS with a total of six *1Ax* alleles and eight *1Ay* alleles (Talini et al., 2020). Availability of DNA markers to efficiently detect the *1Ay* gene and distinguish the three *Glu-A1* alleles in common wheat increases the potential of utilisation of the gene for improving end-use quality (Dong et al., 2017). *T. urartu*

accessions have also shown a high genetic variability in the i-type (Luo et al., 2015, Ahmadi et al., 2018, Cuesta et al., 2017), m-type and s-type (Luo et al., 2015, Cuesta et al., 2017) LMW-GSs at the *Glu-A3* locus compared with the *Glu-A3* of cultivated wheat. An s-type gene *TuA3-460* was the first to be identified at the *Glu-A3* locus of *T. urartu* (Luo et al., 2015). A total of 11 novel alleles associated with *Glu-A3-1* genes, showing differences with those of common wheat have been reported (Cuesta et al., 2015).

1.3.7 Photosynthetic traits in *T. urartu* accessions

Photosynthetic traits are determinants of biomass production and subsequent grain yield (Richards, 2000). Wild relatives can be utilised for improvement of photosynthetic traits because of the high degree of natural variation in key photosynthetic traits at the accession level (McAusland et al., 2020). A high rate of flag leaf photosynthesis was reported in *T. urartu* compared to its tetraploid and hexaploid relatives. Difference in flag leaf photosynthesis was attributed to ploidy level with diploid wheats having the highest rate followed by tetraploids and then hexaploids (Austin et al., 1982). High photosynthetic rates were also identified in *T. urartu*, when photosynthesis was expressed per unit leaf area, and a much higher rate compared to hexaploid wheat when photosynthesis was expressed on a leaf dry weight basis (Austin et al., 1986). *T. urartu* also shows a high photosynthetic rate expressed in terms of high values of stomatal conductivity, high values of intercellular CO_2 content and high values of chlorophyll content in the flag leaf (Chunyan et al., 2008).

1.3.8 Starch synthesis

Variability evaluation of *Wx* (waxy protein) genes of *T. urartu* and einkorn wheat (*T. monococcum* L. ssp. monococcum) accessions with the *Wx-A1a* allele of bread wheat revealed four different novel alleles (*WxA^u1b, -A^u1c, -A^u1d and -A^u1e*) in *T. urartu* accessions (Ortega et al., 2014). Waxy proteins are enzymes responsible for the accumulation of amylose during development and synthesis of starch granules in wheat (Zi et al., 2018). A full coding sequence (Guzman and Alvarez, 2012) and partial sequences (Yan and Bhave, 2000) of waxy protein genes have been reported in *T. urartu*. Recently, a novel basic zipper (Bzip) transcription factor *TubZIP28* (TRIUR3_00571) on the short arm of the group 2 chromosome of *T. urartu* was reported to be expressed in the endosperm throughout grain filling. Overexpression of

TubZIP28 in wheat increased starch content by up-regulating transcription and activity of a starch synthesis related gene; cytosolic *AGPase*. Knockout of the *TubZIP28 T. aestivum* homologue (*TabZIP28*) using the CRISPR/Cas9 system, resulted in reduction of total starch of mature grains and reduction in thousand kernel weight (Song et al., 2020).

1.3.9 Drought, salinity and other stress tolerant traits in *T. urartu* accessions

Drought, salinity and temperature stresses are among the abiotic stresses that cause substantial losses in wheat production across the globe. Abiotic stress usually affects plant growth and development and grain yield and quality of wheat (Kamal et al., 2010). *T. urartu* and other diploid species exhibited drought tolerant traits based on physiological and chlorophyll fluorescence responses at the seedling stage. Under drought stress conditions, *T. urartu* showed high relative water content (RWC), highest values in stomatal conductance, drought stress tolerant index and decreased maximum quantum yield of PSII and maximum primary yield of photochemistry PSII (Pour-Aboughadareh et al., 2017).

Recently, a pathogenesis related hybrid protein gene (*Pr-1-rk*) and a related pseudogene (*TuA-1-rKP*) were identified in 30 and 39 *T. urartu* accessions respectively. Transcriptional analysis revealed that *TuPr-1-rk* was expressed in response to salinity stress and pathogen attack (Lu et al., 2018). Novel Vesicle-Inducing Protein in Plastids 1 (VIPP1) genes; *TuVipp1* and *TuVipp2* were also cloned from *T. urartu* accessions (Gao et al., 2017). *VIPP1* are proteins that help to maintain membrane integrity of chloroplasts under heat (Zhang et al., 2016b) and salt stress (Huang et al., 2006). In hexaploid wheat, *TuVipp1* and *TuVipp2* were reported to be induced at a rate greater than normal under light, salt, mannitol and cold treatment (Gao et al., 2017).

1.3.10 Introgression of *T. urartu* into wheat

Interspecific and intergeneric hybridisation through controlled pollination remains the primary way in which novel genes are introduced into cultivated crops (Dempewolf et al., 2017, Zhang and Batley, 2020). Despite some minor hybridisation challenges (Box 1), *T. urartu* has been successfully introgressed into diploid (Ma et al., 1997, Fricano et al., 2014, Valkoun, 2001, Johnson and Dhaliwal, 1976), tetraploid (Ahmed et al.,

2014, Rodriguez-Suarez et al., 2011, Rafique et al., 2012, Alvarez et al., 2009), and hexaploid wheat (Grewal et al., 2018a, Qiu et al., 2005, Grewal et al., 2021). Direct hybridisation of *T. urartu* with hexaploid or tetraploid wheat (Figure 1, key Figure) is possible because *T. urartu* and cultivated wheat belong to the primary gene pool of wheat whose species can inter-mate freely to produce fertile hybrids (Chaudhary et al., 2014, Harlan and de Wet, 1971). Furthermore, the A^u chromosomes of *T. urartu* are homologous to the A chromosomes of hexaploid wheat (Chapman et al., 1976) maintaining synteny and macro-collinearity in majority of linkage groups with the exception of the 4A/5A/7B translocation and subsequent inversions in 4A found in the hexaploid and tetraploid wheat (Devos et al., 1995, Dvorak et al., 2018). *T. urartu* genome and many other Triticeae and wild relative species also have the 4A/5A translocation suggested to have originated from a common ancestor (King et al., 1994, Ling et al., 2018, Dvorak, 1978). However, the subsequent translocation involving chromosome 7B and inversions in chromosome 4A may make it difficult *for T. urartu* introgression from linkage group 4 to occur in the wheat background (Grewal et al., 2018a, Grewal et al., 2021). Although no cross incompatibility has been observed in the hybridisation of *T. urartu* and other wheats, a number of studies have demonstrated high levels of infertility in the F_1 generation depending on whether *T. urartu* was used as the male or female parent. A cross between *T. monococcum and T. urartu* produced mostly sterile F_1 plants when *T. urartu* was used as a pollen donor while *T. urartu* as a female failed to produce viable F_2 plants (Fricano et al., 2014). Hexaploid wheat-*T. urartu* recombinant lines generated using the wheat *ph1/ph1* approach showed a high level of infertility with low cross fertility percentage of F_1 generation and normal fertility with every round of backcrossing (Grewal et al., 2018a). Increasing the number of pollinated heads and embryo rescue can increase hybridisation success rate (Valkoun, 2001, Qiu et al., 2005).

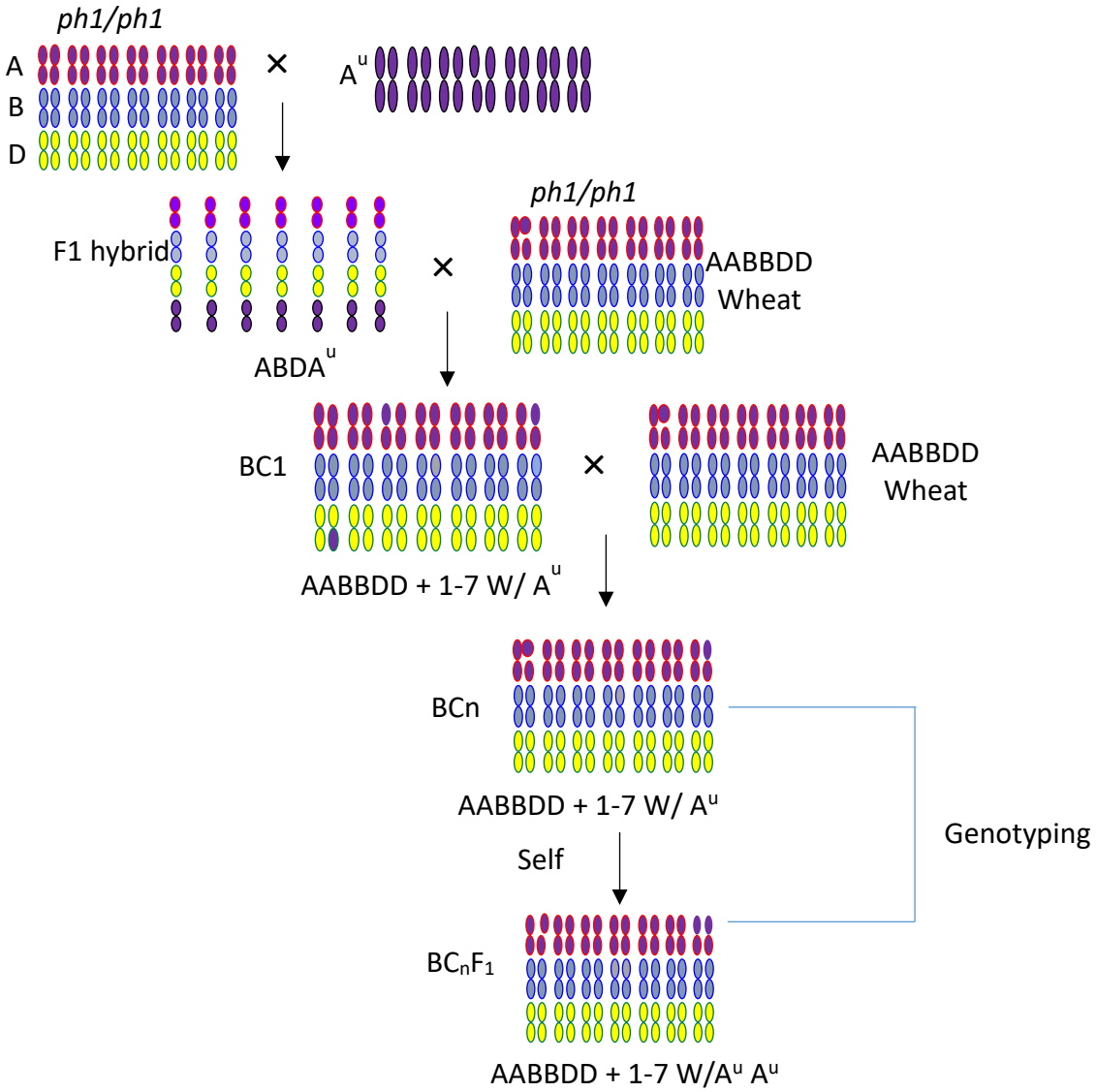

Figure 1.8: A pre-breeding crossing program for developing wheat-*T. urartu* introgression lines using the *ph1* mutant approach. W/A^u = heterozygous wheat/*T. urartu* recombinant, W/A^{u}A^u = wheat/*T. urartu* homozygous recombinant

1.3.11 *T. urartu* introgression lines with disease resistant traits

Although a number of screening studies have identified disease resistance traits in *T. urartu* accessions, only a few of these traits have been transferred to cultivated wheat. A powdery mildew resistance gene from *T. urartu* was successfully transferred into hexaploid wheat from a cross between *T. urartu* and Chinese Spring. Expression of the gene in the hybrids showed full resistance to 15 *B. graminis* isolates. Using microsatellite markers a powdery resistance resistant gene (*PmU),* was mapped on the distal region of the chromosome 7A^{u}L (Qiu et al., 2005). Additionally, *T. urartu*

introgression lines have shown resistance to different races of powdery mildew at different growth stages (Rafique et al., 2012, Valkoun, 2001, Ahmed et al., 2014, Ma et al., 1997).

1.3.12 Introgression lines with quality traits

Introgression lines derived from the amphiploid between durum wheat and *T. urartu* carrying the *1Ax + Ay* subunits from *T. urartu* showed higher values of gluten strength. Gluten strength was also associated with grain colour where red grains had high gluten strength while yellow grains showed soft gluten (Alvarez, 2009). The *1Ay* subunit from *T. urartu* also increased glutenin content in transgenic barley lines although it resulted in reduced gliadin content and failed dough formation due to the lack of the x-type HMW-GS and reduced number of subunits (Yang et al., 2019).

In recent years, genetic biofortification has become an important quality objective in wheat breeding. Preliminary results of variation in grain zinc content of *T. urartu* introgression lines have shown higher grain zinc concentration of introgression lines compared to their parents (Fricano et al., 2014).

1.3.13 Drought and heat stress resistance lines

An introgression line derived from a cross between *T. urartu* and durum wheat showed higher yield potential and drought tolerance index compared to their recurrent parent under drought stress conditions (Aberkane et al., 2021).

1.3.14 Doubled Haploid (DH) introgression lines

Recently, a number of doubled haploid lines with different chromosome segments of *T. urartu* were developed for trait analysis. The *T. urartu* DH lines were developed by crossing *ph1* mutant hexaploid wheat with different *T. urartu* accessions. The F_1 interspecific hybrids were backcrossed with the recurrent parent and the advanced BC lines were subjected to the DH procedure. Initial studies resulted in the development of a panel of 17 wheat-*T. urartu* recombinant lines with introgressed segments covering the whole genome of *T. urartu* (Grewal et al., 2018a). Further work resulted in the generation of 86 stably inherited wheat-*T. urartu* introgression lines (Grewal et al., 2021).

1.3.15 Molecular characterisation of *T. urartu* segments in introgression lines

In the past, different molecular markers such as microsatellites (Rodriguez-Suarez et al., 2011, Qiu et al., 2005), RAPDs (Vierling and Nguyen, 1992, Chabane and Valkoun, 1998) and amplified fragment length polymorphisms (AFLPs) (King et al., 1994) were used to detect *T. urartu* introgressions in a wheat background. However, these markers were not able to fully cover the *T. urartu* genome (Rodriguez-Suarez et al., 2011). The identification of SNPs between different varieties of hexaploid wheat and between hexaploid wheat and related species and progenitor species (Winfield et al., 2016) was a step change in the identification and characterisation of wild relative introgressions. The development of a high-throughput Axiom® Wheat-Relative SNP Genotyping Array has been a useful tool for identifying and tracking introgressions from different species among them *T. urartu* (Winfield et al., 2016, Burridge et al., 2017). Grewal *et al*. (Grewal et al., 2018a) used the Axiom wheat-relative array to detect *T. urartu* introgressions in a hexaploid wheat background. Marker analysis resulted in the generation of a genetic map of *T. urartu* with 368 SNP markers across the seven chromosomes of *T. urartu*. Further studies showed that the Axiom wheat-relative array could not effectively distinguish between heterozygotes and homozygotes in a self-fertilised population. Therefore, SNPs were converted to Chromosome-specific Competitive Allele-Specific (KASP) assays (Grewal et al., 2020a) that can distinguish heterozygotes from homozygotes and provide information on their site of introgression. The markers have been successfully used to detect *T. urartu* introgressions in a doubled haploid population (Grewal et al., 2021).

1.3.16 Cytogenetic characterisation

Cytogenetic techniques such as fluorescence *in situ* hybridisation (FISH) and **genomic *in situ* hybridisation (GISH)** have been widely used to detect the presence of alien chromatin of different species in a wheat background (He et al., 2017, King et al., 2019a), and to study evolutionary chromosome rearrangements in wheat (Zhang et al., 2021). GISH probes obtained from *T. urartu* accessions can distinguish chromosome segments of *T. urartu* translocated to the B and D genomes of hexaploid wheat, but are unable to distinguish between the A and A^u genomes in an A/A^u translocation (Grewal et al., 2018a, Grewal et al., 2021).

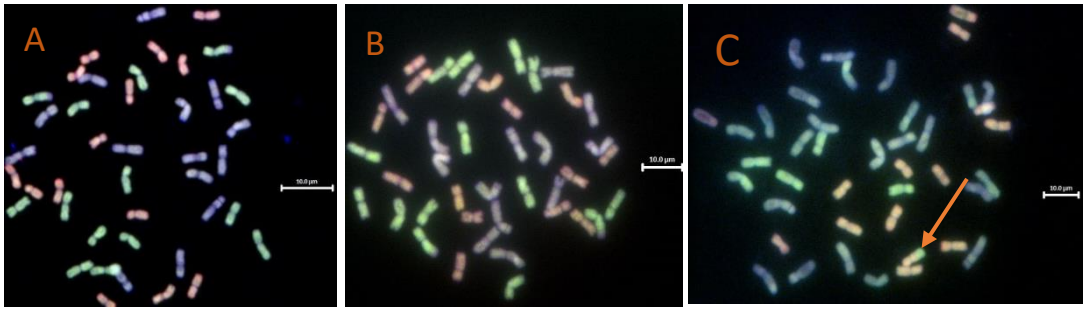

Figure 1.9: GISH from metaphase spreads showing comparisons between chromosomes of wheat and two different wheat-*T. urartu* recombinant lines. Image A shows the chromosomes of hexaploid wheat. Image B shows chromosomes of an F_1 hybrid derived from hexaploid wheat and wheat-*T. urartu* doubled haploid line carrying a heterozygous introgression on the A chromosome. The A chromosome cannot be distinguished from the A^u segments because *T. urartu* is the A genome donor. Image C shows chromosomes of an F_1 hybrid derived from hexaploid wheat and wheat- *T. urartu* DH lines carrying a heterozygous introgression on a D chromosome. A chromosomes = green, B chromosomes = blue, D chromosomes = red, A^u genome = Green

Similarly, FISH probes showing hybridisation sites on the A genome chromosome of *T. urartu* (Zhang et al., 2021, Adonina et al., 2015) cannot clearly distinguish between all A genome chromosomes in hexaploid wheat and cannot distinguish between the A and A^u genomes (Adonina et al., 2015). Despite these challenges, GISH provides a visual way of counting chromosome numbers to check if introgression lines have maintained normal chromosome numbers.

1.3.17 Text box

1.3.17.1 Introgression breeding

Introgression breeding involves the transfer of genetic material from one species into the gene pool of another through hybridization and repeated backcrossing (Anderson, 1953, Thórsson et al., 2001). Introgression lines are generated when homoeologous chromosomes of the two species recombine during meiosis. The polyploid nature of wheat makes it possible to introgress genetic material from its progenitors and wild relatives due to a genetic buffering effect (Dubcovsky and Dvorak, 2007, Hao et al., 2020). Two approaches can be employed in introgression breeding; a whole genome approach or transfer of targeted chromosome segments for specific regions of the genome carrying target genes (Moore, 2014). A transfer of leaf rust resistance genes

from *Ae. umbellulata* into wheat through irradiation was among the earliest introgression breeding reported (Sears, 1956). Further reports on successful introgressions in wheat date back to the 1940s, 1950s and 1970s (Tsunewaki, 1964, Schlegel and Korzun, 1997, Zeller et al., 1973, Riley and Chapman, 1958, O'Mara, 1947) when rye (*Secale cereale* L.) segments were transferred into wheat (Rabinovich, 1997). Important traits such as high yield (Villareal et al., 1995) and resistance to powdery mildew, stem rust and leaf stripe (Rabinovich, 1997, McIntosh et al., 2011) have been associated with the 1BL.1RS translocation**.** Since the successful transfer of *Ae. umbellulata* and rye segments into wheat, several other alien genome segments have been transferred into wheat. King *et al.* (King et al., 2019a) used the whole genome approach to transfer *Am. muticum* segments into wheat which have been exploited for resistance to different species of leaf rust, stem rust and yellow rust (Fellers et al., 2020). A major challenge to introgression breeding is the low level of pairing and recombination between wheat and wild relative chromosomes due to the presence of the *Ph1* (*Pairing Homoeologous 1*) locus located on the long arm of chromosome 5B (Riley and Chapman, 1958, Sears and Okamoto, 1958). *Ph1* controls the pairing of homologues to form bivalents during the process of meiosis (Riley and Chapman, 1958). In introgression breeding, *Ph1* results in univalent chromosomes at metaphase 1 (Gill et al., 1993). Switching off or deletion of the *Ph1* locus was shown to induce homoeologous recombination between wheat chromosomes and related species (Riley and Chapman, 1958). Chinese Spring with the *Ph1* gene deleted has been successfully used in different introgression programs globally. Special cases of *Ph1* suppression have been reported in *Ae. speltoides* and *Am. muticum* (Dover and Riley, 1972, Dvorak et al., 2006). Linkage drag is also another challenge in introgression breeding because it results in the transfer of non-desirable traits (Hospital, 2001). With repeated backcrossing, and shortening of the introgression segments the complexity of transferring alien genetic material into cultivated varieties due to linkage drag can be overcome (Summers and Brown, 2013).

Table 1.12: Some species in the wheat primary, secondary and tertiary gene pool that have been used to exploit specific traits in introgression breeding

Species	**Genome**	**Wheat gene pool**	**Target trait**	**Reference**
Aegilops. umbellulata	U	Secondary	Leaf rust resistance	(Sears, 1956)

Aegilops. tauschii	D	Primary	High grain zinc concentration	(Singh et al., 2017)
Aegilops speltoides	S	Secondary	Powdery mildew resistance	(Hsam et al., 1998)
Aegilops caudata	C	Secondary	High grain zinc concentration	(Wang et al., 2011a)
Thinopyrum intermedium	$J^{vs}J^{r}St$	Tertiary	End use quality	(Li et al., 2013)
Secale cereale	R	Tertiary	Stripe rust resistance	(Li et al., 2020)

1.3.18 Conclusion and future prospects

A narrow genetic base of cultivated wheat is a major challenge limiting wheat improvement and prospects to satisfy future global food demand, which is projected to be 840 million tonnes of wheat by 2050, from current production level of ~700 million tonnes. In addition, effects of climate change and the emergence of new races of pathogens of major diseases of wheat pose further threats to wheat production. Historically, wheat wild species and progenitor species have significantly contributed to resistance genes of important wheat diseases. More recently, the D progenitor (Table 1) of wheat has contributed to traits that increase micronutrient uptake in wheat. Increasing yield to meet future food and micronutrient demand under a changing climate will require development of more cultivars that can adapt to both biotic and abiotic stresses. Good processing, baking and high nutritional value are also important considerations for wheat improvement. With the successes in the transfer of introgressions, and availability of cheaper and high throughput technologies to track these introgressions, *T. urartu* presents a promising genetic resource for tetraploid and hexaploid wheat improvement. Pre-breeding of lines with different *T. urartu* introgressions is a step forward in the utilisation of its variability; however more efforts should be focused on phenotyping these lines under different stress conditions, selecting lines that have desirable traits and further genetic and mapping studies to identify genes or quantitative trait loci (QTLs) underpinning different phenotypic traits (see outstanding question box). More efforts will also be required to transfer the diversity into varieties that are more adaptable (see outstanding questions). Further characterisation and utilisation of the traits reported in this review could potentially contribute to the development of cultivars with novel agronomic and quality traits that will in turn contribute to global food and nutrition security.

1.3.19 Outstanding questions box

- Establishing and maintaining pre-breeding programs for progenitor/wild species of wheat demands substantial financing for activities such as sequencing, genotyping and molecular cytogenetic techniques. To what extent are funding bodies willing to invest in such programmes?
- Are there enough experts or programs to build the capacity of scientists in the field of introgression breeding?
- With a number of introgression lines developed and availability of preliminary testing results published, to what extent are breeders and institutions willing to phenotype the materials for different traits?
- How much effort has the research community put into gene discovery in introgression lines that shows significant phenotypic effects?
- To what extent will genetic engineering be used to fast track the transfer of genes of interest from wheat-*T. urartu* pre-breeding materials to adapted varieties?

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CHAPTER 2

2 General materials and methods

This chapter is a brief overview of the materials and methods for the experiments in this PhD thesis. Detailed description of the methods have been outlined in each chapter's materials and methods section.

2.1 Germplasm

Four different sets of germplasm were used in this study, and these were:

1) Different accessions of wheat progenitors and wild relatives
2) Doubled haploid (DH) lines derived from the wheat progenitor *Triticum urartu* and the wild relative *Amblyopyrum muticum*
3) Three Malawian wheat varieties and three UK reference wheat varieties
4) Malawian wheat/*T. urartu* and Malawian wheat/*Am. muticum* introgression lines.

The wheat wild relatives and progenitors are maintained at the Nottingham *BBSRC* Wheat Research Centre (WRC) at the University of Nottingham, and these were obtained from the Genetic Resource Unit (GRU), at the John Innes Centre (Norwich, UK) and the National Centre for Genetic Resources Preservation, United States Department of Agriculture (Fort Collins, Colorado). The Nottingham WRC developed the *Am. muticum* and *T. urartu* DH lines, for screening of different traits. The *Am. muticum* DH lines were developed by crossing hexaploid wheat cv. Paragon with *Am. muticum* (accessions 2130004, 2130008, and 2130012). The F_1 interspecific hybrid carrying the *Am. muticum*/wheat recombinant chromosome were grown to maturity, and backcrossed as females to Paragon wheat to produce BC_1 plants. The BC_1 plants were recurrently pollinated with Paragon up to BC_3. BC_3 plants were then crossed to maize and the embryos treated with colchicine to produce the *Am. muticum* DH lines, which are homozygous for different *Am. muticum* introgressions (King et al., 2019a, King et al., 2017). *T. urartu* DH lines were developed by crossing hexaploid wheat (*ph1/ph1*) with *T. urartu* (accessions 1010001, 1010002, 1010006, and 1010020). DH lines were produced following the same procedure as *Am. muticum* DH lines (Grewal et al., 2018a).

The hexaploid wheat varieties Paragon, Chinese Spring and Pavon 76 were also obtained at Nottingham WRC, whilst *Kadzibonga*, *Kenya Nyati* and *Nduna*, were obtained from Lilongwe University of Agriculture and Natural Resources in Malawi. These varieties were further sourced from wheat farmers in Tsangano Extension Planning Area (EPA) in Ntcheu district.

The Malawian wheat/*T. urartu* and the Malawian wheat/*Am. muticum* were developed in this study (Chapter 5) by crossing DH-348 (wheat/*Am. muticum*) and DH-254 (wheat/*T. urartu*) with the three Malawian varieties (*Kadzibonga*, *Nduna* and *Kenya nyati*).

2.2 Generating a segregating population

2.2.1 Seed germination and vernalisation

Seeds germination was undertaken in the glasshouse, in module trays using Levington Advance Seed and Modular + Sand (F2 + S) compost (ICL, Suffolk, United Kingdom). For the crossing program, the Malawi varieties were germinated from week one through to week eight while the DH lines were germinated from week five to six. Plants were placed in vernalisation for 4 weeks at 6^0C with a photoperiod of 12 hours (6 am-6 pm). After vernalisation, the seedlings were taken out, potted into 2 litres pots using John Innes compost No. 2 (Westland Horticulture Limited, Dungannon, Northern Ireland), and left under glasshouse conditions (25^0C with a photoperiod of 16 hours light and 8 hours dark).

2.2.2 Emasculation

Emasculation was done before the spikes completely emerged from the flag leaf. All three anthers were removed from the florets to avoid self-pollination. Removal of anthers was done carefully with a pair of forceps to avoid damaging the stigma. After emasculation, the spike was labelled with the name of parent (male and female), and date of emasculation. The emasculated spike was covered with a glassine bag and crossing information was recorded in a crossing notebook.

2.2.3 Pollination

Two days after emasculation, the stigma was checked and it was ready for pollination when it was fluffy/feathered. Bright yellow anthers were collected from a male parent

for pollination of the emasculated spike. Pollen was released onto the stigma using a pair of forceps and the spike was again covered by a glassine bag to protect from any other pollen.

2.3 Genotyping

2.3.1 DNA extraction for genotyping

Three pieces of 1.5×0.5cm leaves were collected into Qiagen sample collection tubes in a 96 well plate. The samples were freeze dried overnight (16 hours) with the tube lid open. A steel ball was added to each sample before crushing with a TissueLyser (QIAGEN Schwingmuhle TissueLyser II, Germany) for 4 minutes. After 4 minutes, the sample blocks were removed and turned through 180 degrees for another 4 minutes shake. 300µl of template preparation solution (TPS) buffer (Appendix 1) was added to each tube and mixed thoroughly. The samples were incubated at 65^0C for 20-30 minutes before ice cooling for 10 minutes. 450µl of isopropanol was added into the tubes to allow DNA to precipitate, and the plate was left in the -20 freezer for 30 minutes. The plate was then centrifuged at 4700rpm for 10 minutes to pellet the DNA. A vacuum was used to remove the supernatant from each row of collection tubes. 200µl of 70% ethanol was added to each collection tube and the DNA plate was spun at 4700rpm for 10-15 minutes and the supernatant removed using a vacuum. The DNA pellet was incubated at 70^0C to remove excess ethanol and the DNA re-suspended in 100ul of sterile water. The samples were placed at -20^0C for 20 minutes to allow the DNA dissolve. DNA was diluted in an AB0765 midi plate using a dilution factor of 1:20.

2.3.2 Adding DNA and primers to 384 plates

DNA was dispensed into 384-well plates using a Gilson pipette max 268 (Gilson, INC. 3000 Parmenter St. Middleton, WI 53562). The DNA plates were centrifuged and dried at 80^0C for 30 minutes. Dried DNA plates were stored at room temperature inside a sealed bag. A primer master-mix was made using water, primers, PACE mix and ROX (3CR BIOSCIENCE, Essex, UK). The master-mix was vortexed and spun briefly to avoid bubbles. 74.1µl of mastermix was dispersed for four markers per plate (4mpp) per well of a source plate, whilst 55µl was dispersed for eight markers per plate (8mpp).

2.4 Genomic *in situ* hybridization (GISH)

2.4.1 Collecting root tips and nitrous oxide treatment

Seeds for GISH were germinated on wet tissues in petri dishes at room temperature, until the roots grew to 2-3cm. Roots (1 cm) were collected in a water moist 0.5ml tube with a hole in the lid. The samples were placed in a nitrous oxide chamber at a pressure of 10 bar for 2 hours. Treated roots were fixed with 90% acetic acid for 10-15 minutes and then washed three times with de-ionised water (dH_2O).

2.4.2 Preparation of metaphase spreads

Metaphase spreads were prepared from root tips using a nitrous oxide-enzymatic maceration method (Kato, 1999). Root tips (1-2 mm) were cut from the nitrous oxide treated roots into a 0.5ml tube containing 20µl of enzyme solution Pectolyase 1% and Cellulase 2% (Yakult Pharmaceutical Ind. Co., LTD. Japan). The samples were digested at 37^0C for 50 min. After digestion, the root tips were washed three times with 70% ethanol and the root tips were then kept in 50µl of 70% ethanol on ice. A dissection needle was used to carefully crush the root tips into a very fine cell suspension. Cell pellets were formed following centrifugation at 6000 rpm for two minutes. The cell pellets were briefly dried and re-suspended in 15-30µl of 100% acetic acid before leaving on ice for 10 minutes to 2hours. 6-7µl of cell suspension was carefully dropped on a glass slide placed in a moist cardboard box. The cardboard was closed to allow the suspension to dry gradually. Slides with good metaphase spreads were chosen using a light microscope.

2.4.3 DNA extraction for GISH and sequencing

Genomic DNA was isolated from young leaves of the three progenitors of wheat (*T. urartu*, *A. speltoides*, *Ae. tauschii*) and *Am. muticum*. Six pieces of young leaf (2-3cm) were collected in a 2ml tube on ice. The samples were freeze dried overnight (16 hours) with the tube lid open. A steel ball was added to each sample, and the samples were crushed using a TissueLyser (QIAGEN Schwingmuhle TissueLyser II, Germany) for 6 minutes at 25/second. 600-800µl of extraction buffer (0.1M Tris-HCl (pH 7.5), 0.05M EDTA (pH 8.0), 10% SDS and water) was added to each tube, and the samples were incubated at 65^{o}C for 1 hour. After 1 hour, the samples were shaken thoroughly and left on ice for 10 minutes to cool down. 300-400µl of ice-cold 6M

ammonium acetate (appendix 1) was added and the samples kept on ice for 15 minutes. The samples were then spun down at 13000 rpm for 10 minutes and 300µl of phenol/chloroform (1:1 V/V) was added to the supernatant. The samples were centrifuged for 5 minutes at 13000 rpm and then 400µl isopropanol was added to allow DNA to precipitate on ice for 15 minutes. The tubes were spun for five minutes at 13000 rpm and the supernatant discarded. The DNA pellet was washed twice with 200-300µl of 70% ethanol, air-dried at 37^{o}C for 5 minutes and re-suspended in 30-50µl dH_2O depending on the size of the DNA pellet

2.4.4 Assessment of DNA quality

DNA quality was checked using a Qubit 2.0 fluorometer (Invitrogen^{TM}) and was estimated in ng/µl. Dilutions were then made using the formula N1 x V1 = N2 x V2, where N is the normality and V is the volume and one and two are the initial and final values respectively.

2.4.5 Probe preparation for multi-colour GISH

2.4.5.1 Nick translation reaction (20ul)/ Probe labelling

Nick translation was carried out using the components in table 3. Reaction volumes for each species was based on DNA concentration.

Table 2.1: Nick translation components

Components	**Volume**
dH_2O	Xµl to 20µl
DNA (Plasmid DNA 3-100kb insert)	2µg
10x Nick translation buffer or 10x buffer2 (NEB)	2.0µl
Non-labelled dNTPs (2mM each, mixed)	2.0µl
Labelled dNTP (1mM)	0.5µl
DNA polymerase I (10U/ul)	5.0µl
DNase (100 mU/ul) diluted (5 ul of 2U/ul DNase	0.8µl

2.4.6 Ethanol precipitation of probes

160µl of single stranded DNA (SS DNA) working solution (Appendix 1) was added to 20µl nick translation probe reaction mix (Table 2.1) and vortexed. 500µl of 3M sodium acetate (Appendix 1) solution ($C_2H_3NaO_2$) was added to the reaction mixture before incubating at -20°C overnight. The tubes were centrifuged at 12,000 rpm for 30 minutes at 4°C and the pellets washed with 70% ethanol. The tubes were centrifuged for another five minutes at 12,000 rpm and the ethanol discarded. The probes were air-dried in the dark for 5-10 min, and the pellet dissolved in 20µl of 2x SSC+1x TE buffer (Appendix 1). The probes were stored at -20°C.

2.4.7 Multicolour GISH

Selected metaphase slides were treated twice with ultraviolet (UV) light at 0.125 Joules to cross-link the chromatin on the slide. A probe mix was prepared using the components below:

Table 2.2: GISH Probe mix (10µl per slide)

Probe mix components	**Volume (X1)**
2xSSC in 1x TE	(X µl make up to 10µl)
T. urartu probe (Alexa fluor-488)	1.5µl
Ae. speltoides probe (Alexa flour 405)	1.5µl
Ae. tauschii probe (Alexa fluor-594)	2.0µl
Am. muticum probe (Alexa Fluor-546)	0.3µl

To probe the slides, 10µl of probe mix (Table 2.2) was added to each slide and covered by a plastic cover slip (22mm x 22mm). Slides were left in a tray in a water bath at 75-80°C for five minutes, and then incubated at 55°C overnight. The slides were dipped into 2x SSC to remove the cover slips and a tissue was used to carefully dry excess liquid. One drop of Vectashield (H-1200, Vector Laboratories, Inc. Burlingame, CA 94010) with 4′, 6-diamidino-2-phenylindole (DAPI) or 1:2 diluted with 1x phosphate buffered saline (PBS) was added to each slide and the slides were covered with 24x50mm glass cover slips.

2.4.8 Capturing GISH images and analysis

A multi-filter Zeiss (microscope) Axio imager (Carl Zeiss Microscopy, Germany) was used to analyse the samples for DAPI, Alexa Fluor-594 (Red), Alexa Fluor-488 (Green) and Alexa Fluor-546 (Gold). Images were captured using a PhotoFluor camera (PhotoFluor LM-75, 89 North Inc. USA) attached to the microscope. The images were analysed using Metafer (automated metaphase image capture) and ISIS (image processing) software (Metasystems GmbH, Altlussheim, Germany).

2.5 Mineral analysis

2.5.1 Sample digestion

Grain samples were digested using a Multicube-48 hot block acid digestion system (Anton Paar Gmbh, Graz, Austria). The digestion block was set at 105^0C for 2h. Samples were diluted with milliQ water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK) up to 50mls. Straw samples were digested using a microwave system comprising a Multi-wave 3000 platform with a 41-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria). The digestion vessels were made up of perfluoroalkoxy (PFA) tubes in polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). 6mls of nitric acid (HNO_3) PrimarPlus-Trace analysis (Fisher Scientific Loughborough, UK) was used to digest the samples. Two tubes of operational blanks, two tubes of certified reference material (CRM-Wheat flour 1567b, NIST, Gaithersburg, MD, USA) and one tube of Laboratory Reference Material (LRM) wheat (Paragon) were included in the digestion run. The samples were digested for 60 minutes at the microwave setting of: 175^0C ramp for 20 minutes, 175^0C hold for 20 minutes, 55^0C for 10 minutes cooling and power 1500 W. After digestion, each tube was made up to a final volume of 24mls by adding 18mls of Milli-Q water, then transferred to a 25ml universal tube (Sarstedt Ltd., Numbrecht, Germany) and stored at room temperature. The samples were further diluted using a dilution factor of 1:10 (1 ml digested sample and 9mls milli-Q water) prior to multi-elemental analysis**.**

2.5.2 Multi-element analysis.

Grain and straw multi-element analysis was undertaken using inductively coupled plasma mass spectrometry as described by Gashu et al. (2021) and Khokar et al. (2019) (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany).

Thirty elements, Zn, Fe, Ca, Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sr, Ti, Tl, U, V and Zn, were analysed. A helium collision-cell (He-cell) operation mode with kinetic energy discrimination was used to reduce polyatomic interferences. Samples were introduced (flow rate 1.2 mL min^{-1}) from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo Fisher Scientific, Bremen, Germany). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 μg L^{-1}), Rh (10 μg L^{-1}), Ge (10 μg L^{-1}) and Ir (5.0 μg L^{-1}) in 2% TAG HNO3 (Primar plus grade; Fisher Scientific UK Ltd). An external multi-element calibration standard (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) was used to calibrate Ag, Al, As, B, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, Tl (semi-quant), U, V and Zn, in the range 0–100 μg L^{-1} (0, 20, 40, 100 μg L^{-1}). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used to create Ca, K, Mg and Na standards in the range 0–30 mg L^{-1}. B, P and S calibration utilised in-house standard solutions (KH_2PO_4, K_2SO_4 and H_3BO_3). Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required. Se was determined separately using a triple quadrupole ICP–MS (iCAP TQ; Thermo Fisher Scientific) using an oxygen cell to mass shift the isotope ^{80}Se to *m*/*z* 96 ($^{80}Se^{16}O$) to reduce interference from the ^{40}Ar dimer. Drift correction was achieved using Rh as an internal standard; calibration used the CLMS-2 multi-element standard (Certiprep).

2.6 Soil analysis

2.6.1 Aqua-regia hot-plate acid digestion

4 grams of soil samples were weighed into digestion tubes and placed on the heating blocks, along with two blanks and one certified reference material (CRM-WEPAL Calc-ISE 850), and a laboratory reference material (Ethiopian soil). 3mls of HNO_3 (trace metal grade) was added into each digestion tube and the samples were incubated overnight. Following the overnight incubation, 9mls of hydrochloric (HCl) acid-trace metal grade (Fisher Scientific, Loughborough, UK) was gently added and the samples were left for 1 hour. Watch glasses were used to cover the digestion tubes and the

heating blocks were set at 108˚C for 2h. The samples were later diluted with milliQ water up to 50 mls. Samples were further diluted in ICP tubes using a dilution factor of 1:10. Multi elemental analysis was performed using ICP-MS.

2.6.2 DTPA-Extractable zinc and iron

10mls of DTPA-TEA solution (appendix 1) was added into 5g of soil in falcon tubes. The suspension was shaken on an head-over-head shaker for 2 h. After shaking, the samples were centrifuged for 10 min at 3000 rpm. The suspension was filtered with 0.22 µm syringe filter and the samples diluted to 1:10 using 2% nitric acid for analysis with ICP-MS.

2.6.3 Determination of total nitrogen- Kjeldahl digestion method

4.4 ml of the Kjeldahl digestion solution (Appendix 1) was added into 0.2 gram of soil in 50 ml digestion tubes. The tubes were placed on the digestion block along with five standards of known concentration. The samples were digested at 360 degrees for 2-3hours and left to settle overnight after diluting with distilled water to the 50 ml mark. 0.5 ml of the samples were added into small vials and 5 ml of N1 solution (appendix 1) was added. After 10 minutes, 5 ml of N2 solution (appendix 1) was added and the samples were left on the bench for 1 hour for colour development. Absorbance was read on UV at a wavelength of 655nm.

2.6.4 Available phosphorus-Mehlich 3 extraction method

25mls of Mehlich 3 extraction (appendix 1) solution was added to 2.5 g of soil in 50 ml centrifuge tubes. The samples were shaken for 5 minutes, and filter papers were used to filter the samples. 1 ml from the filtrate was poured into 20 ml glass vials, 8 ml of the P working solution (appendix 1) was added into the samples, and the samples were left on the bench for 30 min for colour development. Absorbance was read using a Thermo Seotronic Uv-vis spectrophotometer (Helios Alpha, England) at a wavelength of 860.

2.6.5 Determination of soil potassium- Mehlich 3 extraction method

25mls of Mehlich 3 (Appendix 1) solution was added into 2.5g of soil in 50ml centrifuge tubes. The samples were shaken for 5 minutes before filtering with filter papers. To determine the concentration of potassium, 0.5 ml of the sample was diluted

with 19.5mls strontium chloride ($SrCl_2$). The concentration was read on the Atomic absorption spectrometer (Virian Spectra AA20, Australia).

2.6.6 Organic matter or carbon -Walkley and Black method

10mls of potassium dichromate ($K_2Cr_2O_7$) was added into 1g of soil in a conical flask. To speed up the oxidation process, 10mls of concentrated sulfuric acid was added and the samples were left on the bench for 30 minutes for oxidation to take place. 100mls of distilled water was added to the samples to dilute the acids. For titration, 1ml of the diphenylamine indicator (Appendix 1) was added and the samples were titrated with ammonium ferrous sulfate ($FeNH_4SO_4$) until a green color developed. A blank was added to the samples and initial and final readings were used to calculate percentage organic matter using the formula below.

$N_1V_1\ (K_2Cr_2O_7) = N_2V_2\ (FeNH_4SO_4)$

$$N_2 = \frac{N_1V_1}{V_2}$$

The volume of potassium dichromate used in the soil $V_s = (V_{blank} - V_2)\ N_2$

% C in the sample given 1 ml 1 N $K_2Cr_2O_7$ reacts with 0.003 g C

$$\%C = \frac{V_s \text{ X } 0.003 \text{ X } 100}{\text{Wt. of soil used}} \text{ X } 1.33$$

$$\%OM = \%C \text{ X } 1.774$$

N.B: N_1 & V_1 = Normality and volume of potassium dichromate respectively.

N_2 & V_2 = Normality and volume of ferrous ammonium sulphate respectively.

V_{blank} = Volume of ammonium ferrous sulphate used in the blank

2.6.7 Particle size distribution (texture)

70ml of Calgon solution was added into 50 g of soil in shaking bottles. The samples were shaken for 5 minutes, transferred into 1-litre cylinders, and distilled water was added to fill the bottles to the 1-litre mark. The samples were shaken for I minute, and after shaking, a hydrometer was left in the cylinder where clay (< 0.002 mm) and silt (0.05 - 0.002 mm) readings were taken after 5 minutes. After another 5 minutes, readings for sand (2.00 - 0.05 mm) were taken. The samples were left on the bench for 3 hours and a second reading for clay was taken. Readings were taken in a room with

a temperature between 19.5 and 20^0C. Percentage sand, silt, clay and textural class was calculated using the formulas below:

$$\text{Sand/ clay \%} = \frac{\text{particle content}}{\text{Weight of soil}} \times 100$$

Silt % = 100 - (percentage clay + percentage sand)

2.7 Field experiments

Both field experiments were conducted under irrigation at Lilongwe University of Agriculture and Natural Resources (LUANAR -14.18'S 33.76' E), Lilongwe, Malawi. Test lines were planted along with three UK checks (Chinese Spring, Paragon and Pavon 76) and three Malawian wheat checks (*Nduna*, *Kenya nyati* and *Kadzibonga*). The three UK varieties were used as checks as they were used to generate the DH introgression lines. Basal dressing fertiliser 23:10:5 +6S +1Zn (SuperFert Fertilisers, Harare, Zimbabwe) was applied 14 days after planting at a rate of 200kg ha^{-1}. The Malawi government recently approved the NPK (23:10:5 +6S +1Zn) basal fertiliser with 1% Zn due to severe deficiencies (< 2 mg kg^{-1}) of soil Zn across the country (IFDC et al., 2018). Thus, all basal fertiliser blends for selected cereals and legumes in Malawi have 1% Zn. UREA (46% N) was applied three weeks later as top dressing, at a rate of 100kg ha^{-1}. Both basal and top dressing were applied according to the Malawi Guide to Agriculture Production (GAP, 2020). First weeding was done four weeks after planting and subsequent weeding as soon as weeds appeared. Insect pests were controlled by applying Profex Super (Profencfos 40% + Cypermenthrin 4% EC –Kewalram Chanrai group). All the plants were harvested at physiological maturity.

CHAPTER 3

3 Variation in grain micronutrient concentrations of wheat-wild relative accessions, and grain and straw micronutrient concentrations of introgression lines derived from *Amblyopyrum muticum* and *Triticum urartu*

3.1 Abstract

Mineral nutrient deficiencies particularly zinc (Zn), iron (Fe), calcium (Ca) and selenium (Se) are widespread in low and middle-income countries of Sub-Saharan Africa. Genetic biofortification of food crops is considered a sustainable and cost-effective approach for alleviating these deficiencies. Availability of substantial variability in a crop genepool is prerequisite for a successful biofortification program. Thirty-one wild relative accessions from the genus *Triticum*, *Aegilops*, *Thinopyrum*, *Ambryopylum* and *Secale* and five cultivated modern wheat varieties (3 Malawian varieties, Chinese spring and Paragon) were screened for mineral concentration to determine the natural variation between wheat and wild relatives. Grain Zn, Fe, Ca and Se were determined using inductively coupled plasma mass spectrometry (ICP-MS). Mean grain Zn, Fe and Ca concentration varied from 31.3 to 328.1, 26.3-135.7 and 244-2531 mg kg $^{-1}$ respectively, whilst grain Zn, Fe and Ca content varied from 0.4-3.9, 0.3-2.4, 7.9-46.5 µg seed $^{-1}$ respectively. Se concentration varied from 1.7-70.0 µg kg $^{-1}$, whilst Se content varied from 0-0.003 µg seed $^{-1}$. Diploid *Am. muticum* and *T. urartu* accessions had the highest mean grain Zn concentration with five and three-fold higher concentration than cultivated wheat respectively. *Am. muticum* also had high Fe, Ca and Se concentration. Grain Zn and Fe negatively and significantly correlated with grain size, and grain Zn also positively and significantly correlated with grain Fe. Based on the results of the wild relatives, 95 pre-breeding introgression lines (doubled haploids) derived from *T. urartu* and *Am. muticum* accessions were grown in ear rows under field conditions in Malawi. The lines carry different TT and A^uA^u chromosome segments in a Paragon background. Preliminary analysis of mineral elements of 48 lines that grew to maturity showed that grain Zn, Fe and Ca concentration varied from 29.1-88.1, 35.1-105.3, 521-1258 mg kg $^{-1}$ respectively. Se varied from ~0-29.40 µg kg $^{-1}$. 25% and 41% of the lines had higher grain Zn and Fe respectively, compared to Paragon wheat, and this suggestive of the effects of the wild

chromosome segments on mineral concentration. Grain Zn positively correlated with grain Fe and Ca but negatively correlated with grain Se. Correlation analysis suggest that an increase in grain Zn concentration will have a positive effect on grain Fe and Ca.

3.2 Introduction

Inadequate intake or malabsorption of essential micronutrients (minerals and vitamins) by the human body results in malnutrition, also known as "hidden hunger"(Von Grebmer *et al*., 2014). Globally, an estimated 2 billion people are affected by micronutrient deficiencies such as zinc (Zn), iron (Fe), iodine and vitamin A (Bailey et al., 2015, Bhutta, 1998). Fe deficiency, affects around 42% of children and 40% of pregnant women globally (WHO, 2020), and over 17% of the global population are at risk of inadequate Zn intake (Wessells and Brown, 2012). Fe and Zn deficiencies are widespread among women and children in low- and middle-income countries (Gupta *et al.*, 2020), and they are among the major causes of anaemia, stunting, cognitive impairment, adverse pregnancy outcomes, increased susceptibility to diseases and increased child mortality and morbidity rates (Sandstead, 2000, Bailey *et al*., 2015). In 2020, the World Health Organisation (WHO) estimated that ~ 149 million under-five children were stunted, 45 million wasted (FAO, 2022), while 14.6% of children were born with low birthweight in 2015 (Blencowe *et al*., 2019, FAO, 2022). In Sub-Saharan Africa, 3.1% of under five children were stunted in 2019, representing 40% of all stunted children globally (FAO, 2022). Globally, undernutrition has been associated with 45% of deaths among under five children (WHO, 2021) and an economic impact of US$3.5 trillion annually (Panel, 2016a). In Africa, the economic burden of malnutrition is between 3 and 16% of GDP annually (Panel, 2016b).

The estimated prevalence of inadequate intake of other essential micro and macronutrients such as Selenium (Se) and Calcium (Ca) also remain prevalent. In 2011, 3.5 billion people were estimated to be at risk of Ca deficiency (Kumssa *et al*., 2015). In 46 African countries, Ca deficiency risk was estimated at 54% of the population, with 16 of the 46 countries having <95% deficiency risks (Joy *et al*., 2014). Se deficiency in Africa was estimated at 28% and at regional level, deficiency risks of up to 52% were reported (Joy *et al*., 2014). In Malawi, for instance, an estimated 97% of the population are at risk of dietary Ca deficiency (Kumssa et al., 2015), >80% Se deficiency (Hurst *et al*., 2013), and among women of reproductive age, Se deficiency risks varies between 34-62% depending on social economic status (Phiri *et al*., 2019).

Unfortunately, limited diversity in diets and heavy reliance on cereals and roots and tubers in low- and middle -income countries exacerbate micronutrient deficiency risks

(Ruel and Bouis, 1998). It is well documented that cereals and root and tuber crops provides enough calories to meet energy needs of the people globally (Chandrasekara and Josheph Kumar, 2016, Lafiandra *et al*., 2014). However, these are unable to deliver adequate essential nutrients required by the human body, because they are inherently low in micronutrient concentration, and, they have high level of anti-nutritional factors that affect Zn and Fe bioavailability (Ruel and Bouis, 1998, Velu *et al.*, 2014). For example, tropical maize (*Zea mays L*.) varieties grown in three diverse agro-ecologies in West Africa showed less than 20 mg kg $^{-1}$ grain Zn and not more than 20 mg kg $^{-1}$ Fe (Oikeh *et al*., 2003). Cassava (*Manihont esculenta*) storage roots from cassava clones showed a range of 4-18 mg kg $^{-1}$ Zn (Maziya-Dixon et al., 2000), while most modern cultivated wheat varieties have a baseline Zn concentration of 25 mg kg $^{-1}$ (Bouis and Welch, 2010). In countries that depend on cereals and roots and tubers crops for staple food, these concentrations are inadequate to meet the WHO estimated average requirement (EAR) for Zn (~7-11 mg/d) in both children and pregnant and non-pregnant adults (Gibson *et al*., 2016).

Wheat is one of the target cereal crops for Zn biofortification (Bouis and Welch, 2010). Interestingly, increasing grain Zn concentration could potentially increase Fe concentration, as they have previously shown to have pleiotropic effects (Velu *et al*., 2017a, Tiwari *et al*., 2009a, Velu *et al.*, 2019, Crespo-Herrera *et al.*, 2016, Wang *et al*., 2021). One key element for a successful genetic biofortification program is availability of genetic variation within the gene pool of a target crop. Wheat domestication, polyploid speciation and constant selection, particularly related to yield, resulted in loss of genetic diversity in modern wheat (Dubcovsky and Dvorak, 2007, Matsuoka, 2011). However, its progenitors and wild species are a major target (Monasterio and Graham, 2000, Guzman et al., 2014, Velu et al., 2014). Studies have shown significantly higher levels of Fe and Zn in a number of progenitors and wild species compared to modern wheat varieties. For instance, *Aegilops* and wild *Triticum* species contain significantly higher grain Zn and Fe compared to cultivated wheats (Chhuneja et al., 2006, Rawat et al., 2009). Similarly, other wheat/wild relative derivatives and synthetic hexaploid wheats from *Triticum* durum and *Aegilops tauschii* were reported to have high levels of grain Zn concentration compared to their wheat parents (Calderini and Ortiz-Monasterio, 2003, Tiwari et al., 2010a, Farkas et al., 2014, Singh et al., 2017). Although different wheat progenitors and wild species have been

exploited for grain Zn and Fe concentration, the gene pool of wheat contains a lot more species in different accessions that could potentially be utilised as a source of novel alleles for increasing essential micro and macronutrients in modern cultivated wheat.

Therefore, the aim of this work was to:

(i) Analyse the grain mineral concentration of different wild species accessions maintained at Nottingham BBSRC Wheat Research Centre.

(ii) Assess the differences in grain mineral concentration between cultivated wheat and wheat wild species as a basis for improving modern cultivated wheat varieties.

(iii) Phenotype derivatives of the wild species showing high mineral concentration in objective (1), in order to identify lines with high mineral concentration under field conditions.

3.3 Materials and methods

3.3.1 Exploiting the natural variation of wheat and wild species - germplasm

Thirty-one different wild relative accessions of *Triticum timopheevii*, *Secale cereale*, *Triticum urartu*, *Aegilops speltoides*, *Amblyopyrum muticum*, *Aegilops caudata*, *Thinopyrum ponticum*, *Thinopyrum elongatum*, *Thinopyrum bessarabicum* and *Aegilops tauschii* (Table 3.1) were analysed for grain mineral concentration along with three Malawian wheat varieties (*T. aestivum* vars. *Kadzibonga, Kenya nyati and Nduna*) and two UK reference materials (*T. aestivum*, cvs. Chinese Spring and Paragon). Malawian wheats were sourced from Lilongwe University of Agriculture and Natural Resources (LUANAR) and these were further sourced from farmers in Tsangano extension planning area (Tsangano, Ntcheu). The wild relatives maintained at the Nottingham BBSRC Wheat Research Centre (WRC) at the University of Nottingham were obtained from the Genetic Resource Unit (GRU), at the John Innes Centre (Norwich, UK) and the National Centre for Genetic Resources Preservation, United States Department of Agriculture (Fort Collins, Colorado). The species were multiplied once or twice under glasshouse conditions at the University of Nottingham. Briefly, Paragon, Chinese Spring and the wild species and were germinated in moduled trays using Levington Advance Seed and Modular + Sand (F2 + S) compost (ICL, Suffolk, United Kingdom). After 7 days, seedlings were placed into vernalisation for 8 weeks at 6˚C with a photoperiod of 12 hours (6 am-6 pm). After vernalisation, the seedlings were taken out, potted into 2 litres pots using John Innes compost No. 2 (Westland Horticulture Limited, Dungannon, Northern Ireland) and left under glasshouse conditions (25˚C with a photoperiod of 16 hours light and 8 hours dark).

Table 3.1: Description of the wild relatives

Genus	Species	Source	No. of accessions	Genome	Ploidy level
Triticum	*T. timopheevii*	USDA	6	A^tG	Tetraploid
Triticum	*T. urartu*	USDA	4	A^u	Diploid
Aegilops	*Ae. speltoides*	JIC	3	S	Diploid
Aegilops	*Ae. caudata*	JIC	2	C	Diploid
Aegilops	*Ae. tauschii*	USDA	1	D	Diploid
Thinopyrum	*Th. bessarabicum*	USDA	1	E^b/J^b	Diploid
Thinopyrum	*Th. ponticum*	USDA	2	$E^b/E^e/E^x/J$	Decaploid
Thinopyrum	*Th. elongatum*	USDA	3	$E^eE^jE^xE^{St}E^{St}$	Decaploid
Amblyopyrum	*Am. muticum*	JIC	3	T	Diploid
Secale	*S. cereale*	USDA	6	R	Diploid

3.3.2 Field experiment- germplasm

Thirty-five *T. urartu* and 60 *Am. muticum* doubled haploid (DH) lines were sourced from the WRC. The lines were developed by crossing *ph1/ph1* hexaploid wheat (var., Paragon) with *Am. muticum* (accessions 2130004 and 2130012) and *T. urartu* (accessions 1010001, 1010002 and 1010006) respectively. The F_1 interspecific hybrid carrying the *Am. muticum*/wheat and the *T. urartu*/wheat recombinant chromosome were backcrossed as females to Paragon up to the BC_3 generation, which was used to produce the DH lines (King et al., 2017, Grewal et al., 2018a). The introgression lines were selected based on availability of seed at WRC seed store. Three UK reference materials; *T. aestivum* cvs. Paragon and Chinese Spring and three Malawian wheat varieties; *T. aestivum* vars. *Kadzibonga*, *Kenya nyati* and *Nduna* were used as checks.

3.3.3 Experimental design and trial management

The experiment was conducted under field conditions at Lilongwe University of Agriculture and Natural Resources (LUANAR -14.18'S 33.76' E), Lilongwe, Malawi. Sixty-two wheat/*Am. muticum* DH lines and thirty-three wheat/*T. urartu* DH lines were planted along with two UK and three Malawian wheat checks. Planting was done

in ear rows of 10 seeds, spaced 10 cm apart with a row spacing of 15 cm. Basal dressing fertiliser 23:10:5 +6S +1Zn (SuperFert Fertilisers, Harare, Zimbabwe) was applied 14 days after planting at a rate of 200kg/ha, and 3 weeks later UREA (46% N) was applied as top dressing at a rate of 100kg/ha. Basal and top dressing were applied according to the Malawi guide to agriculture production (GAP, 2020) guidelines. First weeding was done 4 weeks after planting and subsequent weeding as soon as weeds appeared. Insect pests were controlled by applying Profex Super (Profencfos 40% + Cypermenthrin 4% EC –Kewalram Chanrai group). All the plants were harvested at physiological maturity. Agronomic and phenological data was collected from the six middle plants in the ear rows. Data collected included; Days to heading (DH), days to flowering (DF) and tiller numbers. Number of tillers were collected from three randomly selected plants in the net plot, and the average was calculated.

3.3.4 Mineral analysis

3.3.4.1 Sample preparation and digestion

All the wild accession samples were threshed manually and handled carefully to avoid contamination. For the field experiment, clean and unbroken grain samples were carefully packaged in small brown envelopes after threshing. Straw samples were oven dried (Memmert oven 100-800, Memmert GmbH Co.Kg) at 75 °C for 24 hours. Each sample was then ground using a laboratory mill (Petern LM 3610, Hagersten, Sweden), which was wiped clean before and after adding each sample. After grinding, each sample was packed in small zip lock bags. Both grain and straw sample from the field experiment were shipped to the University of Nottingham.

Wild relative accessions and straw samples from the field experiment were digested using a microwave digestion platform in 2019 and 2021 as outlined in Gashu et al. (2021). Briefly, 0.2 g of each of the grain samples and 0.2 g each of finely ground straw samples was weighed in pressure-activated venting vessels (56-ml 'SMART VENT', Anton Paar). Two reference materials (RMs) for grain samples (CRM-wheat flour 1567b and LRM-Paragon wheat), three RMs for straw samples (CRM-Tom-1573a, BCR-Hay 129 and LRM-cabbage) and two operational blanks were also included in each run. 6 ml of >68 PrimarPlus trace-analysis-grade nitric acid (HNO_3) was added to the samples, and the samples were digested in a Multiwave PRO

microwave with 41-vessel digestion rotor (41HVT56). For digestion, the microwave was set at 1,500 W, 10 min heating to 140 °C, 20 min holding at 140 °C, and 15 min cooling to 55 °C. Following digestion, each tube was made up to a final volume of 24ml by adding 16 ml Milli-Q water, then transferred to a 25-ml universal tube (Sarstedt).

Grain samples from the field experiment were digested in 2021 using a 48 multicube hot block acid digestion system (Anton Paar Gmbh, Graz, Austria). ~0.4 g of each of the grain samples along with certified reference material (wheat flour 1567b-CRM) and laboratory reference material (Paragon wheat-LRM) were weighed into 50ml Anton Paar digestion tubes. The digestion tubes together with 2 operational blanks were placed on a Multicube 48 digestion block (Anton Paar Gmbh, Graz, Austria). The samples were soaked overnight (16 hours) in 8mls of nitric acid (HNO_3). For digestion, the digestion block was set at 105˚C for 2h. Samples were left to cool down for 10 minutes before adding milliQ water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK) up to 50mls. Both grain and straw samples were further diluted in ICP tubes using a dilution factor of 1:10 (1ml sample and 9mls milliQ water) before analysis.

3.3.4.2 Multi-elemental analysis

Grain and straw multi-element analysis was undertaken using inductively coupled plasma mass spectrometry as described by Gashu et al., 2021 and Khokar et al., 2019 (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany). Thirty elements including; Zn, Fe, Ca, Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sr, Ti, Tl, U, V and Zn were analysed. In the first experiment, 36-grain samples (wheat and wheat wild relatives) were analysed along with blanks and CRMs. In the field experiment, 52-grain samples and 52-straw (not replicated) samples along with blanks and CRMs were analysed. The Zn, Fe, Se and Ca-specific recovery from CRMs for wild relative grain samples, was 83%, 88%, 93% and 95% respectively. The Limit of Detection (LOD) values for grain Zn, Fe, Ca and Se were 2.1, 1.2, 3.6 and 0.0016 respectively. For the field experiment, the mineral-specific recovery values from CRMs for grain samples was 90% Zn, 79% Fe, 95% Se and 95% Ca, and the LOD values were 0.8, 0.7, 9.2 and 0.005 respectively. For straw samples, Zn and Se-specific recovery from CRMs was 92% and 94%

respectively, and the LOD values were 0.3 and 0.072 respectively. Mineral content was calculated on dry weight basis, and final content was converted to µg/seed.

3.3.5 Grain area, length, width and seed weight

Grain area, length and width were measured at Rothamsted Research using a MARVIN- Digital Seed Analyzer SN 176 (Marvitech—Germany). One hundred and fifty seeds from each accession were analysed. Seed weight was measured using am EK-3000i digital balance (A and D Instruments LTD, Oxford, UK)

3.4 Data analysis

Descriptive statistics and Pearson correlation analyses were performed in XLSTAT 2022.3.1 (Addinsoft, 2022). Graphs were plotted in Rstudio 2022.07.1-554 (RStudioTeam, 2020).

3.5 Results

3.5.1 Mineral concentration and content of grain samples of cultivated wheat and wheat-wild relative accessions

Thirty different mineral elements were determined using ICP-MS. This chapter captures data for three microelements (Zn, Fe, and Se) and one macro element (Ca) that are of particular importance to human nutrition, and whose deficiency risks are particularly high in low and middle-income countries of Sub-Saharan Africa.

3.5.1.1 Grain zinc

Grain Zn concentration of the wild species varied from 46.6 to 328.1 mg kg $^{-1}$, while grain Zn concentration of the cultivated wheats varied from 31.3-90.9 mg kg^{-1}(Table 3.2). *Am. muticum* accessions had the highest grain Zn ranging from 179.1 to 328.1 mg kg $^{-1}$ with accession 213008 having the highest concentration. *T. urartu* accessions had the second highest grain concentration, which varied from 141.1-102.0 mg kg $^{-1}$. Accessions of *Ae. speltoides*, *Ae. caudata* and two accessions of *T. timopheevii* (427998 and 538512) also had high grain Zn concentration with each species having up to 100 mg kg $^{-1}$. Among the wild relatives, *Th. bessarabicum* and two *S. cereale* accessions had the lowest grain Zn concentration (< 50 mg kg $^{-1}$). Among the cultivated wheat, Malawian wheat varieties had the lowest mean grain Zn ranging from 31.3-41.4mg kg^{-1}. Paragon and Chinese Spring had a concentration of 66.6 and 90.9 mg kg^{-}

^{1} respectively. Zn content of the wild species varied from 0.4 u to 2.7 µg seed $^{-1}$, while grain Zn content of the cultivated wheats varied from 1.4-3.9 µg seed $^{-1}$ (Table 3.2). Among the wild relative accessions, *S.cereale* 426170, *S. cereale* Blanco, *T. timopheevi* P99-95-1-1, *Am. muticum* 2130008, *T. urartu* 101002 and *T. timopheevi* 427998 had the highest grain Zn content with 2.7, 2.4,2.3, 2.2.2.2 and 2.1 µg seed $^{-1}$ respectively. *T. ponticum 531737* (0.5 µg seed $^{-1}$) and *T. bessarabicum* (0.4 µg seed $^{-1}$) had the lowest grain Zn content

3.5.1.2 Grain iron

Grain Fe concentration of the wild species varied from 26.3 to 135.7 mg kg^{-1}, while Fe concentration of the cultivated wheats varied from 40.7 to 56.3 mg kg^{-1} (Table 3.2). As with grain Zn, *Am. muticum* accessions had the highest Fe concentrations ranging from 104.1 to 135.7 mg kg $^{-1}$, and accession 213008 had the highest Fe concentration. *Ae. tauschii* accession P95-81-1-1 had Fe concentration of 80.2 mg kg $^{-1}$, and it was the second highest grain Fe concentration from the *Am. muticum* accessions. Mean Fe concentration of *T. urartu*, *Ae. speltoides* and *Ae. caudata* accessions were 67.9, 61.8 and 60, 2 mg kg $^{-1}$ respectively. Among the wild relative accessions, *Th. bessarabicum* and three-*S. cereale* (Palazzo, Brassetto and 426170) accessions had the lowest mean grain Fe concentration (26.3-30.5mg kg $^{-1}$). Among the cultivated wheat, *Kenya nyati* and *Nduna* had the lowest concentration, while *Kadzibong*a, Chinese Spring and Paragon had the highest concentration.

Grain Fe content of the wild species varied from 0.3- 2.3 µg seed $^{-1}$, while Fe content for the cultivated wheat varied from 1.8-2.4 µg seed $^{-1}$ (Table 3.2). Among the wild species, *T. timopheevi* P99-95-1-1 (2.3 µg seed $^{-1}$) and *S.cereale* 426170 (2.1 µg seed $^{-1}$) had the highest grain Fe content, whilst *T. ponticum 531737* (0.4 µg seed $^{-1}$) and *T. bessarabicum* (0.3 µg seed $^{-1}$) had the lowest grain Fe content.

Table 3.2: Variation in grain Zn and Fe concentrations and content of cultivated wheat varieties and wheat wild relative accessions. The accessions have been ordered according to grain Zn concentrations (highest to lowest)

Genotypes	Zn concentration (mg/kg)	Zn content (µg /seed)	Fe concentration (mg/kg)	Fe content (µg /seed)
Am. muticum 2130008	328.1	2.2	135.7	0.9
Am. muticum 2130012	184.2	1.0	111.5	0.6
Am. muticum 2130004	179.1	1.1	104.1	0.6

T. urartu 101006	141.1	1.7	71.4	0.9
T. urartu 101002	140.5	2.2	69.3	1.1
T. urartu 101001	132.0	1.8	73.6	1.0
T. urartu 1010020	102.0	1.0	57.1	0.5
Ae. speltoides 2140020	121.0	1.0	74.1	0.6
Ae. speltoides 2140018	113.4	1.1	67.1	0.7
Ae. speltoides 2140008	100.6	1.0	44.3	0.4
Ae. caudata 2090002	122.2	1.3	59.0	0.6
Ae. caudata 2090001	95.3	0.9	61.4	0.6
T. timopheevii 427998	106.5	2.1	62.4	1.2
T. timopheevii 538512	104.4	1.3	61.2	0.8
T. timopheevii 355452	88.2	1.4	78.9	1.3
T. timopheevii 427414	78.1	1.1	60.0	0.8
T. timopheevii 289752	64.4	1.4	53.0	1.1
T. timopheevii P95-99-1-1	54.3	2.3	53.9	2.3
Th. elongatum 401007	96.7	0.9	68.5	0.6
Th. elongatum 401008	73.7	0.7	64.9	0.6
Th. elongatum 40116	69.1	0.8	56.5	0.6
T. tauschii P95-81-1-1	77.7	0.8	80.2	0.8
Th. ponticum 547312	74.6	0.7	65.9	0.6
Th. ponticum 531737	62.7	0.5	54.5	0.4
S. cereale 426170	89.2	2.7	70.7	2.1
S. cereale Blanco	77.0	2.4	43.0	1.3
S. cereale 428373/107	65.1	1.5	55.7	1.3
S. cereale 390382	59.8	1.9	26.3	0.8
S. cereale Brassetto	46.6	1.9	35.0	1.4
S. cereale Palazzo	38.7	1.5	34.3	1.3
Th. bessarabicum 531711	46.6	0.4	30.7	0.3
Chinese spring	90.9	3.9	55.4	2.4
Paragon	66.6	2.4	56.3	2.1
Kenya nyati	41.4	1.8	42.3	1.8
Kadzibonga	38.9	1.8	52.2	2.4
Nduna	31.3	1.4	40.7	1.8
Minimum	31.3	0.4	26.3	0.3
Maximum	328.1	3.9	135.7	2.4
Median	83.2	1.5	59.5	1.1
Mean	94.5	1.3	62.0	0.8
Standard deviation (n-1)	54.5	0.7	21.8	0.6

3.5.1.3 Grain calcium

Grain Ca concentration of the wild species varied from 244 to 2531 mg kg $^{-1}$ (Table 3.3). *Am. muticum* accessions had the highest Ca concentration ranging from 1878 to 2530 mg kg $^{-1}$ with accession 2130012 showing the highest concentration. Although

most of the species in the *Thinopyrum* genus had lower Zn and Fe concentration compared to the other wild species, their Ca concentration was much higher. *Th. ponticum* 531717 and 547312 had 2020 and 1379 mg kg $^{-1}$ respectively. All three *Th. elongatum* accessions had grain Ca above1500 mg kg $^{-1}$ while *Th. bessarabicum* 1464 mg kg $^{-1}$. Among the wild species, *T. timopheevii* P95-99-1-1 and the majority of the *S. cereale* accessions had the lowest Ca concentration [1]. Interestingly, the Malawian wheat varieties had high Ca concentration ranging from 900 to 1013 mg kg $^{-1}$ while the UK reference materials had the lowest Ca concentration ranging from 281 to 354 mg kg $^{-1}$.

Grain Ca content for the wild species varied from 7.4 – 17.1 µg seed $^{-1}$, whilst Ca content for the cultivated wheat varied from 12.2-46.5 µg seed $^{-1}$ (Table 3.3). Among the wild relative accessions, *Th. elongatum* 40116, *S. cereale* Blanco, *Th. Ponticum* 531737 and *Th. elongatum* 401008 had the highest grain Ca content with 17.1, 17.0,16.2 and 15.5 µg seed $^{-1}$ respectively. *Ae. speltoides* 2140020 (8.8 µg seed $^{-1}$), *T. timopheevi* 5385120 (8.0 µg seed $^{-1}$) and *T. urartu* 1010020 (7.4 µg seed $^{-1}$) had the lowest grain Zn content.

3.5.1.4 Grain Selenium

Selenium concentration was generally low in all the accession varying from 1.7 – 68.3 µg kg $^{-1}$ (Table 3.3). Malawian varieties had the highest Se concentration ranging from 18-70 µg kg $^{-1}$ with variety Nduna showing the highest grain Se concentration. Among the wild relatives, *Ae. caudata* 2090001 and *Am. muticum* 2130012 had the highest grain selenium concentration with 63.8 and 42.0 µg kg $^{-1}$ respectively. A number of *S. cereale* accessions, *Ae. caudata* 2090001, *T. urartu* 100020. *T. timopheevi* 538512, *Th. ponticum* 547312 and *Th. bessarabicum* 531711 had less than five µg kg $^{-1}$ respectively. Paragon and Chinese Spring had a selenium concentration of 3.7 and 11.1 µg kg $^{-1}$ respectively. Se content was very low in all the grain samples, although Malawian varieties, *Kenya nyati* (0.0031 µg seed $^{-1}$) and *Nduna* (0.0030 µg seed $^{-1}$) had the highest Se content.

Table 3.3: Variation in grain Ca and Se concentrations and content of cultivated wheat varieties and wheat wild relative accessions.

Genotypes	Ca concentration (mg/kg)	Ca content (µg/seed)	Se concentration (µg/kg)	Se content µg/seed
Am. muticum 2130008	1872	12.5	30.2	0.0002
Am. muticum 2130012	2531	13.5	42.0	0.0002
Am. muticum 2130004	2060	12.4	26.9	0.0002
T. urartu 101006	778	9.3	29.9	0.0004
T. urartu 101002	921	14.1	21.9	0.0003
T. urartu 101001	647	9.1	33.9	0.0005
T. urartu 1010020	790	7.4	3.8	0.0000
Ae. speltoides 2140020	1012	8.8	13.6	0.0001
Ae. speltoides 2140018	1169	11.7	6.8	0.0001
Ae. speltoides 2140008	914	9.1	27.9	0.0003
Ae. caudata 2090002	1211	12.9	4.9	0.0001
Ae. caudata 2090001	1093	10.2	63.8	0.0006
T. timopheevii 427998	741	14.8	16.9	0.0003
T. timopheevii 538512	631	8.0	3.1	0.0000
T. timopheevii 355452	886	14.2	14.2	0.0002
T. timopheevii 427414	752	10.5	5.9	0.0001
T. timopheevii 289752	458	9.8	18.4	0.0004
T. timopheevii P95-99-1-1	244	10.4	23.5	0.0010
Th. elongatum 401007	1580	14.7	26.1	0.0002
Th. elongatum 401008	1553	15.5	13.9	0.0001
Th. elongatum 40116	1513	17.1	22.8	0.0003
T. tauschii P95-81-1-1	982	9.8	12.4	0.0001
Th. ponticum 547312	1379	12.9	1.9	0.0000
Th. ponticum 531737	2020	16.2	32.6	0.0003
S. cereale 426170	356	10.7	14.0	0.0004
S. cereale Blanco	553	17.0	1.7	0.0001
S. cereale 428373/107	621	14.5	9.0	0.0002
S. cereale 390382	316	10.1	1.8	0.0001
S. cereale Brassetto	341	13.6	2.5	0.0001
S. cereale Palazzo	379	14.4	3.9	0.0001
Th. bessarabicum 531711	1464	13.7	7.2	0.0001
Chinese spring	281	12.2	11.1	0.0005
Paragon	354	13.0	3.7	0.0001
Kenya nyati	900	39.0	17.6	0.0008
Kadzibonga	993	46.3	67.0	0.0031
Nduna	1074	46.5	70.0	0.0030
Minimum	244	7.4	1.7	0.000
Maximum	2531	46.5	70.0	0.003
Mean	907	14.9	14.1	0.000
Median	982	12.9	19.6	0.000
Standard deviation (n-1)	560	9.3	18.0	0.001

3.5.2 Relationship between grain Zn, Fe, Ca and Se concentration and content of cultivated wheat varieties and wheat wild relative accessions

Regression analysis (Figure 3.1) did not show any association between grain Fe (R^2 = 0.044, P = 0.219) and Zn (R^2 = 0.007, P = 0.629) content and concentration, although a few accessions showing high Zn and Fe concentration also showed high Zn and Fe content.

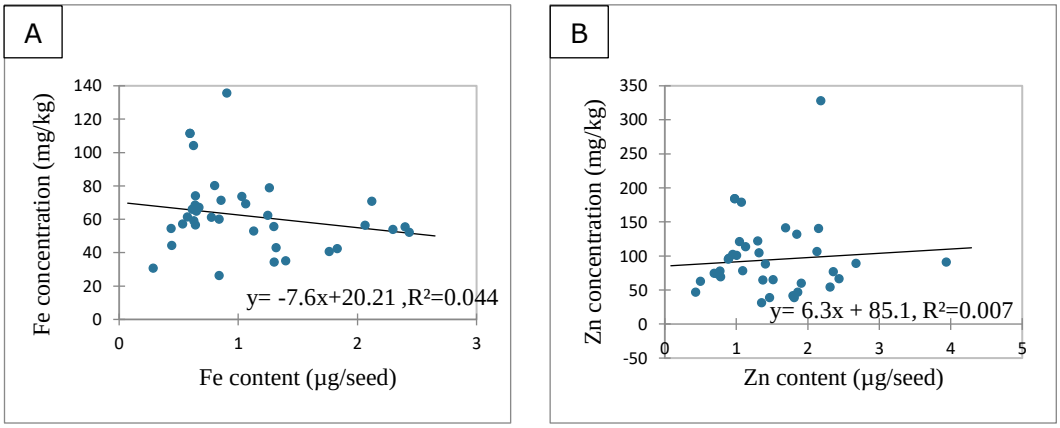


Figure 3.1: Regression analysis between (A) grain Fe concentration and content (B) and Zn concentration and content of wild relative accessions and cultivated wheat

Grain Se concentration showed a significant association (Figure 3.2) with grain Se content (R^2 = 0.571, P = <0.0001), whilst grain Ca concentration did not show any association with grain Ca content (R^2 = 0.009, P = 0.574).

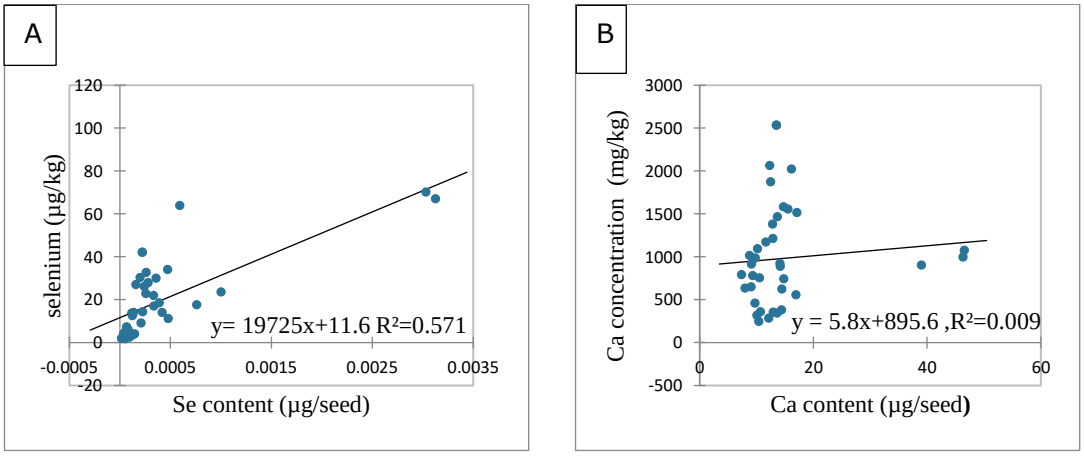


Figure 3.2: Regression analysis between (A) grain Se concentration and content (B) and Ca concentration and content of wild relative accessions and cultivated wheat

3.5.3 Seed size analysis

Grain area, length and width of the wild accessions and cultivated wheat varied from 9.0-21.2, 5.3-8.8 and 2.2-3.1 mm^2 respectively (Table 3.4). *Am. muticum* accessions had the smallest area (9.0-9.6 mm^2), followed by *Th. ponticum* accessions (11.0-11.5) and *Th. bessarabicum* (11.8 mm^2). Seed length for the *Am. muticum* accessions ranged from 5.3-5.5 mm^2 and 6.9-8.0 for *Th. ponticum* and *Th. bessarabicum* accessions. Grain area for all the *Ae. speltoides* accessions, three *T. urartu accessions, one Ae. tauschii accession* and two *Th. elongatum* accessions ranged from 12.0-13.0 mm^2 with a seed length of 6.0-8.0 $mm^{2.}$ The cultivated wheat varieties, five *S. cereale* accessions and two *T. timopheevi* accessions had the biggest grain area, ranging from 17-21 mm^2. Grain length for these genotypes varied from 6.4-8.9 mm^2. *Am. muticum* accessions also had the lowest grain weight, varying from 0.8-1.0 g and grain weight for the cultivated wheat varieties, five *S. cereale* accessions and two *T. timopheevii* varied from 4.8-7.0 g.

Table 3.4: Grain area, length, width and weight of 31 wild relative accessions and 5 cultivated wheat varieties. The accessions have been ordered according to mean area (lowest to highest)

Species	Observations	Area(mm²)		Length(mm)		Width(mm)		Weight (g)
		Mean	Standard deviation (n-1)	Mean	Standard deviation (n-1)	Mean	Standard deviation (n-1)	
Am. muticum 2130004	54 (150)	9.0	3.3	5.3	0.8	2.2	0.6	0.9
Am. muticum 2130012	124 (150)	9.5	1.7	5.5	0.8	2.3	0.4	0.8
Am. muticum 2130008	114 (150)	9.6	1.6	5.4	0.6	2.3	0.4	1.0
Th. ponticum 531737	150	11.0	1.7	6.9	0.8	2.0	0.3	1.2
Th. ponticum 547312	150	11.5	1.5	7.2	0.7	2.0	0.4	1.4
Th. bessarabicum 531711	150	11.8	2.4	8.0	1.0	1.9	0.4	1.4
Ae. tauschii P95-81-1-1	150	12.0	2.1	6.1	0.7	2.6	0.4	1.5
Ae. speltoides 2140020	150	12.0	2.1	7.1	0.9	2.1	0.2	1.3
Ae. speltoides 2140007	150	12.1	2.3	6.9	0.8	2.2	0.3	1.3
Ae. speltoides 2140018	150	12.5	2.3	7.2	0.7	2.1	0.4	1.5
Th. elongatum 401007	150	12.5	1.7	7.2	0.7	2.1	0.3	1.4
T. urartu 100006	150	12.5	2.1	7.2	0.8	2.3	0.4	1.8
Th. elongatum 401008	150	12.7	2.6	7.6	1.1	2.1	0.6	1.5
T. urartu 100020	150	12.7	2.2	8.0	0.3	2.1	0.7	1.4
T. urartu 100001	150	13.0	2.2	7.4	0.6	2.3	0.3	2.1
Ae. speltoides 2140008	150	13.3	2.6	7.3	0.8	2.3	0.4	1.5
T. urartu 100002	150	13.4	2.6	7.4	0.8	2.4	0.4	2.3
Th. elongatum 40116	150	13.8	2.7	7.3	0.9	2.3	0.4	1.7
Ae. caudata 2090001	150	14.4	2.3	8.5	0.9	2.1	0.4	1.4
S. cereale 248373/107	150	14.6	2.3	7.1	0.6	2.5	0.3	3.5
Ae. caudata 2090002	150	14.7	3.1	8.5	0.9	2.2	0.5	1.6
T. timopheevii 355452	150	15.0	2.2	8.5	0.8	2.2	0.2	2.4
T. timopheevii 538512	150	15.5	2.6	9.0	1.0	2.2	0.2	1.9

S. cereale 426170	150	16.3	3.7	7.1	1.0	2.9	0.4	4.5
T. timopheevii 427998	150	16.6	3.7	8.8	1.1	2.4	0.4	3.0
T. timopheevii 427414	150	16.9	3.5	9.5	1.3	2.3	0.3	2.1
T. timopheevii 289752	150	17.1	2.6	8.1	0.7	2.8	0.3	3.2
S. cereale Blanco	150	17.3	3.1	7.4	0.8	2.9	0.3	4.6
T. aestivum cv Paragon	150	17.6	3.1	6.4	0.5	3.5	0.5	5.5
T. aestivum cv Chinese Spring	150	17.6	3.3	6.3	0.6	3.5	0.4	6.5
T. aestivum cv *Nduna*	150	18.1	2.9	6.4	0.5	3.6	0.4	6.5
T. aestivum cv *Kenya nyati*	150	19.1	4.7	6.9	0.7	3.5	0.6	6.5
S. cereale Palazzo	150	20.1	3.6	8.2	0.7	3.0	0.4	5.7
S. cereale Brassetto	150	20.1	3.7	8.1	0.7	3.1	0.5	6.0
T. aestivum cv Kadzibonga	150	20.9	3.7	7.1	0.6	3.8	0.4	7.0
S. cereale 390382	150	21.0	3.8	8.9	1.1	3.0	0.4	4.8
T. timopheevii P95-991-1-1	150	21.2	5.4	8.8	1.2	3.1	0.5	6.4

Note: *Each Am. muticum* accession had 150 seeds. However, due to the small size of the seeds, the Marvin only measured 54 seeds for accession 2130004, 124 seeds for accession 2130012 and 114 for accession 2130008. Therefore, seed weight was based on the 150 seeds and not the Marvin measured number of seeds.

3.5.4 Relationship between grain zinc, iron and grain area, length, width, and weight

There was a significant negative correlation (Table 3.5) between grain Zn and grain area (r = -0.633, P = <0.0001), grain weight (r = -0.544, P = 0.001), grain length (r = -4.38, P = 0.007) and grain width (r = -3.85, P = 0.007). Correlation analysis also showed a highly significant positive correlation between grain Zn and grain Fe (r = -0.869, P = <0.0001). Grain Fe also negatively and significantly correlated with grain area (r = -0.677, P = <0.0001), grain weight (r = -0.545, P = 0.001), grain length (r = -5.43, P = 0.001) and grain width (r = -3.85, P = 0.020). Grain area positively and significantly correlated with grain weight (r = 0.895, P = <0.0001, grain width (r = 0.793, P = <0.0001) and grain length (r = 0.483, P = <0.003).

Table 3.5: correlation analysis between grain Zn, Fe and grain area, length, width and weight of wheat wild relatives and cultivated wheat

Variables	GAR	GLE	GWI	GWE	G Zn	G Fe
GAR	**1**					
GLE	**0.483**	**1**				
GWI	**0.793**	-0.130	**1**			
GWE	**0.895**	0.087	**0.945**	**1**		
G Zn	**-0.633**	**-0.438**	**-0.413**	**-0.544**	**1**	
G Fe	**-0.677**	**-0.543**	**-0.385**	**-0.545**	**0.869**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

GAR = grain area, GLE = grain length, GWI = grain width, GWE = grain weight, G Zn = grain zinc G Fe = grain iron

3.5.5 Field experiment

Higher mineral concentration obtained in the *Am. muticum and T. urartu* accessions above, resulted in the selection of pre-breeding introgression lines derived from the two species, for mineral phenotyping under field conditions in Malawi. Due to unavailability of enough seed, preliminary results from un-replicated ear row trial are reported. Although variations due to genotypic differences cannot be conclusive because of lack of replicates, the results give an overall idea of the mineral concentration in the introgression lines and form a basis for further investigations.

3.5.5.1 Grain Zinc and Iron

Out of the initial 95 DH lines planted, only 48 (19 wheat/ *T. urartu* and 29 wheat/ *Am. muticum*) were able to grow to maturity and produce seed. Mineral analysis showed that grain Zn concentration varied from 29.1 to 88.1 mg kg $^{-1}$ with a mean of 50.8 mg kg $^{-1}$ and a median of 47.1 mg kg $^{-1}$ (Table 3.6). DH-191, DH-8 and DH-96 had the highest grain Zn concentration with 88.1, 81.4, and 81.1 mg kg $^{-1}$ respectively. Twelve lines (DH-191, DH-8, DH-96 DH-304, DH-210, DH-339, DH-273, DH-129, DH-271, DH-29, DH-144 and DH-94) had grain Zn above Paragon, while 81% of the DH lines had high grain Zn above the Malawian check (*Nduna*) with the lowest grain zinc concentration, and ~29% above the Malawian check (*Kenya nyati*) with the highest Zn concentration.

Grain Fe varied from 35 -105.3 mg kg $^{-1}$ with an average of 58.7 mg kg $^{-1}$ and a median of 53.3 mg kg $^{-1}$ (Table 3.6). DH-339 and DH-193 had the highest Fe concentrations with 105.3 and 103.3 mg kg $^{-1}$ respectively. Twelve lines (DH-339, DH-193, DH-8, DH-94, DH-96, DH-271, DH-144, DH-273, DH-129, DH-210, DH-191 and DH-29) had grain Fe concentrations above 70 mg kg $^{-1}$. Forty-four percent of the DH lines had higher grain Fe concentrations above the UK check; (Paragon). Malawian check (*Kadzibonga*) and DH-131 had the lowest Fe concentrations.

3.5.5.2 Grain Selenium and Calcium

Se concentration varied from 0-29.4 µg kg $^{-1}$ with a mean 11.7µg kg $^{-1}$ and a median of 11.3 µg kg $^{-1}$ (Table 3.6). Among the DH lines, DH-288 and DH-316 had the highest Se concentrations of 29.4 µg kg $^{-1}$ and 21.5 µg kg $^{-1}$, respectively. Three checks: Paragon, *Nduna* and *Kenya nyati* had high Se concentration compared to all the DH

lines, with exception of DH-288 and DH-316. The Se concentration in these checks varied from 21.4-25.7 µg kg $^{-1}$.

Table 3.6: Variation in mineral concentration of 48 DH lines derived from *Am. muticum* and *T. urartu* accessions. The DH lines have been ordered according to grain Zn concentrations (highest to lowest)

Genotype	Zn (mg kg^{-1})	Fe (mg kg^{-1})	Se (µg kg^{-1})	Ca (mg kg^{-1})
DH-191	88.1	72.2	0.0	1258
DH-8	81.4	93.5	0.0	1103
DH-96	81.0	83.6	12.2	567
DH-144	79.7	75.6	17.0	744
DH-94	76.4	87.1	19.9	610
DH-29	73.0	70.6	8.8	981
DH-271	72.5	78.6	6.2	1456
DH-129	72.3	74.5	17.6	497
DH-273	69.3	74.6	0.0	605
DH-339	69.2	105.3	0.0	1848
DH-210	65.1	74.0	9.6	1193
DH-304	64.6	64.8	12.8	746
Paragon	61.6	58.9	25.7	392
DH- 357	60.3	60.8	7.1	559
DH-54	55.1	62.4	0.0	1320
DH-318	53.9	60.2	11.2	1386
Kenya nyati	53.9	47.3	22.8	655
DH-81	53.5	57.7	12.7	656
DH-1	52.0	55.2	15.9	565
DH-314	51.2	69.6	11.3	429
DH-202	51.0	53.7	14.6	967
DH-139	50.6	54.3	5.4	734
DH-270	50.5	61.5	13.1	1358
DH-285	50.4	57.9	11.3	785
DH-193	49.7	103.3	10.6	1340
DH-294	48.2	52.8	11.6	762
DH-20	47.5	63.3	11.1	392
DH -316	47.1	67.7	21.5	432
Kadzibonga	45.8	35.1	21.4	218
DH-321	44.7	61.6	10.0	520
DH-288	44.6	47.5	29.4	485
DH-306	43.9	47.0	15.2	315
DH-179	42.6	45.3	9.4	732
DH-141	42.6	48.8	14.9	775
DH-233	42.3	47.4	10.3	691
DH-244	41.7	49.3	13.2	647
DH-85	40.8	53.7	6.4	886

DH-18	40.4	56.0	17.8	517
DH-66	40.2	46.2	14.4	745
DH-126	39.7	52.8	9.0	733
DH-259	37.6	51.7	15.4	743
DH-295	37.3	42.8	8.0	606
DH-91	36.5	54.3	14.0	545
Nduna	36.5	40.1	8.1	542
DH-76	36.3	44.4	11.0	688
DH-134	36.2	41.1	6.4	660
DH-278	36.1	40.9	11.7	531
DH-138	35.2	41.5	11.3	675
DH-84	35.1	46.5	3.6	655
DH-291	34.8	41.4	9.9	614
DH-131	32.5	39.5	10.0	531
Pavon 76	30.6	45.8	11.6	480
DH-15	29.1	49.1	17.9	521
Minimum	29.1	35.1	0.0	218
Maximum	88.1	105.3	29.4	1848
Mean	50.8	58.7	11.7	743
Median	47.1	53.3	11.3	660
Standard deviation (n-1)	15	16.9	6.3	327

Ca concentration varied from 217.98 mg kg $^{-1}$ to 1847.51 mg kg $^{-1}$ with an average of 737.88 mg kg $^{-1}$. DH-339 had exceptionally high Ca concentration (1847.51 mg kg $^{-1}$) compared to all the lines, and nine lines including DH-191, DH-193 and DH-8 had Ca concentrations > 1000 mg kg $^{-1}$. Ninety-six percent of the DH lines had high grain Ca above the UK check Paragon, which had Ca concentration of 392.44 mg kg $^{-1}$. Fifty-eight percent of the DH lines showed higher grain Ca compared to the Malawian check (*Kenya nyati*) with the highest Ca concentration.

3.5.6 Mineral analysis of straw samples of wheat/*Am. muticum* and wheat/*T. urartu* DH lines

Mineral elements for straw were measured to determine if high straw uptake of mineral elements is associated with high grain concentrations. Thirty different mineral elements were determined. However, this report will concentrate on Se and Zn. Results for Fe, revealed sample contamination because of the higher than normal values obtained. Sample contamination might have occurred during harvesting or milling.

3.5.6.1 Straw zinc

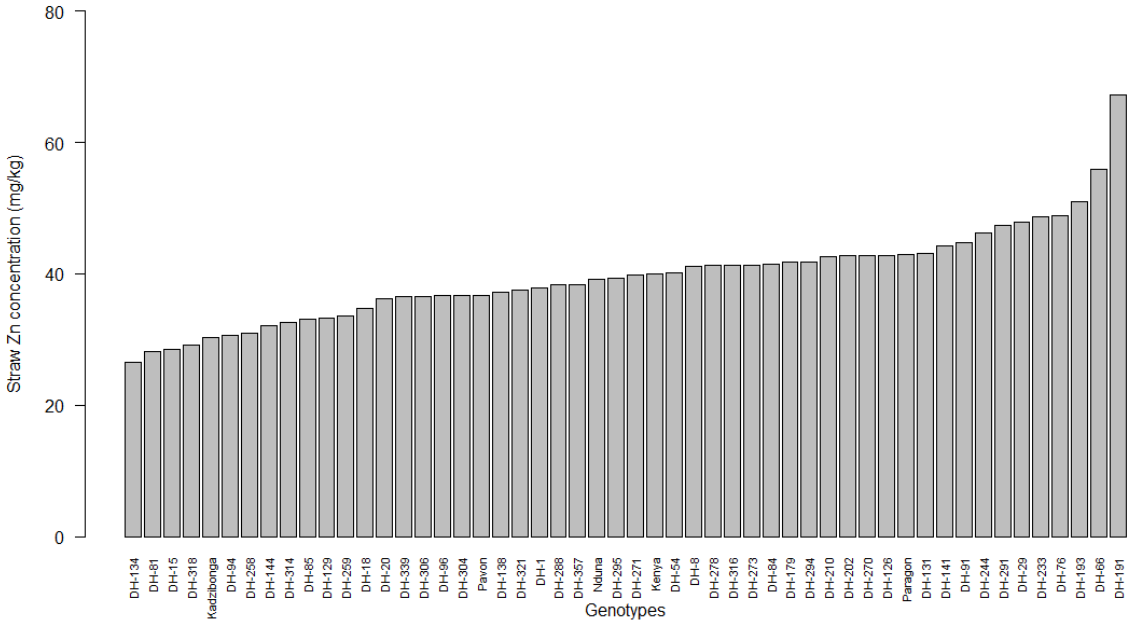


Figure 3.3: Variation in straw zinc concentration of 48 DH lines derived from *Am. muticum* and *T. urartu* accessions

Straw Zn for the field trial varied from 17.63 mg kg $^{-1}$ to 67.28 mg kg $^{-1}$ with an average of 38.87 mg kg $^{-1}$ (Figure 3.3). DH-191 had the highest straw Zn followed by DH-66, DH-134 and DH-193 with 67.28, 55.90, 51.00 and 50.90 mg kg $^{-1}$ respectively. In total, ten lines had high straw Zn compared to the cultivated wheat, Paragon. All Malawian checks had straw Zn concentration > 30 mg kg $^{-1}$ while four of the DH lines (DH-134, DH-81, DH-15 and DH-318) had Zn concentration below 30 mg kg^{-1}.

3.5.6.2 Straw Selenium

Selenium concentration in straw samples varied from 13.05 to 70.19 µg kg $^{-1}$ with an average of 30.08 µg kg $^{-1}$ and a median of 25.08 µg kg $^{-1}$ (Figure 3.4). DH-144 had the highest Se concentration of 70.19 µg kg $^{-1}$. Four DH lines (DH-144, DH-259, DH-244 and DH-210) had Se concentration >50µg kg $^{-1}$ while 44% of the DH lines had >30 µg/kg $^{-1}$ straw Se concentration. Among the checks, Pavon 76 and *Kenya nyati* had the highest Se concentration.

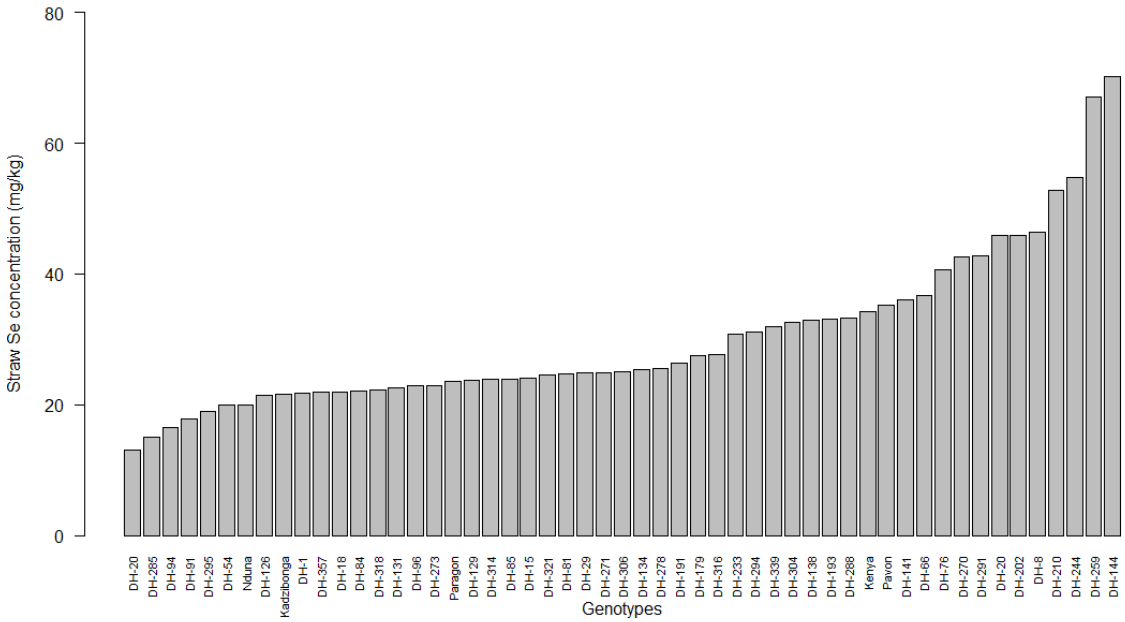


Figure 3.4: Variation in straw Selenium concentration of 48 DH lines derived from *Am. muticum* and *T. urartu* accessions

3.5.7 Phenotypic data

Phenological data was collected for days to heading, days to flowering and number of tillers (Table 3.7). Flowering and heading data revealed that most of the DH lines were long duration as compared to the checks, specifically *Nduna*, *Kadzibonga* and Pavon 76. Among the DH lines, DH-191, DH-233, DH-8, DH-81 and DH-96 had the longest heading and flowering duration. Number of tillers ranged from 2-11. DH-15, DH-18 and DH-8 had the highest number of tillers while majority of the DH lines had a maximum of three tillers.

Table 3.7: Phenotypic data for 48 *Am. muticum* and *T. urartu* DH lines

Statistic	No. of tillers	Heading	Flowering
No. of observations	53	53	53
Minimum	2	70	74
Maximum	11	130	134
1st Quartile	3	102	109
Median	4	119	116
3rd Quartile	4	117	122
Mean	4	108	114
Standard deviation (n-1)	1.95	11.46	11.69

3.5.8 Correlation analysis of grain micro and macro-elements and phenotypic data of *Am. muticum and T. urartu* DH lines

Correlation analysis (Table 3.8) showed that grain Zn concentration positively and significantly correlated with grain Fe ($r = 0.776$, $P = <0.0001$) and moderately correlated with grain Ca ($r = 0.401$, $P = 0.003$). Grain Fe also showed a positive correlation and a moderate correlation with Ca ($r = 0.555$, $P = <0.0001$). Grain Se negatively correlated grain Zn, and positively but insignificantly correlated with all the parameters tested. Straw selenium was also negatively correlated with all the parameters, with the exceptions of grain Ca, straw Zn and the number of tillers, which were positively but insignificantly correlated. Grain Zn, Fe and Ca concentration also showed a significant, but not strong, positive correlation with days to flowering and days to heading. Grain Zn, Fe, Ca, and straw Zn were negatively correlated to number of tillers. However, there was a very strong positive correlation ($r = 0.987$, $P = <0.0001$) between days to flowering and days to heading.

Table 3.8: Correlation coefficients between grain micro and macro-elements and phenotypic data of *Am. muticum and T. urartu* DH lines grown under field conditions

Variables	G Zn	G Fe	G Ca	G Se	S Zn	S Se	NT	DH	DF
G Zn	1								
G Fe	**0.776**	**1**							
G Ca	**0.401**	**0.555**	**1**						
G Se	-0.004	0.052	0.113	**1**					
S Zn	0.087	0.034	0.248	0.198	**1**				
S Se	-0.059	-0.056	0.027	0.051	0.133	**1**			
TN	-0.122	-0.095	-0.153	-0.163	-0.072	0.052	**1**		
DH	**0.403**	**0.417**	**0.298**	0.158	0.139	-0.151	-0.051	**1**	
DF	**0.410**	**0.428**	**0.308**	0.184	0.132	-0.165	-0.038	**0.987**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

G Zn = grain Zn, G Fe = grain iron, G Se = grain selenium, G Ca = grain calcium S Se = straw selenium, S Zn = straw zinc, TN = number of tillers, DH = days to heading and DF = days to flowering

3.6 Discussion

3.6.1 Variability in mineral concentrations of wheat and wild relative accession

Substantial genetic variability in the wheat gene pool is very useful for breeding for high mineral dense wheat grains. Considering that the mineral concentration of modern cultivated wheat varieties is low, and the genotypic variation is relatively narrow, wild relatives are a potential resource for genetic variability. In this study, wild relative accessions in the genus *Triticum, Aegilops, Thinopyrum, Amblyopyrum* and *Secale,* and pre-breeding introgression lines derived from *T. urartu* and *Am. muticum* accessions were analysed for mineral concentration, as a basis for enhancing the nutrient density of Malawian wheat varieties.

Among the wild relatives studied, grain mineral concentration varied widely, and significantly exceeded that of Malawian and UK cultivated wheat varieties. Grain Zn concentration of diploid *Am. muticum* was five and six fold higher than the mean Zn concentration of UK wheat and Malawian wheat varieties respectively. Mean grain Zn concentration of *T. urartu*, *Ae. speltoides* and *Ae. caudata* showed three-fold higher Zn concentration than the average Zn concentration of Malawian wheat. Mean Fe

concentration of *Am. muticum* and *Ae. tauschii* accessions also showed ~three-fold higher concentration above the Malawian wheat. These results suggest that *Am. muticum, T. urartu, Ae. speltoides* and *Ae. caudata* accessions could potentially be useful genetic resources for improving grain Zn, and *Am. muticum* and *Ae. tauschii* grain Fe. Although not much is known about the mineral concentration of *Am. muticum* and *T. urartu*, the C chromosome of *Ae. caudata* has previously been associated with genes for high Fe and Zn (Wang 2011). Similarly, *Ae. tauschii* accessions (Chhuneja et al., 2006) and addition lines (Monasterio and Graham, 2000) have consistently shown higher Fe than Zn concentration.

Significant variation, particularly in grain Zn than Fe concentrations, have been reported in collections of wild tetraploid *Triticum turgidum* ssp. dicoccoides and wild diploid wheat *ssp* boeticum (Cakmak *et al*., 2004, Monasterio and Graham, 2000, Cakmak *et al*., 2000)**.** The present study also found more variation in Zn than Fe. The means and range of Zn concentration obtained in the above studies are also similar to what was obtained for the wild species in this study, with the exception of *Am. muticum* accessions, which had exceptionally high mean grain Zn concentrations of up to 328 mg/kg^{-1}. Cakmak *et al*. (2000) suggested that the high concentrations of Zn in seeds of wild diploid wheats are partly related to smaller seed weight/size. However, a different study showed that accessions with the highest concentrations of Zn and Fe also had the highest total Zn content indicating that the high concentrations of Fe and Zn in seeds were not associated with small seed size (Cakmak *et al*., 2004). In the present study, some wild relative accessions such as *Am. muticum* accessions and *T. urartu* accessions showed both high seed concentration and content, particularly for grain Zn and Fe. Although seed size analysis showed that *Am. muticum* and *T. urartu* accessions had smaller sizes, and correlation between grain Zn and Fe, and seed size parameters showed a significant negative correlation, their high mineral concentrations cannot be attributed to their seed size. Some studies on wild relative derivatives have demonstrated that mineral concentration in wild species may not be attributed to seed size, considering that derivatives with bigger seeds still maintain the high mineral concentrations detected in their wild parents (Rawat *et al*., 2008, Tiwari *et al.*, 2008, Gomez-Becerra *et al*., 2010b, Neelam *et al*., 2012). In this study, some wild relative accessions and cultivated wheat seeds with bigger sizes, showed higher mineral contents and lower mineral concentrations, particularly for Zn and Fe. Regression analysis showed a non- significant association between grain Zn and Fe concentrations

and content. However, a significant association was shown for grain Se concentration and content.

Grain Zn and Fe concentration obtained in this study mostly exceed the values reported in field experiments of other wild relatives such as *Ae. tauschii* (Chhuneja *et al*., 2006, Monasterio and Graham, 2000), *Ae. kotschyii* (Chhuneja *et al*., 2006) and *T. timopheevii* accessions (Hu et al., 2017). The gap in mineral concentration could be attributed to differences in environmental conditions (glasshouse/greenhouse versus field conditions), as shown in previous studies (Cakmak et al., 2004, Srinivasa et al., 2014a). Correlation analysis has shown a highly significant positive association between Zn and Fe indicating that the two minerals can be improved simultaneously. Similar results were reported previously (Cakmak *et al*., 2004, Zhao *et al*., 2009)

3.6.2 Selenium and Calcium

The results show that grain Se concentration in both the wild accessions and cultivated wheats was very low, and can be classified as deficient to moderate (Hawkesford and Zhao, 2007, Tan, 1989). The mean Se concentration of the wild species in this study are not comparable to results on other wild relatives from previous studies (Lyons et al., 2005, Genc et al., 2005, Zhao et al., 2009), and are generally less than the average grain Se concentration in UK wheat (Adams et al., 2002). Studies have shown that Se concentration in plant tissues is very sensitive to a number of soil factors, particularly availability of soil Se, plant species and soil pH (Hawkesford and Zhao, 2007, Zhao 2009 and Lyons, 2005). It is therefore difficult to ascertain if the wild relatives in the present study are not genetically variable for grain Se, or if soil factors had an effect on the concentrations. The Malawian wheats had the highest mean grain Se (51.67µg kg^{-1}), and this could be because the samples were collected from farmers who grew them under different environmental conditions than Paragon, Chinese Spring and the wild species. In Malawi, spatial variability in soil Se was shown to affect grain Se concentration in maize (Chilimba et al., 2011, Hurst et al., 2013, Kumssa et al., 2015, Gashu et al., 2021).

Calcium concentration in wheat wild species has not been widely studied. Previous studies mostly focused on grain Zn and Fe, due to the availability of data and reports on global Zn and Fe deficiency risks. In this study, the mean Ca concentration of *Am. muticum* significantly exceeded all the other genotypes. The results show that

Amblyopyrum and *Thinopyrum* genera are a potential source of variation for grain Ca. Previously, accessions in the genus *Aegilops* showed high grain Ca concentration compared to the wild *Triticum* species and cultivated wheat (Bálint et al., 2001). Interestingly, the mean Ca concentration obtained for the *Aegilops* species in that study are similar to the present findings. A large variation in Ca concentration was also reported in *T. turgidum* spp *dicoccoides* (Gomez-Becerra et al., 2010a), with the range in Ca concentration comparable to the results in the present study.

3.6.3 Field experiment

3.6.3.1 Zinc and Iron

To reach 80% of EAR of Zn and Fe for an adult male, targets for Zn and Fe biofortification in wheat grain were set at 40 and 60 mg kg^{-1} respectively. These results show that 77% and 41% of the introgression lines had grain Zn and Fe concentration above the set targets respectively. Interestingly, 25% and 41% of the lines had higher grain Zn and Fe compared to Paragon wheat respectively. The introgression lines screened in this trial were developed from crossing *Am. muticum* and *T. urartu* accessions with Paragon/Chinese Spring (Grewal et al., 2021, King et al., 2019a). Assuming no variability in soil and environmental conditions at the trial site, an increase in mineral concentration of the introgression lines above the wheat parent suggests the effect of the introgressed chromosome segments. For grain Zn, DH-191, DH-8 and DH-96 had grain Zn concentration above 80 mg kg^{-1}. These lines are *Am. muticum* derivatives with TT segments on wheat chromosome 2D, 4D, 7B and 7D. DH-271 with A^uA^u segments on wheat chromosome 5A and 3A had the highest Zn concentration among the *T. urartu* introgression lines. *Am. muticum* introgression lines DH-339 and DH-193 with the highest Fe concentration have segments TT segments on chromosome 2D, 7B and 7D, while wheat/*T. urartu* lines with high Fe concentration have A^uA^u segments on wheat chromosomes 2A, 3A, 5A and 3D.

Overall, introgression lines with >50 mg Zn kg^{-1} have one or more TT segments recombined with wheat chromosomes 1B, 1D, 2A, 2D, 4D, 6A, 6B, 6D, 7A, 7B and 7D, and A^uA^u segments on chromosomes 1A, 2A, 3A, 3D, 5A, 5D and 6A. Introgression lines with >60 mg Fe kg^{-1} have one or more TT segments on wheat chromosomes 1A, 1B, 2A, 2D, 3A, 4B, 4D, 6B, 6D, 7A, 7B and 7D and A^uA^u segments on chromosomes 1A, 2A, 5A, 5D and 6A. This background suggests that the

wild chromosome segments from *T. urartu* and *Am. muticum* could potentially have QTLs associated with grain Zn and Fe. In a *T. aestivum/T. spelta* RIL population, QTLs for grain Zn were mapped on chromosomes 2A, 2B, 3D, 6A and 6B, QTLs for Fe on chromosomes 2A, 2B, and three QTLs on 1A (Srinivasa et al., 2014a). In a wild tetraploid/hexaploid RIL mapping population, Velu et al. (2017), also identified two major QTLs (1B, 6B) associated with grain Zn. Other studies have also identified QTLs for grain Zn and Fe on chromosomes 1B, 1D,2 A, 2B, 2D, 3A, 4B, 5A,6A, 6B, 7A and 7B and 1B, 1D, 2A, 2B, 2D, 3B, 5A, 6A, 6B and 7A respectively (Rathan et al., 2021, Peleg et al., 2009, Crespo-Herrera et al., 2016, Tiwari et al., 2009b). The introgression lines in this study have the TT and A^uA^u segments on most of the chromosomes reported in these studies, with the exception of chromosomes 1D and 2D for zinc, and 1B and 3B for Fe. The introgression lines also have TT segments on wheat chromosome 4D, 6D and 7D, and none of the previous work has identified QTLs on these chromosomes.

A number of studies have found a positive correlation between Zn and Fe (Velu et al., 2019, Khokhar et al., 2018, Rathan et al., 2021, Krishnappa et al., 2017, Crespo-Herrera et al., 2016, Velu et al., 2017b). As expected, the results of this study are consistent with these studies. This implies that an increase in grain Zn is likely going to have a positive increase in grain Fe.

3.6.3.2 Selenium

Se concentration of all the samples was very low compared to the food and fodder Se requirements (50–100 µg kg^{-1}) for animals and humans (Zhao et al., Gissel-Nielsen et al., 1984). The results are consistent with previous reports on estimates of Se concentration in plant samples of commonly consumed crops grown in non-calcareous soils in Malawi (Kumssa et al., 2015). The study showed that concentration of minerals including Se are influenced by soil type. The results in the present study could be a reflection of the status of soil Se and other soil factors on the experimental site. Across Malawi, variation in soil Se concentration with higher concentration in eutric vertisols has been reported (Chilimba et al., 2011). Maize grain samples grown on calcareous soils have shown higher Se concentration than those from the low-pH soils (Chilimba et al., 2011, Kumssa et al., 2015).

Se concentration was higher in straw samples than in grain samples, and there was a very weak positive correlation between the two parameters showing that although grain Se and straw Se increase in response to each another, the level of response is minimal.

3.6.3.3 Calcium

The mean and range of Ca concentration of DH lines determined in this study (743.3 and 217.98-1847.51 mg kg^{-1}) were higher than previously reported in cultivated bread wheat (Pandey et al., 2016), wild emmer RIL population (Peleg et al., 2009) and synthetic bread wheat (Calderini and Ortiz-Monasterio, 2003). Ca concentration of wheat/*Am. muticum* DHF1-339 far exceeded all the DH lines, and this result is in agreement with the results of the wild relative screening, where the mean Ca concentration of *Am. muticum* accessions far exceeded all the other accessions. DH lines with >1000 mg kg^{-1} have TT segments on chromosomes 2D, 6B, 7B and 7D, and A^uA^u segments on chromosomes 2A, 3A, 5A, 5D and 6A. Previously QTLs associated with grain Ca were mapped on chromosomes 1A, 2B, 4A, 4B, 6A, 6B and 7D (Peleg et al., 2009) and 11 marker-trait association (MTAs) for Ca concentration were identified on chromosomes 1B, 3A, 3B, 3D, 6B and 7A (Bhatta et al., 2018). Similarities in QTLs previously found on chromosomes 3A, 6A, 6B and 7D could suggest potential Ca QTLs in the wild segments.

A number of studies have found a positive correlation between wheat grain Ca, Fe, Ca and Zn (Pandey et al., 2016, Bhatta et al., 2018, Khokhar et al., 2018). However, Balint et al. (2001) did not find any correlation between grain Zn and Ca. In this study, grain Ca had a moderate positive correlation with grain Zn and Fe.

3.7 Conclusion

Diploid *Am. muticum* and *T. urartu* could potentially be useful genetic resources for improving grain Zn and Fe concentration in modern cultivated wheat. *Am. muticum* and species in the *Thinopyrum* genera could also be useful genetic resources for grain Ca concentration. A number of pre-breeding DH lines derived from *Am. muticum* and *T. urartu* have shown high Zn, Fe and Ca concentration above their wheat parent and the Malawian checks. *Wheat/Am. muticum* DH-191 and DH-339 stood out in their level of Zn, Fe and Ca concentration while DH-271 stood out among the *T. urartu* lines. Further replicated and multi-locational trials will be useful to validate these

results. Lines with high mineral concentration can be crossed with adapted cultivated wheat varieties to transfer the introgressions.

CHAPTER 4

4 Soil type affects grain and straw zinc, iron, selenium and not calcium concentrations of wheat/*Amblyopyrum muticum* and wheat/*Triticum urartu* doubled haploid lines

4.1 Abstract

The concentration of mineral nutrients in the edible parts of a plant is associated with bioavailabilities of soil mineral nutrients, which are regulated by various soil physio-chemical properties. A pot experiment was conducted to investigate the effects of soil type on grain and straw Zn, Fe, Se and Ca concentration of wheat/*Am. muticum* and wheat/*T. urartu* double haploid lines. A set of 42 treatments in a factorial combination with two soil types (Ngabu soils and Chitedze soils) and 21 genotypes was laid in a randomised complete block design (RCBD) in three replicates. Soil analysis showed that the two soils had similar texture, but different mineral concentration, pH levels and percentage organic matter. Grain samples were analysed using inductively coupled plasma-mass spectrometry (ICP-MS). Analysis of variance (ANOVA) revealed a ~two-fold higher grain Zn concentration in low pH, high Zn soils (Chitedze soils) compared to high pH, low Zn soils (Ngabu soils). Variation in grain Zn concentration was associated with the genotypes ($p = 0002$), soil type ($p = <0.0001$), and the interaction between soil and genotypes ($p = 0.035$). Grain Fe was 1.3-fold higher in low pH than in high pH soils. Variation in grain Fe was influenced by the factors: genotypes ($p = < 0.0001$) and soil type ($p = <0.0001$). Grain Se was highly associated with soil type ($p = <0.0001$), and it was 30-fold higher in high pH than in low pH soils. Variation in grain Ca was independent of soil type, but highly influenced by genotypes ($p = < 0.0001$), and partly by the interaction between genotype and soil type ($p = 0.018$). The findings demonstrate the significance of soil physio-chemical properties in a breeding program genetic biofortification.

4.2 Introduction

Mineral nutrient deficiencies (MNDs), particularly zinc (Zn), iron (Fe), selenium (Se) and calcium (Ca), remain widespread in low-income countries of sub-Saharan Africa(Kumssa et al., 2015, Joy et al., 2014, Phiri et al., 2019, Ligowe et al., 2020). In humans, Zn and Fe are essential micronutrients for growth, development and maintenance of the immune system (Walker et al., 2005). Se is essential for hormone regulation and immune system functioning (Avery and Hoffmann, 2018),while Ca is essential for skeletal structure, smooth muscle contraction, and neuronal signalling (Bourassa et al., 2022). In many African countries, cereals including wheat (*Triticum aestivum*), maize (*Zea mays*), rice (*Oryza sativa*) and teff (*Eragrostis tef*) are a major source of dietary Zn, Fe and Se (Joy et al., 2014, Abdu et al., 2022). For example, cereals contribute approximately 52, 56 and 57% of the total Se, Fe and Zn supply respectively (Joy et al., 2014). This compared to developed countries such as the UK, where cereals and cereal products contribute approximately 25% of dietary Zn, 39% of dietary Fe (Bates et al., 2016), and 22% of dietary Se (Rayman, 2000). Conversely, the greatest contribution of Ca supply in African countries is fish and dairy products, although fruits, vegetables and roots and tubers contribute the majority of dietary Ca supply in specific African regions (Joy et al., 2014). Cereals such as finger millet and teff have also shown to be potential sources of dietary Ca in Africa (Gashu et al., 2021).

Wheat is an important cereal crop providing approximately 20% of dietary energy globally (Shiferaw et al., 2013). Traditionally wheat was not a dominant staple cereal in sub-Saharan Africa. However, several factors including increased urbanisation, population growth and change in eating habits over the years has resulted in a shift in the demand for wheat and wheat products (Tadesse et al., 2018, Guwela et al., 2021). Previous studies have established that cultivated wheat is inherently low in grain mineral nutrients, particularly Zn and Fe (Cakmak et al., 1999, Genc et al., 2005, Zhao et al., 2009). In addition, wheat has a high percentage of anti-nutritional factors limiting its bioavailability (Cakmak et al., 1999, Kutman et al., 2010, Cakmak and Kutman, 2018, Welch and Graham, 2004). Additionally, there is evidence that mineral concentration in grains is associated with soil mineral concentration, soil chemical properties, and plant uptake and remobilisation, which is largely associated with plant genetic make-up (Graham and Rengel, 1993, Chilimba et al., 2011, Kumssa et al., 2015, Manzeke et al., 2019, Liu et al., 2019).

Grain mineral concentration is affected by plant genetic variation as some genotypes accumulate more mineral nutrients compared to others. For example, grain Zn and Fe concentration varies widely between modern cultivated wheat varieties and wild relatives (Graham et al., 1999, Zhao et al., 2009). In wheat, significant variation in genotype efficiency in Zn uptake and utilisation has been studied (Graham et al 1992, Kalayci et al 1999, Cakmak et al., 1997). However, no study has shown any correlation between genotype efficiency and grain Zn concentration. Lyons et al., 2004 also reported variation in grain Se concentration among commercial, advanced breeding lines and diploid ancestral wheat species grown in sites with spatial variation in soil Se.

Soil pH is a major soil factor affecting solubility of zinc in the soil, subsequently affecting availability for plant uptake (Alloway, 2009, Rengel, 2015). High soil pH is associated with a high presence of calcium carbonate ($CaCO_3$) and a high content of bases, especially calcium and magnesium (Virmani et al., 1982, Alloway, 2009). $CaCO_3$ increases adsorption of Zn thereby reducing its availability for plant uptake (Alloway, 2009). Similarly, the concentration of iron in the soil solution decreases sharply as the soil pH increases. This is because Fe is readily oxidized, and is predominately in the form of insoluble ferric oxides in high pH soils, whilst in low pH soils, the ferric Fe is freed from the oxide, and becomes more available for plant uptake (Morrissey and Guerinot, 2009, Tsai and Schmidt, 2020). Conversely, bioavailability of Se decreases with decreasing pH (Gissel-Nielsen et al., 1984, Stroud et al., 2010), although generally, availability of Se in the soil depends on the properties of the parent rock (Wells 1967, Gupta and Subhas, 2000, Pan et al, 2023). Soil pH also determines the predominant species of Se in the soil, with some species being high in low pH, while others are more in high soil pH (Elrashidi et al., 1989, Broadley et al., 2006, Sharma et al., 2015). Se species subsequently affects Se bioavailability as aluminium oxides and hydroxides adsorb some speciations more than others (Hawkesford and Zhao, 2007, Sharma et al., 2015). Availability of Ca for plant uptake is also affected by soil pH, with more availability in alkaline soils compared to acidic soils (Flis, 2019).

Mineral concentrations in plants are also affected by soil organic matter. High organic matter content results in low available Zn because of the high level of Zn adsorption by organic ligands and components (Alloway, 2009). This is different with soil Fe,

where organic matter improves its availability by combining with soil Fe, thereby reducing chemical fixation or precipitation of Fe as ferric hydroxide, resulting in higher concentrations of Fe in the soil solution available for plant uptake (Schulte, 2004). For Se, natural organic matter plays an important role in speciation which is linked to mobility of Se species for plant uptake (Tam et al., 1999).

Soil mineral concentration substantially affects concentrations of minerals in grains, particularly Zn. For example, post-anthesis Zn accumulation in wheat grains was associated with an increase in diethylene triamine penta-acetic acid (DTPA) - Zn concentration above 7.15 mg kg^{-1} (Liu et al., 2019). A positive correlation between grain Zn and soil available zinc in wheat-maize growing areas has been reported (Huang et al., 2019). Se in edible parts of the plant is also determined by soil Se phytoavailability (White, 2016). However, Chilimba et al. (2011) found no obvious link between grain and soil Se concentrations while Zou et al (2019) found a weak positive correlation. Availability of the soil mineral nutrients for plant uptake, translocation and remobilisation is therefore associated with soil type, determined by different soil physio-chemical properties. For example, Zn is usually deficient in strongly weathered, deep tropical soils, saline/sodic soils, greysols and calcareous soils such as vertisols (Alloway, 2008).

The specific aims of the study were to:

(i) Investigate the effects of soil type on grain Zn, Fe, Se and Ca concentration of wheat/*Am. muticum* and wheat/*T. urartu* double haploid lines

(ii) Understand the relationship between grain concentration of the four mineral elements (Zn, Fe, Se and Ca), straw concentration and phenotypic and phenological traits of wheat/*Am. muticum* and wheat/*T. urartu* double haploid lines grown under two different soil types

4.3 Materials and methods

4.3.1 Germplasm

Wheat/*T. urartu* and wheat/*Am. muticum* doubled haploid (DH) lines were developed at Nottingham BBSRC Wheat Research Centre (WRC) at Nottingham University, UK. Chapter 3 gives a brief description of the development and characterisation of the DH lines. Two checks (Paragon and Chinese Spring) were sourced from the WRC while three checks (*Kadzibonga, Kenya nyati and Nduna*) were sourced from Lilongwe University of Agriculture and Natural Resources (LUANAR), Lilongwe, Malawi.

4.3.2 Soil collection

Topsoil samples (0 – 20 cm) were collected from a field in Ngabu (16° 45′ S and 34° 89′ E), Chikwawa, Malawi and Chitedze Research Station (13° 98′ S and 33° 65′ E), Lilongwe, Malawi. Previous work has shown that some micro and macronutrients such as Zn, Fe and Ca are higher in the top soil, and decrease with depth (Gupta et al., 2008, Jobbágy et al., 2001). The soils collected were previously described as vertisols (Lowole, 1985, Botoman et al., 2020). Approximately 1000 kg of each soil type was collected using hand hoes. The soils were transported to LUANAR in 50 kg sacks. For analysis, samples were collected across the two selected fields using a zigzag sampling pattern. Soil samples collected from different points were mixed in a bucket to form a composite sample.

4.3.3 Soil sample preparation and analysis

Soil samples were air-dried, crushed with a pestle and mortar before passing them through a 2 mm sieve. 200 grams of each of the composite soil sample was transferred into zip-loc bags, labelled and shipped to the University of Nottingham for analysis in the laboratory. Soil pH was determined using a Mettler Toledo calibrated pH meter (Scientific Laboratory Supplies, Leicester, United Kingdom) following suspension of 5g of soil sample in 12.5mls Milli-Q water (18.2 MΩ cm; 1:2.5 m/v) and shaking for 1 hour on an end-over-end shaker. The DTPA extraction method was used to analyse extractable Zn and Fe (Lindsay and Norvell, 1978). 5 g of soil sample was mixed with 10 mls of 0.005 M DTPA, 0.1 M triethanolamine (TEA) and 0.01 M $CaCl_2$ at pH = 7.3 for 2 h on an end-over-end shaker. The soil suspensions were then centrifuged and filtered (<0.22 μm) before analysis using inductively coupled plasma mass

spectrometry (ICP-MS; iCAP Q; Thermo Fisher Scientific, Bremen, Germany). Samples included two reagent blank samples, three random sample duplicates and three certified reference materials (CRMs).

Ca and Se were analysed using aqua-regia hot plate acid digestion and analysis using ICP-MS (Crosland et al., 1995). Briefly, ~0.4g of sample was analysed along blanks, WEPAL Calc-ISE 850 (Wepal-Quasimeme, NL-6700 EC Wageningen, Netherlands) certified reference material and a laboratory reference material (Ethiopian soil). Total N was analysed using the Kjeldahl digestion method (Kjeldahl, 1883) following digestion of 0.2g of soil samples in a hydrogen peroxide, lithium sulfate and sulfuric acid digestion solution. The samples along with two standards (N1 solution: sodium salicylate, sodium citrate, sodium tartrate and sodium nitroprusside and N2 solution: sodium hydroxide and sodium hypochlorite) were read on a UV spectrophotometer at a wavelength of 655 to get absorbance. Organic matter was determined using the Walkley and Black method (Walkley and Black, 1934). Approximately 1 g of dried soil was oxidised in 10 ml of 0.167-mol potassium dichromate (K2Cr2O7) and 10 ml of concentrated sulfuric acid (H_2SO_4). Samples were diluted with 100 ml distilled water. Titration of the solution was done using ammonium ferrous sulfate. A titration solution was prepared by adding 1 ml of diphenylamine indicator.

4.3.4 Experimental design and trial management

The experiment was conducted in the winter season of 2021 (May-September) at Lilongwe University of Agriculture and Natural Resources (LUANAR -14.18'S 33.76' E), Lilongwe, Malawi. A set of 42 treatments in a factorial combination of two soil types and 21 genotypes was laid out under screenhouse conditions, in a randomised complete block design (RCBD) with three replicates. The genotypes comprised of 12 wheat/*Am. muticum* and four wheat/*T. urartu* DH lines, along with two UK (Chinese spring and Paragon), and three Malawian (Kenya nyati, Nduna and Kadzibonga) checks. Soils were air-dried and sieved before filling 2 litre pots. Distance between pots was 0.2 m, distance between blocks was 1.0 m, while distance between plants from pot to pot ranged from 0.25 to 0.30 m. One seed was sown in each pot, and 14 days later seeds were re-sown in pots where there was no germination. 10 L water-cans were used for irrigation from sowing to maturity at which point irrigation was withdrawn to allow plants to dry. Basal dressing fertiliser 23:10:5 +6S +1Zn

(SuperFert Fertilisers, Harare, Zimbabwe) was applied 14 days after planting at a rate of 200kg/ha, 3 weeks later UREA (46% N) was applied as top dressing at a rate of 100kg/ha. Basal and top dressing were applied according to the Malawi guide to agriculture production (GAP, 2020) guidelines. First weeding was done 4 weeks after planting and subsequent weeding as soon as weeds appeared. Insect pests were controlled by applying Profex Super (Profencfos 40% + Cypermenthrin 4% EC – Kewalram Chanrai group).

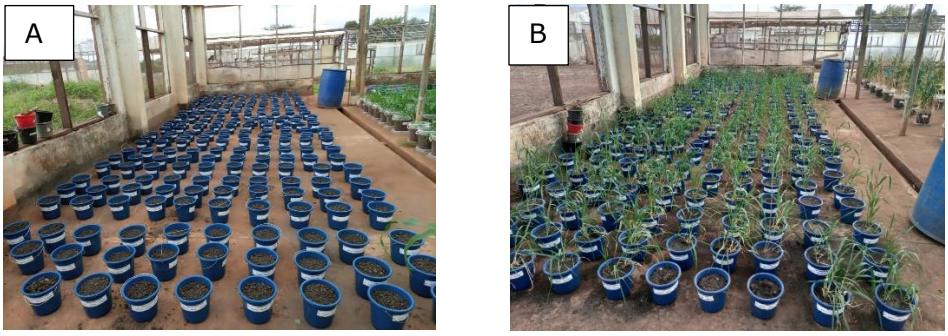


Figure 4.1: A layout of pots filled with Chitedze and Ngabu soils (A) and wheat plants 6 weeks after planting (B)

4.3.5 Grain and straw micronutrient analysis

4.3.5.1 Sample preparation and digestion

Sample preparation and digestion was done as described in chapter 3. Briefly, grain samples were soaked overnight (16 hours) in 8mls of nitric acid (HNO_3) (>68% PrimarPlus-Trace analysis grade- Fisher Scientific, Loughborough, UK). Samples were digested in 2021 using a hot block acid digestion system (Anton Paar Gmbh, Graz, Austria). ~0.4 g of each of the grain samples along with certified reference material (wheat flour 1567b-CRM) and laboratory reference material (Paragon wheat-LRM) were digested using a Multicube 48 digestion block (Anton Paar Gmbh, Graz, Austria). Two operational blanks were added in each run. The digestion block was set at 105˚C for 2h. Samples were diluted with milliQ water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK) up to 50mls.

Straw samples were digested in 2022 using a microwave digestion platform described in chapter 3. 0.2 g of each finely ground sample was weighed in pressure-activated venting vessels (56-ml 'SMART VENT', Anton Paar) along with three reference materials (CRM-Tom-1573a, BCR-Hay 129 and LRM-Cabbage) and two operational

blanks. The samples were digested in a Multiwave PRO microwave with 41-vessel digestion rotor (41HVT56) set at 1,500 W, 10 min heating to 140 °C, 20 min holding at 140 °C, and 15 min cooling to 55 °C. Following digestion, each tube was made up to a final volume of 24ml by adding 16 ml Milli-Q water, then transferred to a 25-ml universal tube (Sarstedt). Both grain and straw samples were further diluted in ICP tubes using a dilution factor of 1:10

4.3.5.2 Multi-elemental analysis

Grain and straw multi-element analysis was undertaken using inductively coupled plasma mass spectrometry as described in chapter 3. Briefly, thirty elements including; Zn, Fe, Ca, Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sr, Ti, Tl, U, V and Zn were analysed. Multi-elemental analysis either of the straw samples showed higher than normal values in some elements particularly Fe, suggesting that they had been contaminated by soil or during milling. A total of 147-grain samples and 152 straw samples (three replicates for each sample) including blanks and CRMs were analysed. The mineral-specific recovery for grain samples from the field experiment was 90% Zn, 79% Fe, 95% Se and 95% Ca. For straw samples, Zn and Se-specific recovery from CRMs was 92% and 94% respectively.

4.3.6 Statistical analysis

Two-way analysis of variance (ANOVA) was performed with Genstat regression using Genstat for windows statistical package, version 21 (VSN, 2022). Regression and correlation analyses were performed in XLSTAT 2022.3.1 (Addinsoft, 2022). The statistical linear model considered the response Y_{ijk} of the ith genotype in the kth replication within the jth soil type expressed as:

$$Y_{ijk}=\mu+\beta_{kj}+\tau_i+\delta_j+(\tau\delta)_{ij}+e_{ijk}$$

Where μ is the grand mean over all genotypes and soil type, β_{kj} is the effect of the kth block/replicate within the jth soil type, τ_i is the effect of the ith genotype, δ_j is the effect of the jth soil type, $(\tau\delta)_{ij}$ is the interaction of the ith genotype in the jth soil type, and e_{ijk} is the average error. Fishers protected least significant difference (LSD, $P < 0.005$) was used to separate means. Correlation analysis was performed using Pearson correlation tests.

4.4 Results

4.4.1 Soil characterisation

Table 4.1 shows the characterisation of the soil samples collected from Chitedze and Ngabu research stations. Although both soils were classified as sandy clay loam, analysis results revealed differences in the soil chemical properties. DTPA-extractable Zn was high in Chitedze soils (1.25 mg kg^{-1}) than in Ngabu soils (0.33 mg kg^{-1}). Fe was also slightly higher in Chitedze soils (1.8 mg kg^{-1}) compared to Ngabu soils (1.5 mg kg^{-1}). In Chitedze soils, soil pH was 5.7 while in Ngabu soils the pH was 7.1. The soils also differed in percentage organic matter, with Chitedze soils having a higher percentage organic matter/carbon than Ngabu soils.

Table 4.1: Physio-chemical properties of soil samples collected from Ngabu and Chitedze Research Stations

Parameter	Chitedze soils	Ngabu soils
Soil pH	5.4	7.1
Organic matter (%)	2.6	1.3
DTPA-Zn (mg/kg)	1.25	0.33
DTPA-Fe (mg/kg)	1.8	1.5
Se (mg/kg)	0.2	0.1
Ca (mg/kg)	1562	1927
Total N (%)	0.187	0.146
Available P (mg/kg)	18	20.6
Silt (%)	10	16
Clay (%)	22	24
Sand (%)	68	60
Textural class	Sandy clay loam	Sandy clay loam

4.4.2 Grain mineral analysis

4.4.2.1 Grain zinc

Analysis of grain samples showed a significant variation in grain Zn concentration (Table 4.2), with 79% of the variation explained by the variables genotypes (p = 0002), soil type (p = <0.0001), and the interaction between soil and genotypes (p = 0.035). Among the three variables, mean grain Zn was highly influenced by soil type, and it was higher in Chitedze soils compared to Ngabu soils (Figure 4.2). In Chitedze soils, mean grain Zn concentration varied from 37.2 to 98.8 mg kg^{-1} with an overall mean of 69.6 mg kg^{-1}, while in Ngabu soils, mean grain Zn concentration varied from 24.1 to

52.8 mg kg^{-1} with an overall mean of 39.4 mg kg^{-1} (Table 4.2). In Chitedze soils, DH-62 and DH-254 with 98.8 and 94.6 mg kg^{-1} respectively, showed significantly higher grain zinc concentration compared to all the other genotypes. In Ngabu soils, DH-62 again showed significantly higher grain Zn compared to all the other genotypes. Overall, all the DH lines with exception of DH-121, showed Zn concentration above all the five checks in Chitedze soils. In Ngabu soils, five DH lines (62, 191, 196, 339, 304 and 254) showed Zn concentration above all the five checks.

4.4.2.2 Grain iron

Variation in grain Fe concentration was explained by the variables genotypes ($p = < 0.0001$) and soil type ($p = <0.0001$), while the interaction between genotype and soil type ($p = < 0.364$) did not have a significant influence on grain Fe concentration (table 2). Mean grain Fe concentration was generally higher in wheat grown in Chitedze soils compared to in Ngabu soils (Table 4.2). For Chitedze soils, grain Fe concentration varied from 43.7 – 116.4 mg kg^{-1} with an overall mean of 78.1 mg kg^{-1}, while with Ngabu soils grain concentrations varied from 40.7 to 70.5 mg kg^{-1} with an overall mean of 58.5 mg kg^{-1}. For Chitedze soils, DH-62 and DH-191 had significantly higher grain Fe concentrations compared to all the other DH lines. Overall, thirteen DH lines showed higher grain Fe concentration above the check Paragon, whilst three lines had higher Zn concentrations above the three Malawian checks and Chinese spring, but lower than Paragon. Similarly, DH-62 and DH-191 had the highest Fe concentration in Ngabu soils, and most of the DH lines showed grain Fe higher than the check paragon. All the Malawian checks and Chinese Spring also followed a similar trend to the Fe concentration in Chitedze soils.

Table 4.2: Mean variation in grain Zn, Fe, Ca and Se concentration of 21 genotypes grown in Chitedze and Ngabu soils in 2021 winter season. The genotypes have been ordered according to grain Zn concentrations (highest to lowest)

	Grain Zn (mg/kg)		Grain Fe (mg/kg)		Grain Ca (mg/kg)		Grain Se (µ/kg)	
Genotypes	**Chitedze soil**	**Ngabu soil**	**Chitedze soil**	**Ngabu soil**	**Chitedze soil**	**Ngabu soil**	**Chitedze soil**	**Ngabu soil**
DH-62	98.8 ^{a}	52.8 ^{ghijklm}	116.4	70.5	625 ^{fghijk}	671 ^{cdefghi}	1.1	32.4 ^{abc}
DH-254	94.6 ^{ab}	42.4 ^{ijklmn}	93.8	64.3	610 ^{fghijk}	614 ^{fghijk}	0.8	29.4 ^{abcde}
DH-1	90.8 ^{abc}	36.7 ^{klmn}	83.4	57.0	577 ^{ghijkl}	606 ^{fghijk}	0.0	20.4 ^{cdefgh}
DH-191	89.2 ^{abcd}	49.4 ^{ghijklm}	101.3	67.9	784 ^{abcde}	810 ^{abc}	0.4	41.1 ^{a}
DH-91	87.3 ^{abcd}	39.1 ^{jklmn}	99.6	64.1	573 ^{ghijkl}	493 ^{klm}	1.5	17.40 ^{efgh}
DH-122	87.2 ^{abcde}	38.9 ^{jklmn}	93.1	62.0	665 ^{cdefghij}	695 ^{bcdefgh}	1.4	32.0 ^{abcd}
DH-339	80.6 ^{abcdef}	43.7 ^{hijklmn}	87.3	65.8	577 ^{ghijkl}	630 ^{fghijk}	0.3	26.0 ^{bcde}
DH-129	72.4 ^{bcdefg}	36.7 ^{lmn}	81.4	57.7	568 ^{ghijkl}	625 ^{fghijk}	1.4	27.5 ^{abcde}
DH-196	70.6 ^{cdefg}	49.2 ^{ghijklm}	87.8	66.2	877 ^{a}	736 ^{abcde}	0.0	23.5 ^{bcdefg}
DH-74	70.4 ^{cdefg}	41.5 ^{ijklmn}	88.3	66.9	531 ^{ijkl}	618 ^{fghijk}	0.0	33.7 ^{abc}
DH-139	69.1 ^{cdefg}	33.2 ^{mn}	83.3	61.0	633 ^{fghijk}	715 ^{bcdefg}	1.6	24.3 ^{bcdef}
DH-314	66.3 ^{defgh}	24.9 ^{n}	66.2	57.8	527 ^{jkl}	607 ^{fghijk}	1.0	31.5 ^{abcd}
DH-144	63.4 ^{efghi}	38.2 ^{jklmn}	79.0	64.6	716 ^{bcdefg}	539 ^{ijkl}	0.0	37.3 ^{ab}
DH-304	60.5 ^{fghij}	42.6 ^{hijklmn}	67.6	60.8	805 ^{abcd}	632 ^{fghijk}	0.0	32.19 ^{abc}
DH-63	60.5 ^{fghljk}	36.3 ^{lmn}	76.9	59.7	610 ^{fghijk}	658 ^{defghij}	0.4	24.0 ^{bcdef}
Paragon	59.4 ^{fghijkl}	40.9 ^{ijklmn}	71.8	59.2	623 ^{fghijk}	695 ^{bcdefgh}	0.0	18.3 ^{defgh}
Kenya nyati	51.2 ^{gjijklm}	30.3 ^{mn}	49.7	45.0	641 ^{efghij}	677 ^{cdefghi}	2.0	7.3 ^{hi}
DH-121	49.3 ^{ghijklm}	39.7 ^{ijklmn}	60.7	50.3	826 ^{ab}	649 ^{efghij}	1.3	10.0 ^{ghi}
Chinese Spring	40.5 ^{ijklmn}	24.1 ^{n}	51.9	42.7	662 ^{defghij}	370 ^{m}	0.0	12.0 ^{fghi}
Kadzibonga	41.8 ^{ijklmn}	37.2 ^{jklmn}	45.7	40.7	549 ^{hijkl}	432 ^{lm}	1.1	8.7 ^{hi}
Nduna	37.2 ^{jklmn}	42.4 ^{ijklmn}	43.7	44.6	661 ^{defhij}	668 ^{cdefghij}	0.9	8.7 ^{hi}
Grand mean	68.3	39.3	76.3	58.3	646	629	0.8	23.7
R^2	0.79		0.77		0.70		0.85	
Genotypes	<0.0001		<0.0001		<0.0001		<0.0001	
Soil type	<0.0001		<0.0001		0.190		<0.0001	

Genotypes*Soil type	0.035	0.304	0.008	0.011
CV%	27.4	18.7	13.6	53.7
LSD (5%)	21.1	13.7	131.4	12.2

Degrees of freedom (df) for replicates = 2, df for genotypes = 20, df for soil type = 1, df for genotypes*soil type = 20

For each variable, means with different superscript letters are significantly different at P<0.05, following ANOVA and Fishers protected LSD tests

4.4.2.3 Grain calcium

Seventy percent of the variation in grain Ca concentration was explained by the variables genotypes (p = < 0.0001) and the interaction between genotypes and soil type (p = 0.018), with the variable genotypes highly influencing the concentration of grain Ca (Table 4.2). Soil type alone did not have any significant influence on grain Ca concentration (p = 0.292). Mean Ca concentration was slightly higher in Chitedze soils compared to Ngabu soils (Figure 4.2). In Chitedze soils, grain Ca varied from 527 – 877 mg kg^{-1} with a mean of 646 mg kg^{-1}, while in Ngabu soils grain Ca varied from 370-810 mg kg^{-1} with an overall mean of 625 mg kg^{-1}. In Chitedze soils, DH-121 and DH- 196 showed exceptionally higher grain Ca concentrations with 877 and 827 mg kg^{-1} respectively. DH-304 and DH-122 also showed high Ca concentrations above the checks Chinese spring and *Nduna*, which had the highest Ca concentrations among the checks. Among the DH lines, eight lines showed high Ca concentration compared to the check Paragon. In Ngabu soils, DH-191 and DH-196 showed significantly higher Ca concentrations with 810 and 736 mg kg^{-1}. Among the DH lines, only three lines (DH-191, DH-196 and DH-139) showed Ca concentration above the check Paragon.

4.4.2.4 Grain selenium

Grain Se concentration varied widely (Table 4.2), with 85% of the variation explained by the variable genotypes (p = 0.012), soil type (p = <0.0001), and the interaction between soil and genotypes (p = 0.007). Among the three variables, grain Se was highly influenced by soil type, and it was higher in wheat grown in Ngabu soils compared to Chitedze soils (Figure 4.2). For Ngabu soils, mean grain Se concentration varied from 7.3 to 41 µ kg^{-1} with an overall mean of 23.7 µ kg^{-1}. DH-191 and DH-144 showed significantly higher grain Se concentrations with 41.1 and 37.3µ kg^{-1} respectively. All the DH lines, with the exception of DH-121 and DH-91, showed high Se concentration above the five checks. In Chitedze soils, mean grain Se concentration was not significantly different (F = 0.586), and it varied from zero to 3.2 µ kg^{-1} with an overall mean of 0.8 µ kg^{-1}. DH-122 was the only line that had a higher Se concentration above *Kenya nyati*, the Malawian check with the highest Se concentration. Seven of the DH lines along with Paragon and Chinese Spring did not have any Se in the grain.

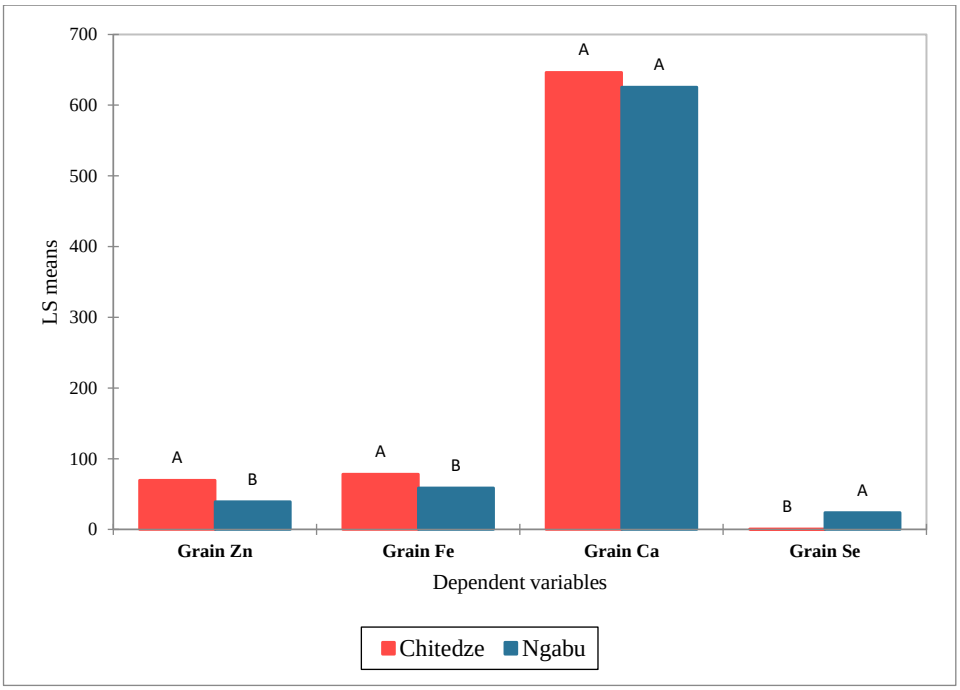


Figure 4.2: Mean values of grain Zn, Fe, Ca and Se concentration for the factor soil type

For each variable, means with different letters are significantly different at P<0.05, following ANOVA and Fishers protected LSD tests.

4.4.3 Straw zinc and selenium

4.4.3.1 Straw zinc

Analysis of straw samples showed significant variation in straw Zn concentrations (Table 4.3), with 75% of the variation explained by the variables soil type (p = < 0.0001) and the interaction between soil type and genotypes (p = 0.001). Among the variables, straw Zn concentration was highly influenced by soil type, while genotypes did not give significant information on the variation (p = 0.059). Mean straw Zn concentration was higher in wheat grown in Chitedze soils than in Ngabu soils (Figure 4.3). In Chitedze soils, straw Zn varied from 18.9 to 39.1 mg kg^{-1} with an overall mean of 27.0 mg kg^{-1}. DH-91 and DH-254 had significantly higher straw Zn with 39.1, and 35.0 mg kg^{-1} respectively. All the DH lines, with exception of DH-304 and DH-121, had a Zn concentration above the check Paragon. Among the Malawian checks, *Kadzibonga* had the highest straw Zn that was statistically comparable to DH-122, DH-139 and DH-196, whilst *Nduna* had the lowest concentration. In plants grown in Ngabu soils, concentration varied from 8.9 to 31.6 mg kg^{-1} with an overall mean of 15.9 mg kg^{-1}. DH-62 and DH-141 had the highest straw Zn concentration with 31.6

and 25.5 mg kg^{-1} respectively. Six DH lines showed high straw Zn concentration above Paragon. Among the Malawi checks, both *Nduna* and *Kenya nyati* had higher straw Zn concentration compared to most of the DH lines.

Table 4.3: Variation in straw Zn and Se of 21 genotypes grown in Chitedze and Ngabu soils in 2021 winter season

	Straw Zn (mg/kg)		Straw Se (µ/kg)	
Genotypes	**Chitedze soils**	**Ngabu soils**	**Chitedze soils**	**Ngabu soils**
DH-62	31.0 abc	25.5 bcdefg	22.5 efghijklmn	41.7 a
DH-254	35.0 ab	10.9 m	18.8 jklmno	29.7 bcdef
DH-1	25.1 bcdefg	8.9 m	17.5 lmno	28.4 bcdefg
DH-191	26.8 bcde	18.1 defghijklm	22.4 efghljklm	26.5 bcdefghi
DH-91	39.1 a	14.6 hijklm	25.5 cdefghijkl	27.8 bcdefgh
DH-122	29.4 abc	14.1 hijklm	18.2 klmno	28.5 bcdefg
DH-339	26.6 bcdef	15.6 ghijklm	21.4 fghijklmn	32.0 bcd
DH-129	25.4 bcdefg	13.0 lm	18.6 klmno	29.0 bcdefg
DH-196	30.0 abc	13.1 lm	19.0 jklmno	28.2 bcdefg
DH-74	27.5 bcd	13.5 klm	17.5 lmno	32.0 bcd
DH-139	29.7 abc	12.4 lm	17.9 lmno	28.4 bcdefg
DH-314	22.0 cdefghijkl	12.6 lm	14.8 no	34.6 ab
DH-144	24.4 cdefgh	31.6 abc	12.7 o	33.3 bc
DH-304	24.2 cdefghi	13.8 jklm	17.8 klmno	32.0 bcd
DH-63	24.3 cdefghi	10.9 m	19.0 jklmno	27.1 bcdefghi
Paragon	23.8 cdefghi	14.1 ijklm	18.9 jklmno	24.6 defghijklm
Kenya nyati	25.2 bcdefg	16.5 fghijklm	17.9 lmno	34.2 ab
DH-121	18.9 defghijklm	24.8 cdefg	19.1 jklmno	29.9 bcde
Chinese Spring	23.4 cdefghij	17.1 efghijklm	18.2 klmno	20.2 hijklmno
Kadzibonga	31.0 abc	18.9 defghijklm	24.8 defghijklm	25.6 cdefghijk
Nduna	23.8 cdefghij	24.8 cdefg	21.3 ghijklmn	17.5 mno
Grand mean	27.0	16.4	19.3	29.1
R^2	0.748		0.769	
Genotypes	0.059		0.041	
Soil type	<0.0001		<0.0001	
Genotypes*Soil type	0.001		0.001	
CV%	27.4		17.5	
LSD (5%)	23.5		7.8	

Degrees of freedom (df) for replicates = 2, df for genotypes = 20, df for soil type = 1, df for genotypes*soil type = 20.

For each variable, means with different superscript letters are significantly different from each other (P <0.05 ANOVA and Fishers protected LSD test).

4.4.3.2 Straw Selenium

Straw Se concentration varied widely (Table 4.3), with 75% of the variation explained by the variables genotypes (p = 0.041), soil type (p = <0.0001) and the interaction between soil and genotypes (p = 0.001). Among the three variables, straw Se was highly influenced by soil type, and it was higher in Ngabu soils compared to Chitedze soils (Figure 4.3). Mean straw Se was higher in Ngabu soils compared to Chitedze soils. In Ngabu soils, straw Se varied from 17.3 to 41.7 µ kg^{-1} with an overall mean of 29.1 µ kg^{-1}. DH-62 had the highest straw Se concentration and it was the only DH line with higher straw Se above the check *Kenya nyati*. Overall, all the DH lines grown in Ngabu soils showed high straw Se concentration above all the checks with the exception of *Kenya nyati*. In Chitedze soils, straw Se varied from 12.7 to 25.5 µ kg^{-1} with an overall mean of 18.8 µ kg^{-1}. DH-91 had significantly higher straw Se concentration and it was the only DH line with higher straw Se above the check *Kadzibonga.* Three DH lines (191, 62 and 339) also showed higher straw Se concentration above the check *Nduna*.

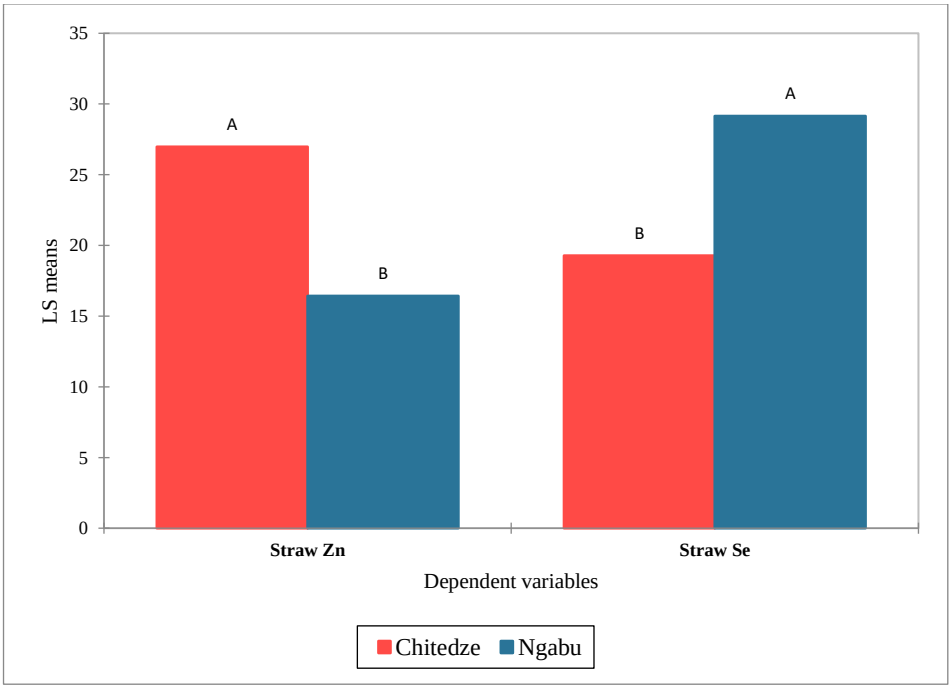


Figure 4.3: Mean values of straw Zn and Se concentration for the factor soil type

For each variable, means with different letters are significantly different from each other at P<0.05 following ANOVA and Fishers protected LSD test

Straw Se concentration varied widely (Table 4.3), with 75% of the variation explained by the variables genotypes (p = 0.041), soil type (p = <0.0001) and the interaction between soil and genotypes (p = 0.001). Among the three variables, straw Se was highly influenced by soil type, and it was higher in Ngabu soils compared to Chitedze soils (Figure 4.3). Mean Straw Se was higher in Ngabu soils compared to Chitedze soils. In Ngabu soils, straw Se varied from 17.3 to 41.7 µ kg^{-1} with an overall mean of 29.1 µ kg^{-1}. DH-62 had the highest straw Se concentration and it was the only DH line with higher straw Se above the check *Kenya nyati*. Overall, all the DH lines grown in Ngabu soils showed high straw Se concentration above all the checks with the exception of *Kenya nyati*. In Chitedze soils, straw Se varied from 12.7 to 25.5 µ kg^{-1} with an overall mean of 18.8 µ kg^{-1}. DH-91 had significantly higher straw Se concentration and it was the only DH line with higher straw Se above the check *Kadzibonga*. Three DH lines (DH-191, DH-62 and DHF1-339) also showed high straw Se concentration above the check *Nduna*.

4.4.4 Regression analysis

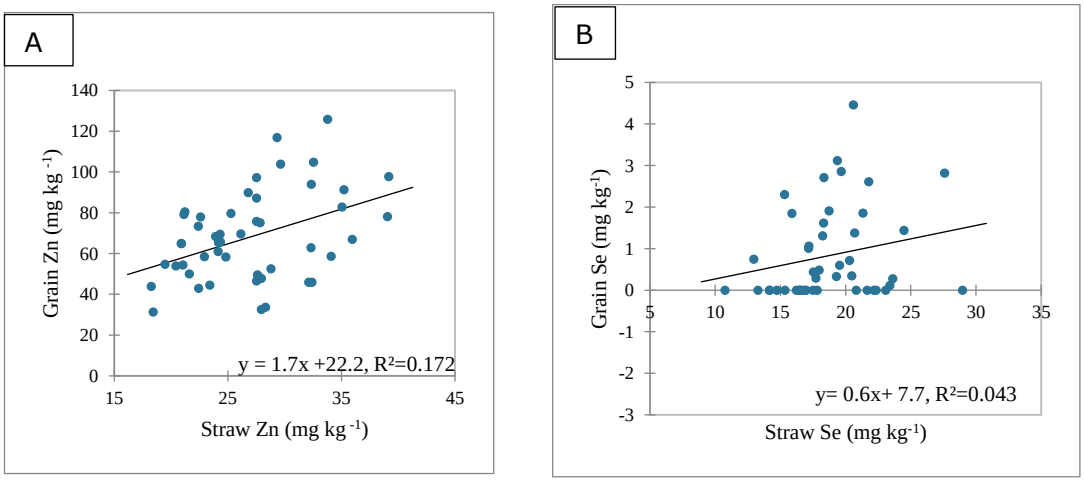


Figure 4.4: Regression analysis between (A) grain and straw and Zn concentration (B) and grain and straw Se concentration when grown in Chitedze soils

In Chitedze soils, a low significant association was observed between straw and grain Zn concentration (R^2 = 0.172, P = 0.004). Regression analysis (Figure 4.4) did not show any association between grain and straw Se concentration (R^2 = 0.043, P = 0.37).

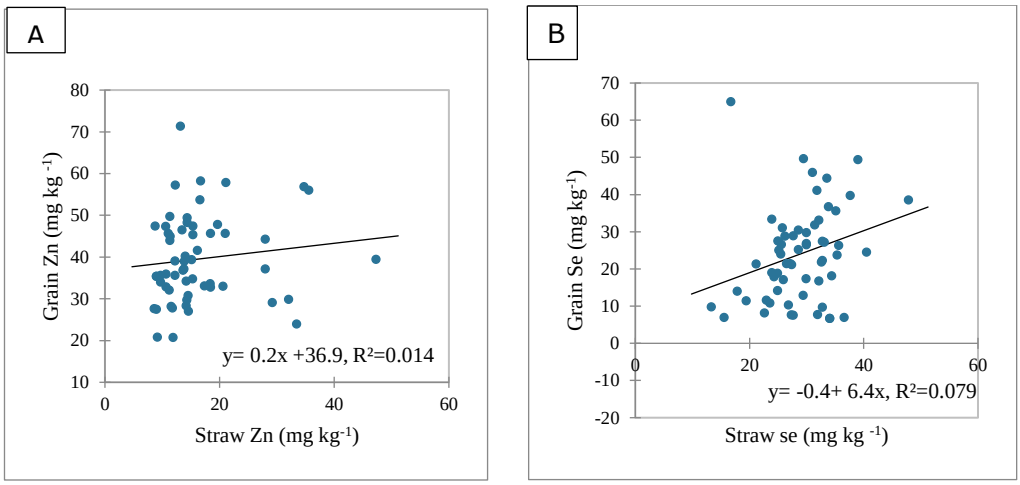


Figure 4.5: Regression analysis between (A) grain and straw Zn concentrations (B) and grain and straw Se concentrations when in Ngabu soils

In Ngabu soils, a low positive association was observed between straw and grain Se concentration (R^2 = 0.079, P = 0.031), while regression analysis (Figure 4.5) did not show any association between grain and straw Se concentration (R^2 = 0.014, P = 0.164).

4.4.5 Phenotypic and phenological traits

Table 4.4: Number of tillers, days to flowering and days to heading of 21 genotypes grown in Chitedze and Ngabu soils in 2021 winter season

Variables	Number of Tillers		Days to Heading		Days to Flowering	
	Chitedze	Ngabu	Chitedze	Ngabu	Chitedze	Ngabu
Min	2	1	86	67	90	75
Max	11	6	123	121	130	126
Grand mean	5	3	105	100	110	106
R2	0.78		0.76		0.77	
Genotypes	<0.0001		<0.0001		<0.0001	
Soil type	<0.0001		0.0002		0.003	
Genotype*soil type	<0.0002		0.001		0.001	
LSD (5%)	9.6		9.7		2.1	
CV%	4.9		5.2		30.0	

Significant variation in the number of tillers, days to heading and days to flowering were influenced by the variables genotype, soil type and the interaction between genotypes and soil type (Table 4.4). 78, 77 and 76% of the variation in the number of tillers, days to heading and days to flower was explained by all the three variables with

both genotypes and soil type having the greatest influence. In Chitedze soils, the number of tillers varied from 2-11 with an overall mean of five. Days to heading varied from 86 to 123 with an overall mean of 105 days and days to flower varied from 90 to 130 with an overall mean 110 days. In Ngabu soils, number of tillers ranged from one to six with an overall mean of three. Days to flowering varied from 75 to 126 with an overall mean of 106 and days to head varied from 67 to 121 with an overall mean of 101 days.

Table 4.5: Correlation coefficients for grain mineral-elements and phenotypic and phenological data of *Am. muticum and T.urartu* DH lines grown in Chitedze soils

Variables	GZn	GFe	GCa	GSe	SSe	S Zn	NT	DH	DF
GZn	**1**								
GFe	**0.897**	**1**							
GCa	-0.154	-0.099	**1**						
GSe	0.195	0.112	0.035	**1**					
SSe	0.139	0.133	0.031	0.208	**1**				
SZn	**0.405**	**0.362**	-0.176	0.196	**0.416**	**1**			
NT	0.035	-0.063	-0.154	-0.148	0.020	0.024	**1**		
DH	**0.374**	**0.349**	-0.042	-0.200	-0.152	0.034	-0.077	**1**	
DF	**0.392**	**0.370**	-0.026	-0.205	-0.151	0.043	-0.097	**0.992**	**1**

Values in bold are significantly different at alpha level =0.05

GZn = grain Zn, GFe = grain iron, GSe = grain selenium, GCa = grain calcium SSe = straw Zn, TN = number of tillers, DH = days to heading and DF = days to flowering

For Chitedze soils (Table 4.5), grain Zn concentration showed a significant and very strong positive correlation with grain Fe (r = 0.897, P = <0.001). Grain Zn also positively and significantly correlated with straw zinc (r = 0.405, P = 0.005), days to flowering (r = 0.392, P =0.007) and days to heading (r = 0.374, P =0.010). Grain Fe showed a positive and significant correlation with days to flowering and days to heading, a weak positive correlation with grain Se, straw Zn and straw Se, and a weak negative correlation with grain Ca and number of tillers. Grain Ca showed a positive but very weak correlation with grain Se and straw Zn, and a weak negative correlation with straw Zn, number of tillers, days to flowering and days to heading. Grain Se showed a positive correlation with straw Zn (r = 0.331, P =0.045), a weak positive correlation with straw Se, and a weak negative correlation with the three phenotypic

traits. Straw Zn positively correlated with straw Se (r = 0.416, P = 0.004), and showed a very weak positive correlation with straw Se (Table 4.5).

Table 4.6: Correlation coefficients for grain mineral-elements and phenotypic and phenological data of *Am. muticum and T.urartu* DH lines grown in Ngabu soils

Variables	GZn	GFe	GCa	GSe	SZn	SSe	TN	DH	DF
GZn	**1**								
GFe	**0.630**	**1**							
GCa	**0.487**	**0.460**	**1**						
GSe	0.232	**0.646**	0.262	**1**					
SZn	0.119	-0.204	-0.083	-0.203	**1**				
SSe	-0.138	-0.068	0.009	0.150	-0.041	**1**			
TN	-0.123	0.214	0.198	**0.311**	-0.077	0.070	**1**		
DH	0.154	**0.319**	-0.107	0.136	-0.132	0.021	0.068	**1**	
DF	0.148	**0.359**	-0.061	0.164	-0.192	0.026	0.155	**0.970**	**1**

Values in bold are significantly different at alpha level =0.05

GZn = grain Zn, GFe = grain iron, GSe = grain selenium, GCa = grain calcium SSe = straw Zn, TN = number of tillers, DH = days to heading and DF = days to flowering

In Ngabu soils, grain Zn concentration showed a significant and strong positive correlation with grain Fe (r = 0.630, P = <0.001), a moderate positive correlation with grain Ca (r = 0.487, P = 0.001) and a weak but insignificant positive correlation with grain Se (r = 0.232, P = 0.095). Grain Fe significantly and positively correlated with grain Ca (r = 0.460, P = 0.001), grain Se (r = 0.646, P = <0.001), days to flowering(r = 0.359, P = 0.008), and days to heading (r = 0.319, P = 0.020). However, grain Fe showed a negative correlation with both straw Zn and Se. Grain Ca showed a weak positive correlation with both grain and straw Se, and a negative and weak correlation with straw Zn. Grain Se positively and significantly correlated to number of tillers (r = 0.311, P = 0.023) and positively but insignificantly correlated with straw Se. For phenotypic traits, days to flowering positively and significantly correlated with days to heading (r = 0.970, P = <0.001), but insignificantly correlated with number of tillers (Table 4.6).

4.5 Discussion

4.5.1 Grain Zinc

Grain Zn concentrations varied considerably among the 21 genotypes and between the two soil types. Between the soils types, the mean grain Zn concentration for Chitedze soils was ~ two-fold higher than that for Ngabu soils. According to the R^2 value from the regression model, variation in grain Zn concentration was highly influenced by soil type, which is likely associated with the differences in soil pH, DTPA-Zn (plant available Zn) and soil organic matter. Soil pH was 7.1 in Ngabu soils while in Chitedze soils pH was 5.4. According to Alloway (2008), an increase in soil pH increases the adsorptive capacity, the formation of hydrolysed forms of Zn, possible chemisorption on calcium carbonate and co-precipitation of Zn in iron oxides, making it unavailable for plant uptake. Previous work has shown that Zn concentration in soil solution decreases by 30 to 45-fold for each unit increase in soil pH range, specifically from pH 5.5 to 7.0 (Marschner, 1993). Previous studies have also shown that soil pH is inversely related to DTPA-Zn (Eyupoglu F, 1994, Cakmak, 2008). In this study, DTPA-Zn was 1.25 mg/kg in Chitedze soils while in Ngabu soils DTPA-Zn was 0.33 mg/kg. In winter wheat, Wang et al. (2017) showed that high DTPA-Zn resulted in increased Zn absorption by the root, high translocation of Zn to shoot and subsequent increase in the straw. Conversely, low soil DTPA-Zn resulted in low absorption, translocation to shoot and low straw uptake (Wang et al., 2017). Grain Zn concentration in cereals and legumes was also shown to increase with increased DTPA-Zn (Manzeke et al., 2019). The findings in the current study suggest that the differences in DTPA-Zn and soil pH between the two soil types significantly contributed to the differences in grain Zn concentration. The trend is also reflected in the straw samples, where mean concentration of straw samples from Chitedze are ~two-fold higher than those from Ngabu soils. Organic matter can have both a positive and a negative effect on the solubility of Zn for plant uptake. Soils with high rapidly decomposable organic matter results in soluble organic Zn complexes, which are mobile and available for plant uptake, while in high non-decomposable organic matter Zn may be low due to the formation of stable organic complexes with the solid-state organic matter (Alloway, 2008, Aghili et al., 2014). In this study, organic matter was higher in Chitedze soils than in Ngabu soils; however, the effect of high organic matter

on grain Zn concentration cannot be conclusive. Both Ngabu and Chitedze soils were previously described as vertisols, which fall in the class of calcareous soils often characterised by high pH, high $CaCo_3$ content and low zinc concentration. Soil characterisation in this study shows that the Ngabu soils fit the vertisols description more than the Chitedze soils. Joy et al. (2015) showed that total zinc concentration of leafy vegetables was significantly higher when grown in non-calcareous soils compared to calcareous soils. These findings are similar to their findings.

Variation in grain Zn concentration was also influenced by the genotypes. DH-62 stood out among the genotypes, with the highest grain zinc concentration when grown in both Chitedze and Ngabu soils and also high straw Zn for both the soils. This finding suggests the efficiency of DH-62 in Zn uptake, and remobilisation of Zn from straw to grain regardless of soil type. A significant positive correlation between grain and straw Zn, particularly in Chitedze soils supports this argument. Regression analysis of grain and straw Zn suggest that some lines were effective in Zn remobilisation to the grain while some were not. Previous studies have shown that root Zn uptake and Zn remobilisation from straw to grain are both essential for grain Zn accumulation (Kutman et al., 2010, Liu et al., 2019). Wheat/*T. urartu* DH-254 and wheat/*Am. muticum* DH-191 also showed exceptionally high grain and straw Zn concentration in both soil types, although DH-254 showed very low straw concentration in Ngabu soils. DH-62 is a wheat/*Am. muticum* DH line with *Am. muticum* segments on wheat chromosome 4D and 7A. DH-254 has two *T. urartu* segments recombined with chromosome 5A of wheat, while DH-191 has *Am. muticum* segments on wheat chromosome 7D. Interestingly, in both the soil types, all the three lines showed high grain Zn concentration above the checks Paragon and Chinese spring, which are the wheat background in the DH introgression lines. This result suggests the effect of the wild relatives segments on grain Zn accumulation. Similarly, the three DH lines, like most of the DH lines, showed high grain Zn concentrations above the three Malawian wheat checks. This shows that the DH lines are potential sources of novel alleles for biofortification of wheat with Zn.

4.5.2 Grain Fe

Variations in grain Fe concentration were also highly influenced by soil type. When grown in Chitedze soils, mean grain Fe concentration was 1.3 fold higher than for

Ngabu soils. This is more likely because of the differences in soil pH and organic matter. Usually, when soil pH is near or above 7.0, plant available Fe becomes limited due to low solubility (Horneck et al., 2007). Low solubility of Fe is more pronounced in calcareous soils where Fe is rapidly converted into unavailable forms, leading to its immobilisation (Ramzani et al., 2017, Ramzani et al., 2016). A previous review has shown that humic substances in organic matter help to form stable complexes with metal micronutrients that help to maintain micronutrients in bioavailable forms at different pH values (Zanin et al., 2019). In this study, Chitedze soils had higher organic matter compared to Ngabu soils, thus creating more bioavailable Fe. Unlike soil Zn concentration, soil Fe was not very different between the two soil types; therefore, organic matter levels less likely affected the variation in grain Fe concentration between the two soil types.

Variation in grain Fe concentration was also explained by the factor genotypes. DH-62 and DH-191 had exceptionally high grain Fe concentration compared to all the genotypes across the soil types. DH-91, DH-254 and DH-122 also had significantly higher grain Fe across the soil type. Interestingly, all these lines also showed significantly higher grain Zn concentration. Correlation analysis between grain Zn and Fe concentration revealed a very strong positive association, suggesting that the two minerals can be improved simultaneously. This finding is in line with previous studies (Velu et al., 2012, Velu et al., 2016, Zhao et al., 2009, Khokhar et al., 2018, Khokhar et al., 2020). Across the soil types, most of the DH lines had a higher grain Fe concentration above Paragon and Chinese Spring, and above the three Malawian checks. According to HarvestPlus, to reach 80% of the estimated average requirement of Fe for an adult male, biofortification programs must aim at increasing grain Fe to 60 mg kg^{-1}. In Chitedze and Ngabu soils, 94 and 69% of the DH lines had Fe concentration above 60 mg kg^{-1} respectively, while all the Malawian checks had less than the EAR, suggesting that the DH lines can be used to improve the Malawian wheats for grain Fe.

4.5.3 Calcium

Variation in Ca concentration was mainly influenced by genotypes and not soil type, suggesting that soil chemical properties did not affect the availability of Ca for plant uptake, shoot accumulation and remobilisation to the grain. The interaction between soil type and genotype showed a significant effect on grain Ca concentration that is

evident in the slightly high concentration of Ca in Chitedze soils. Joy et al. (2015) reported a higher total Ca concentration in maize grain from calcareous soils than those from non-calcareous soils. Their findings are slightly different from the findings in this study, and this could be attributed to the different responses between maize and wheat. Interestingly, grain Ca showed a significant positive correlation with grain Zn and Fe in Ngabu soils and a negligible negative correlation in Chitedze soils. Availability of Ca is thought to increase more in alkaline soils than in acidic soils (Flis, 2019) in the present study, Ngabu soils had higher soil Ca than Chitedze soils. However, it is difficult to ascertain if the difference in correlation is attributed to differences in the soil pH. In *Triticum dicoccoides* grown in different environments, both negative and negligible positive correlations between grain Ca and Fe and Zn were reported (Gomez-Becerra et al., 2009, Gomez-Becerra et al., 2010a). Across the soil types, wheat/*Am. muticum* DH-196 showed the highest Ca concentration. In Chitedze soils, wheat/*Am. muticum* DH-121 and wheat/*T. urartu* DH-304 also had significantly higher Ca concentration than most of the other DH lines, Paragon and Chinese Spring and all the Malawian checks, while in Ngabu soils, wheat/*Am. muticum* DH-191 had significantly high grain Ca. The result suggest the effect of the introgression on the DH lines, and that the lines could potentially be useful for Ca biofortification.

4.5.4 Selenium

Variation in grain Se concentration was highly influenced by soil type, with genotypes grown in Ngabu soils showing a 30-fold higher grain Se than genotypes grown in Chitedze soils. Differences in soil chemical properties, particularly soil pH and organic matter likely influenced the availability of Se for uptake and subsequent translocation to the shoot. Se bioavailability is an important factor controlling Se concentration in wheat grains, and it generally decreases with decreasing pH, increased organic matter and other soil properties (Gissel-Nielsen et al., 1984, Stroud et al., 2010). In maize grains, Chilimba et al. (2011) showed a 10-fold higher grain Se concentration in samples grown in calcareous (Eutric vertisols) soils than in different types of cambisols, luvisols and lixisols. Their study also showed a strong positive correlation between grain Se concentration and soil pH above pH 6·5, which was attributed to decreasing adsorption of inorganic selenate and selenite on iron/manganese oxides (Chilimba et al., 2011). The current study shows that grain Se concentration was high

in high pH soils and almost negligible in low pH soils. These findings are also consistent with other previous studies (Ligowe et al., 2020, Gashu et al., 2020, Chilimba et al., 2019b). Effects of soil type on Se concentration was also reflected in straw samples, with higher straw Se in samples from Ngabu soils than Chitedze soils. Regression analysis showed a significant association between straw and grain Se in Ngabu soils, and a negative association in straw and grain samples grown in Chitedze soils. This could be an indication that the little Se concentration in the straw samples in Ngabu soils was not remobilised to the grain, while in Chitedze soils, some lines were efficient in remobilisation, while others were not. Although the factor genotypes contributed to the overall variation in grain Se concentration, there was no statistical variation in the genotypes in Chitedze soils. In Ngabu soils, DH-191 and DH-144 showed significantly higher grain Se concentration than all other genotypes. All the DH lines, with the exception of DH-121 and DH-91, had higher grain Se than all the checks. However, the grain Se concentration in all the genotypes was below the required level (50-100 µg kg $^{-1}$) for adequate intake for humans (Gissel-Nielsen et al., 1984, Zhao et al., 2005), therefore making it difficult to select them for improvement of wheat for grain Se.

4.6 Conclusion

Differences in soil type results in a substantial variation in grain mineral nutrient concentrations. A ~two-fold higher grain Zn concentration was shown in low pH, high Zn soils (Chitedze soils) compared to high pH, low Zn soils (Ngabu soils). A 1.3-fold higher grain Fe concentration was shown in plants grown in higher Fe, low pH than in lower Fe high pH soils, whilst a 30-fold higher grain Se concentration was shown in high pH than in low pH soils. This study shows that mineral nutrient concentration in the grain is not only influenced by genotypic differences, but also on differences in the soil physio-chemical properties.

CHAPTER 5

5 Developing Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* introgression lines and their molecular and cytogenetic characterisation

5.1 Abstract

Am. muticum and *T. urartu* doubled haploid (DH) lines developed for trait analysis were shown to have potential for increased grain Zn, Fe and Ca concentration above their recurrent parents. Previously *T. urartu* DH-254 and *Am. muticum* DH-348 showed high grain Zn concentration above most of the DH lines. In this study, DH-254 and DH-348 were crossed with low grain Zn Malawian hexaploid wheats, *Kenya nyati, Kadzibonga and Nduna*. The aim of the study was to transfer *Am. muticum* (TT) and *T. urartu (*$A^u A^u$*)* introgressions, potentially increasing grain Zn concentration in the DH introgression lines, into Malawian wheat varieties. The study was also aimed at characterising the introgression lines using chromosome-specific KASP markers. From the F_1 population generated, a few seeds were selected and backcrossed to their respective recurrent parents to obtain the BC_1 population. Analysis of the BC_1 plants with chromosome-specific KASP markers revealed the presence of heterozygous *Am. muticum* segments on wheat chromosomes 4D and 7A, and *T. urartu* introgressions on wheat chromosome 5A. BC_1 introgression lines with *Am. muticum* and *T. urartu* segments were self-fertilised to obtain the BC_1F_1 population. Genotyping of the BC_1F_1 plants detected the presence of homozygous *Am. muticum* and *T. urartu* introgressions in 42 and 17% of Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* introgression lines, respectively. GISH validated the genotyping results of both the BC_1 and BC_1F_1 introgression lines, particularly in *Am. muticum* derived lines. Homozygous introgression lines were advanced to BC_1F_3, where 46,950 and 16,535 Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* seeds were obtained, respectively. Development of lines with high mineral concentration will contribute to efforts of increasing availability of dietary Zn and Fe in the Malawian population.

5.2 Introduction

Wheat is one of the important crops for a majority of people in Sub Saharan Africa. It is cultivated on an estimated 2.9 million hectares of land (FAOSTAT 2021), with an estimated 47 million tonnes consumed per year (Wuletaw Tadesse et al., 2019). Modern cultivated wheat (*Triticum aestivum*) belongs to the triticeae tribe, which has over 500 species in 32 genera (Wang et al., 2014, Feldman and Levy, 2015). It is an allopolyploid with three homoeologous sub-genomes (2n=6x=42, AABBDD), derived from two hybridization events (Dvorak et al., 1993, Liu et al., 2017b). Initial hybridization involved two diploid progenitors *T. urartu* (2n=2x=14, A^uA^u) and an unidentified species (BB genome) related to *Aegilops speltoides* (SS). The result of this hybridization was tetraploid *Triticum turgidum* (2n = 4x = 28, AABB). The second hybridization event involved tetraploid *T. turgidum* and diploid *Aegilops tauschii* (2n =2x = 14, DD), followed by chromosome doubling (Feldman and Levy, 2015, Dubcovsky and Dvorak, 2007). Domestication of wheat by early farmers and further crop improvement using advanced breeding materials resulted in a narrowed genetic base of wheat (Cox, 1997, Valkoun, 2001). Conversely, wheat progenitors and wild species provide a vast and untapped reservoir of genetic variation for potentially most if not all agronomically important traits (Friebe et al., 1996, Qi et al., 2008, King et al., 2019a).

Through introgression breeding, genetic variation from wheat progenitors and wild relatives can be successfully transferred to modern cultivated wheat (Anderson, 1949, Thórsson et al., 2001). Several studies have shown high grain Zn and Fe concentration in introgression lines developed from cultivated hexaploid/tetraploid wheat and several wild species compared to their modern cultivated wheat parents (Rawat et al., 2009, Tiwari et al., 2010b, Neelam et al., 2011, Wang et al., 2011b, Farkas et al., 2014). For example wheat/wild relative derivatives from *Ae. kotschyii* and *Ae. peregrina* have shown up to a five-fold increase in grain Zn concentration above their recurrent parents (Tiwari et al., 2010a, Neelam et al., 2010, Rawat et al., 2011a). Progenitor species, particularly *Ae. tauschii* have also been shown to increase grain Zn and Fe concentration by 20-40% compared to local varieties (Singh et al., 2017). Currently, pre-breeding programs have successfully transferred chromosome segments from a number of wild species into modern cultivated wheat. For example, wild relative chromosome segments from *Ae. speltoides* (King et al., 2018), *Am. muticum* (King et

al., 2019a, Iefimenko et al., 2018), *Triticum timopheevii* (Devi et al., 2019), *Aegilops caudata* (Grewal et al., 2020b), *T. urartu* (Grewal et al., 2021) and other species have been successfully introgressed into modern cultivated wheat.

Previously, characterisation of introgression lines was challenged by lack of high throughput technologies for single nucleotide polymorphism (SNP) discovery (King et al., 2018). The discovery of next-generation sequencing technologies and advancement in bioinformatics procedures have enabled the discovery of large numbers of SNPs, through whole genome sequencing and re-sequencing projects (Akhunov et al., 2009, Uauy, 2017). Next-generation sequencing and re-sequencing protocols have been used to mine thousands of putative SNPs in wheat and its wild relatives (Allen 2011, Winfield et al 2012). Due to their abundance in the wheat genome, SNPs have been used to develop array probes for use in marker-assisted selection (Wilkinson et al., 2020). A number of high-density genotyping arrays have been developed and utilized for marker-assisted breeding following the discovery of SNPs in wheat. The Illumina Wheat 9K iSelect SNP array (Cavanagh et al., 2013) and the Illumina Wheat 90K iSelect SNP genotyping array (Wang et al., 2014) were among the first SNP genotyping arrays developed and utilised for characterisation of genetic variation in allohexaploid and allotetraploid wheat populations. Although these genotyping arrays were successfully utilised, they were not useful for tracking introgressions in wheat/wild-relative inter-specific populations. The development of the Axiom® HD Wheat Genotyping Array (Winfield et al., 2016, Burridge et al., 2017) and the A 35K Axiom® Wheat-Relative Genotyping Array (Allen et al. 2017) were step-changes in the tracking of introgressions in wheat/wild-relative inter-specific populations. For example, the wheat breeder's array has been used to screen for diversity within and between *Aegilops* species and to identify and track *Aegilops* introgressions in hexaploid wheat (Przewieslik-Allen et al., 2019). The array has also been used to genotype hexaploid wheat/*Am. muticum* (King et al., 2017), wheat/*Th. bessarabicum* (Grewal et al., 2018b), wheat/*T. urartu* (Grewal et al., 2018a) and wheat/*Th. intermedium* (Cseh et al., 2019) introgression lines.

Interestingly, SNPs can be easily converted to genetic markers such as Kompetitive Allele Specific PCR (KASP) markers, which are cost-effective and efficient for low-density genotyping (Grewal et al., 2020a). KASP assays have high flexibility for genotyping a small number of specific loci for gene identification, line evaluation and

marker assisted selection (Rasheed et al., 2016). Grewal et al. (2020) used SNPs obtained after PCR and sequence analysis of genomic DNA from potential single-copy regions of the wheat genome in comparison with their orthologous copies from different wild relatives to convert to chromosome-specific KASP assays (Grewal et al., 2020a). These markers have been successfully used to characterise different hexaploid wheat/wild relative introgression lines (King et al., 2019a, Grewal et al., 2020b, Grewal et al., 2021).

In inter-specific populations, wild relative introgressions identified through genotyping can be validated using genomic *in situ* hybridization (GISH). GISH provides a direct, visual method of distinguishing parental genomes as well as intergeneric and interspecific hybrids, allowing the correlation of molecular information of a DNA sequence with its physical location on a chromosome (Uhrin et al., 2012, Schwarzacher et al., 1992). The technique uses total genomic DNA of a species as probe of a progenitor involved in the formation of a hybrid and unlabelled DNA from another progenitor which serves as a blocking DNA, hybridizing with sequences in common with both genomes (Silva and Souza, 2013). *In situ* hybridization was first described in the 1960s (Pardue and Gall, 1969). The initial technique involved localisation of DNA-DNA hybrid molecules in cytological preparations and their detection using autoradiography. This technique used radioactive labelled satellite DNA. A method of using *in- situ* hybridization with non-radioactive labelling was described subsequently (Schwarzacher, 2003, Kato et al., 2004). GISH has been successfully used to detect and characterise alien introgressions in cultivated wheat (Brasileiro-Vidal et al., 2005, Dube E, Yang et al., 2016, King et al., 2019a), maize (Wang et al., 2008), cotton (Tang et al., 2018, Wang et al., 2018) brassica (Kang et al., 2014), potatoes (Gaiero et al., 2017), onion (Yamashita et al., 2005), rice (Abbasi et al., 2010) and tomato (Jacobsen et al., 1995). In wheat, GISH has been used to detect wild relative chromatin in wheat-alien amphiploids, amphidiploids, addition, translocation, substitution and double haploid lines (Uhrin et al., 2012, Yang et al., 2016, He et al., 2017, King et al., 2019a).

The aim of this study was to

1. To transfer *Am. muticum* (TT) and *T. urartu (*$A^{u}A^{u}$*)* introgressions, from doubled haploid introgression lines into Malawian wheat varieties for increased grain mineral concentration
2. To characterise the introgression lines using chromosome-specific KASP markers and genomic *in situ* hybridization (GISH)

5.3 Materials and methods

5.3.1 Germplasm

DH-348 was developed by pollinating hexaploid wheat cv. Pavon 76 with *Am. muticum* accession 2130012, followed by the procedure described in Chapter 3. *T. urartu* DH-254 was developed from a cross between hexaploid wheat cv. Chinese Spring (*ph1/ph1*) and *T. urartu* accession 1010002 followed by the same procedure described in chapter 3. Three Malawian hexaploid wheat varieties, *Kenya Nyati, Kadzibonga* and *Nduna* were obtained from Lilongwe University of Agriculture and Natural Resources (LUANAR) in Malawi, and they represent the wheat varieties that are commonly grown in Malawi.

5.3.2 DNA Sequencing

Genomic DNA (deoxyribonucleic acid) for sequencing was collected from 2-week-old leaf samples. Extraction was performed using extraction buffer (0.1 m Tris–HCl (pH 7.5), 0.05 m EDTA (pH 8.0), 1.25% SDS). Samples were incubated at 65 °C for 1 h before mixing with 6M ammonium acetate (stored at 4°C) for 15 min. The samples were then spun down and the supernatant mixed with isopropanol to precipitate the DNA. To remove RNA in the DNA solution, RNase A was added, and the samples incubated at 37°C for 1 hour. The supernatant was then purified with phenol/chloroform (1:1 V/V) and the isolated DNA was re-suspended in 100ml 1XTE buffer.

Library preparation and DNA sequencing was performed by the Novogene (UK) Company Limited. The DNA sample used for library preparation was prepared following the manufacture's recommendations of NEBNext® DNA Library Prep Kit (New England BioLabs, US). Index codes were added to each sample. Briefly, the genomic DNA was randomly fragmented to size of 350bp. DNA fragments were end polished, A-tailed, ligated with adapters, size selected and further PCR enriched. Then polymerase chain reaction (PCR) products were purified (AMPure XP system),

followed by size distribution by Agilent 2100 Bioanalysis (Agilent Technologies, CA, USA), and quantification using real-time PCR. The library was then sequenced for 10x whole genome sequencing (WGS) on NovaSeq 6000 S4 flow cell with PE150 strategy.

5.3.3 Seed germination

To avoid contamination, the Malawian varieties were sterilised with 5 % sodium hypochlorite solution (ClNaO – sigma Aldrich 017-001-00-1) with 0.1% Tween® 20 (Sigma-Aldrich, Chemie GmbH, Steinheim) before germination. Germination of both the DH lines and the Malawian varieties was performed in module trays using Levington Advance Seed and Modular + Sand (F2 + S) compost (ICL, Suffolk, United Kingdom).

5.3.4 Vernalisation and potting

Seven days after sowing, seedlings were placed into vernalisation. The temperature was set at 6˚C and photoperiod for 12 hours (6 am-6 pm). After 4 weeks, the seedlings were taken to the glasshouse for potting using John Innes Compost No. 2 (Westland Horticulture Limited, Dungannon, Northern Ireland). The plants were left under glasshouse conditions with the photoperiod set at 25˚C, light at 16 hours and 8 hours dark.

5.3.5 Emasculation and pollination

Emasculation was done before the spikes completely emerged from the flag leaf. Emasculated spikes were covered with glassine bags following removal of the anthers. Two days after emasculation, the stigma was checked, and was considered ready for pollination when it appeared fluffy/feathered. Bright yellow anthers were collected from a male parent for pollination of the emasculated spike. Pollen was released onto the stigma using a pair of forceps and the spikes were covered with glassine bags to protect them from foreign pollen (Figure 1).

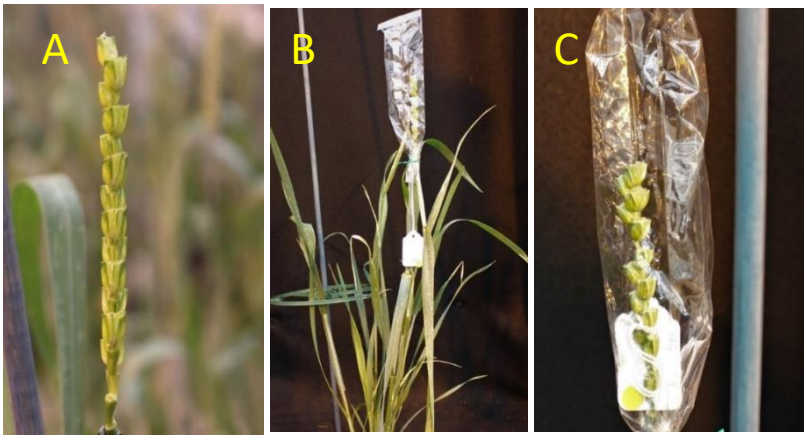


Figure 5.1: Emasculated spike (A), emasculated spike covered with glassine bag (B) and pollinated spike covered with glassine bag (C)

5.3.6 Crossing program

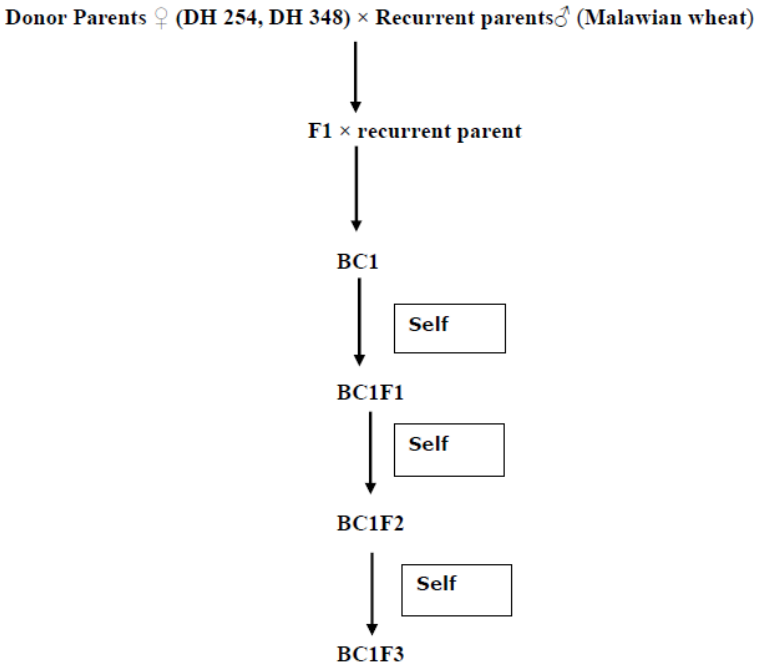


Figure 5.2: A crossing program of three Malawian wheat varieties (*Kadzibonga, Nduna, Kenya nyati*) with DH-348 and DH-254

5.3.7 Harvesting and threshing

All the spikes from the F_1 population were harvested individually and threshed manually, while all the backcross populations were harvested together and threshed mechanically.

5.3.8 Genotyping

Genomic DNA was extracted in a 96 well plate from leaf samples collected from 10 day old seedlings (Thomson and Henry, 1995). Extraction was performed using template preparation solution (TPS) buffer and isopropanol. Malawian wheats, *Kadzibonga*, *Kenya nyati* and *Nduna* alongside wheat/*Am. muticum*, DH 348, wheat/*T. urartu*, DH-254 *Am. muticum* accession 2130012 and *T. urartu* accession 1010002

were used as controls. The KASP assays comprised of two allele specific primers and one common reverse primer (see appendix 2). A final reaction volume of 5µl, which included 1ng genomic DNA, 2.5µl KASP reaction mix (ROX), 0.068µl primer mix and 2.43µl nuclease free water Primer mix, was dispensed into the 386 well plates using Gilson pipette max 268 (Gilson, INC. 3000 Parmenter St. Middleton, WI 53562). Plates were sealed with optical quantitative polymerase chain reaction (qPCR) seals (Sarstedtstr AG & Co. KG, Numbrecht, Germany) following a brief centrifuge. Genotyping was done using ProFlex PCR system (Applied Biosystems by Thermo Fisher Scientific). PCR conditions were set as 15 min at 94°C; 10 touchdown cycles of 10 s at 94°C, 1 min at 65–57°C (dropping 0.8°C per cycle); and 35 cycles of 10 s at 94°C, 1 min at 57°C.

5.3.9 Genomic *in-situ* hybridisation

GISH was performed following a protocol described by Kato *et al*. (2004) and King *et al.* (2017). Genomic DNA was extracted from *Am. muticum* and the three progenitors of bread wheat: *T. urartu*, *Ae. speltoides*, and *Ae. tauschii* using an extraction buffer (0.1 M Tris-HCl, 0.05 m EDTA and 1.25% SDS). Genomic DNAs of *Am. muticum*, *T. urartu*, *Ae. tauschii* and *Ae. speltoides* were labelled by nick translation with ChromaTide Alexa Fluor 546-14-dUTP (Alexa Fluor-546), ChromaTide Alexa Fluor 488-5-dUTP (Alexa fluor-488) [Thermo Fisher Scientific (Invitrogen), Waltham, MA, United States] and Alexa Fluor 594-5-dUTP (Alexa fluor-594) [Thermo Fisher Scientific (Invitrogen), Waltham, MA, United States] and ChromaTide Alexa 405 dUTP, respectively. Metaphase spreads were prepared from root tips using a nitrous oxide-enzymatic maceration method (Kato, 1999). Briefly, root tips were digested in an enzyme solution (Pectolyase 1% and Cellulase 2% -Yakult Pharmaceutical Ind. Co., LTD. Japan) at 37°C for ~50 min following a treatment with nitrous oxide for 2 hours, and root fixation with 90% acetic acid. Digested root tips were pressed into very fine cell suspension, and later centrifuged to form a pellet. Acetic acid (100%) was added to the pellet and left for 10 minutes before dropping on a labelled slide.

Malawian wheat/*Am. muticum* slides were probed using a probe mixture containing 1.5µl of *T. urartu,* 1.5µl *Ae. speltoides*, 2µl *Ae. tauschii* and 0.3µl *Am. muticum* labelled genomic DNA in 2 × SSC and 1 × TE buffer (pH 7.0) to a final volume of 10µl per slide. Malawian wheat/*T. urartu* slides were probed using a similar probe mixture with an exception of *Am. muticum* genomic DNA. Slides were counterstained with

Vectashield mounting medium with 4-6-diamidino-2phenylindole dihydrochloride (DAPI). Analysis was done using a Zeiss Axio ImagerZ2 upright epifluorescence microscope (Carl Zeiss Ltd, Oberkochen, Germany) with filters for DAPI (Ex/Em 358/461 nm, blue), Alexa Fluor 488 (Ex/Em 490/520 nm, green), Alexa Fluor 594 (Ex/Em 590/615 nm, red) and Alexa Fluor 546 (Ex/Em 555/570 nm, yellow). Photographs were taken using a MetaSystems Coolcube 1 m CCD camera.

5.4 Data analysis

The Earlham bioinformatics pipeline (Coombes et al., 2022) was used to analyse the sequencing data (DNA sequence alignment, variant calling, mapping and SNP coverage analysis and data visualization), and this was performed at the Earlham Institute. Florescence detection and data analysis of KASP reactions was performed using Quant Studio Design and Analysis Software V1.5.0 (Applied Biosystems by Thermo Fisher Scientific). GISH analysis was carried out using Meta Systems ISIS and Metafer software (Metasystems GmbH, Altlussheim, Germany).

5.5 Results

5.5.1 Sequencing of the parental lines

The sequence reads from the parental lines, hexaploid wheat cvs. Chinese Spring and Paragon, *Am. muticum* and DH348 were mapped to the wheat reference genome assembly cv. Chinese Spring RefSeq v.1.0 (IWGSC, 2018). Whole genome sequence analysis of DH-348 revealed the presence of two *Am. muticum* segments on wheat chromosomes (Chr) 4D and 7A as shown in the drop in read coverage (red blocks) in Figure 5.3. Analysis of the size of the introgressed segments showed that the segment on Chr 4D is bigger (51.2 Mbp) compared to the segment on Chr 7A (9.1 Mbp). Sequence analysis also revealed a monosomic deletion on the short arm of Chr 5D. GISH analysis of DH-348 (Figure 5.5 A) partially validated the sequencing results, as it showed a pair of recombinant chromosomes with a large D chromosome (labelled red) and a small T segment (labelled gold) at the distal end of the D chromosome. The *Am. muticum* segment visible from the GISH metaphase spread is likely from Chr 4D as the segment on 7A is too small to be detected by GISH.

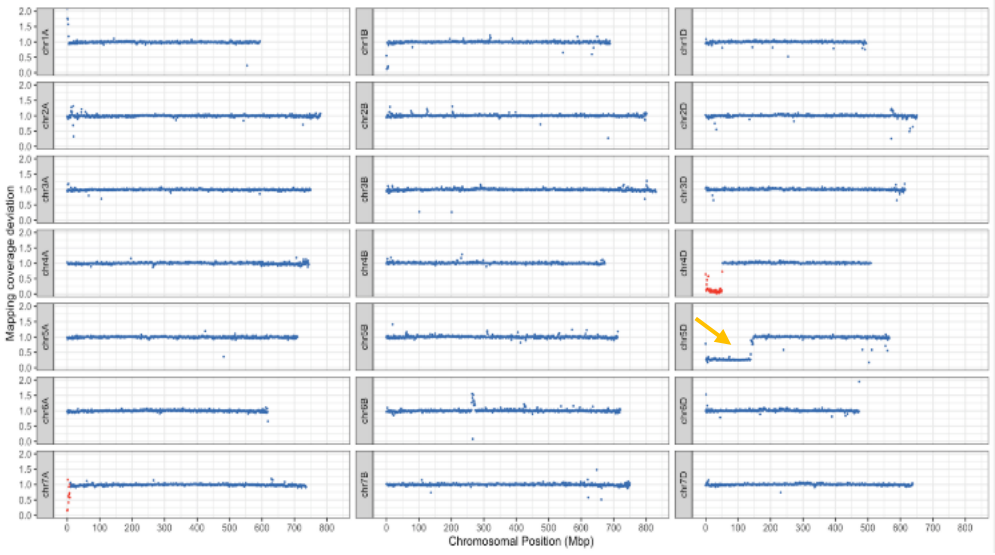


Figure 5.3: Sequencing visualisation of DH-348 showing *Am. muticum* segment introgressed on wheat Chr 4D and on Chr 7A (*Am. muticum* chromosomes in red blocks, wheat chromosomes in blue blocks), and a monosomic deletion on wheat Chr 5D (yellow arrow)

Grewal and Coombes previously sequenced DH-254 (unpublished). Their results showed that two segments of *T. urartu* had recombined with the 5A chromosome of wheat. The size of the two segments are 76.40 and 28.77 Mbps. Sequence analysis also showed that a portion of chromosome 5A had duplicated and translocated to Chr 5D to replace the 5DL chromosome portion.

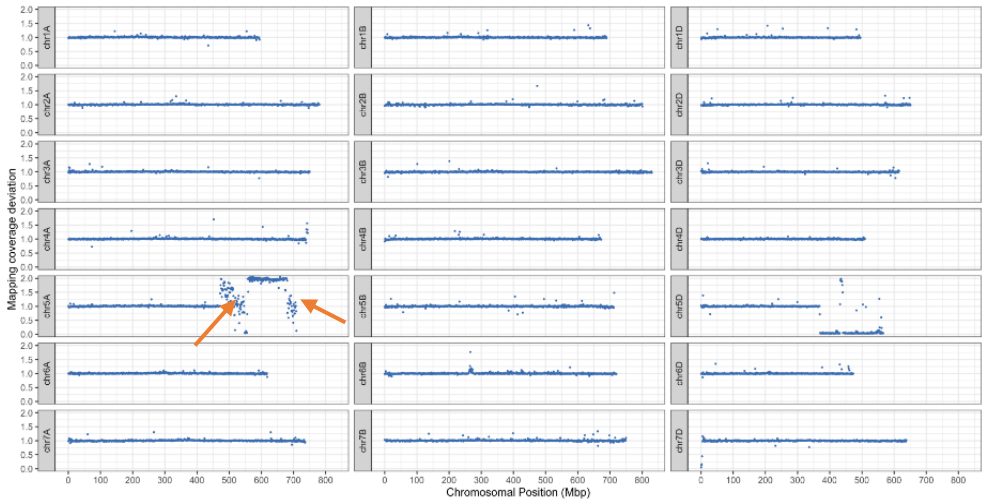


Figure 5.4: A- Sequencing visualisation of DH-254 showing *T. urartu* segments introgressed on wheat Chr5A (shown by orange arrows) and the 5D-5A intergenomic recombination shown by the drop in chromosome block on chromosome 5D

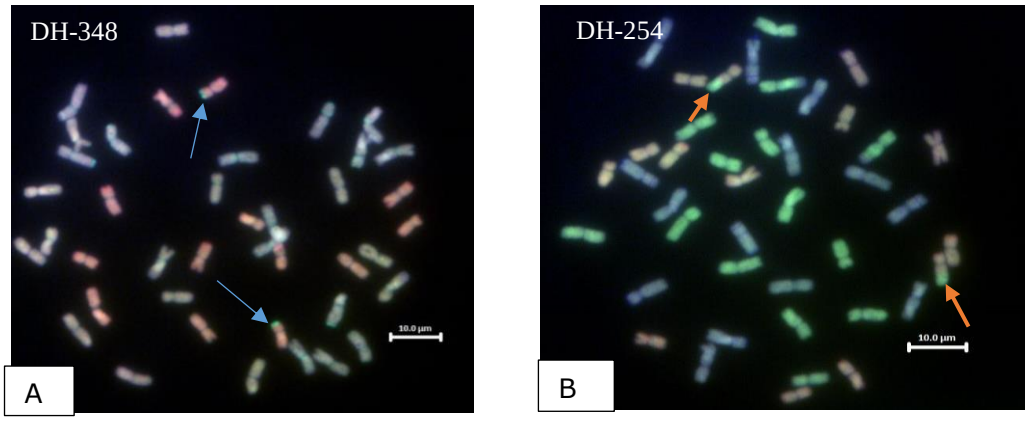


Figure 5.5: A- GISH image of metaphase spreads from roots of DH-348 showing the A, B, D and T genomes (A genome - green, B genome - blue, D genome – red, T genome - gold). The blue arrows indicate the site of *Am. muticum* introgressions. B- GISH image of metaphase spreads from roots of DH-254 showing the A, B and D genomes (A genome - green, B genome - blue, D genome – red. Orange arrows shows the 5A-5D recombinant chromosomes

5.5.2 Generating a segregating population

Hybridisation of the donor (DH-348 and DH-254) and the reciprocal parents (*Nduna*, *Kadzibonga* and *Kenya nyati*) was performed in both directions (as both males and females). In total, six cross combinations were made for each of the DH lines. In the initial round of crossing, 262 and 120 F_1 seeds were obtained for the Malawian wheat/DH-348 and Malawian wheat/DH-254 combinations, respectively (Table 1 and 2). Following harvesting of the F_1 seeds, 6-8 seed from each combination were germinated for backcrossing with the respective recurrent parents. In total, 362 and 190 BC_1 seeds were generated for the Malawian wheat/DH-348 and Malawian wheat/DH-254 combinations, respectively.

Self-fertilisation of the BC_1 plants generated 11,058 Malawian wheat/*Am. muticum* and 5,300 Malawian wheat/ *T. urartu* BC_1F_1 seeds. Further self-fertilisation/bulking of the seeds resulted in the generation of 46,950 Malawian wheat/*Am. muticum* and 16,535 Malawian wheat/*T. urartu* BC_1F_3 seeds. Among the Malawian wheat/*T. urartu* cross combinations, three combinations (DH 254 ×Kadzibonga, Kadzibonga ×DH 254 and Nduna × DH 245) were lost at F_1 and BC_1F_2 due to the inability to produce seed and failure of the selected seeds to germinate, respectively. Among the Malawian wheat/*Am. muticum* cross combinations, only one combination (DH 348 × Kenya nyati) was lost at BC_1F_2 due to failure of the selected seeds to germinate.

Table 5.1: Malawian wheat and *Am. muticum*, DH-348 cross combinations, number of crosses and number of seed produced in the F_1, BC_1, BC_1F_1, BC_1F_2 and BC_1F_3 generations under glasshouse conditions

Cross combination	No. of crosses	F_1 seed	No. of crosses	BC_1	BC_1F_1	BC_1F_2	BC_1F_3
DHF_1 348 x Nduna	10	52	18	121	2,443	721	6,786
Nduna x DHF_1 348	9	33	5	32	1,556	1,471	8,946
DHF_1 348 x Kenya nyati	9	70	2	11	305	-	-
Kenya Nyati x DHF_1 348	14	64	11	63	2,768	1,393	15,382
DHF_1 348 x Kadzibonga	1	17	18	80	2,359	728	7,334
Kadzibonga x DHF_1 348	6	27	7	55	2,077	1,204	8,502
Total	49	263	61	362	11,508	5,517	46,950

Table 5.2: Malawian wheat and *T. urartu* line DH-254 cross combinations, number of crosses and number of seed produced in the F_1, BC_1, BC_1F_1, BC_1F_2 and BC_1F_3 generations under glasshouse conditions

Cross combination	No. of crosses	F_1 seed	No. of crosses	BC_1	BC_1F_1	BC_1F_2	BC_1F_3
DHF_1 254 x Nduna	2	8	7	44	580	163	4,818
Nduna x DHF_1 254	6	33	3	15	504	-	-
DHF_1 254 x Kenya nyati	9	70	19	124	3,622	632	8,065
Kenya Nyati x DHF_1 254	3	9	2	7	594	247	3,652
Total	20	120	31	190	5,300	1,042	16,535

5.5.3 Genotyping Malawian wheat/*Am. muticum* BC_1 and BC_1F_1 plants with chromosome-specific KASP markers

One hundred and eighty-two chromosome-specific KASP markers were tested on the parental line, DH-348, three *Am. muticum* accessions and three Malawian wheat varieties. Eight to ten markers were selected for each linkage group (1A-7A, 1B-7B and 1D-7D) based on their position and results from previous work (King et al., 2019a). The markers were designated codes between WRC1001-WRC1329 (Grewal et al., 2020a) and WRC1330-WRC169 (Grewal et al., 2022). Of the 182 markers tested on the parental lines, only eighteen failed to score the genotypes. Genotyping of 80 wheat/*Am. muticum* BC_1 plants with group 4 (WRC1314, WRC1315, WRC1316 and WRC1784) and group seven markers (WRC2020 and WRC2104) within the region of the segments, detected the presence of heterozygous *Am. muticum* segments on wheat Chr 4D and Chr 7A in 19 lines. The KASP markers were also able to detect 15 lines with the *Am. muticum* segment on Chr 4D only and 13 lines with the *Am. muticum* segment on Chr 7A only. Subsequent genotyping of 85 BC_1F_1 plants for the 4T and 7T segments detected 25 lines homozygous for the segment on Chr 4D, 14 lines on Chr 7A and 2 lines on both Chr 7A and Chr 4D. Further analysis showed that 26 lines remained heterozygous for the segment on Chr 4D while the rest of the lines had lost the segments (Table 5.3).

Table 5.3: A list of Malawian wheat/*Am. muticum* BC_1 and BC_1F_1 lines showing number of *Am. muticum* segments detected by chromosome-specific KASP markers and their location on the wheat genome.

Cross combination	BC_1 code	BC_1F_1 code	No. of T segments	Location on wheat chromosome
DHF1 348 x Nduna	BC_1 605-2	BC_1F_1 64-2	2	4D,7A
	BC_1 606-1	BC_1F_1 62-1	1	4D
	BC_1 603-3	BC_1F_1 67-4	1	7A
	BC_1 606-3	BC_1F_1 63-2	1	4D
	BC_1 603-3	BC_1F_1 67-2	1	4D
DHF1 348 x Kadzibonga	BC_1 597-2	BC_1F_1 78-1	1	4D
	BC_1 599-1	BC_1F_1 72-2	1	4D
	BC_1 600-3	BC_1F_1 70-1	1	4D
	BC_1 600-1	BC_1F_1 71-1	1	7A
	BC_1 600-1	BC_1F_1 71-3	1	7A
	BC_1 598-3	BC_1F_1 75-1	1	7A
	BC_1 599-4	BC_1F_1 73-2	1	4D

	BC_1 600-4	BC_1F_1 123-3	1	7A
Nduna x DHF1 348	BC_1 607-4	BC_1F_1 60-1	1	4D
	BC_1 607-4	BC_1F_1 60-2	1	4D
	BC_1 608-3	BC_1F_1 59-2	1	4D
	BC_1 608-2	BC_1F_1 58-1	1	7A
	BC_1 610-1	BC_1F_1 54-1	1	7A
	BC_1 607-3	BC_1F_1 61-2	2	4D,7A
	BC_1 609-3	BC_1F_1 56-1	1	4D
	BC_1 609-3	BC_1F_1 56-2	1	4D
	BC_1 605-3	BC_1F_1 113-2	1	4D
	BC_1 607-1	BC_1F_1 116-2	1	7A
	BC_1 606-1	BC_1F_1 62-1	1	4D
	BC_1 606-1	BC_1F_1 62-3	1	7A
Kadzibonga x DHF1 348	BC_1 612-3	BC_1F_1 50-1	1	4D
	BC_1 611-1	BC_1F_1 51-1	1	4D
	BC_1 612-2	BC_1F_1 49-1	1	4D
	BC_1 611-4	BC_1F_1 53-1	1	7A
	BC_1 612-3	BC_1F_1 50-2	1	4D
Kenya Nyati x DHF1 348	BC_1 618-1	BC_1F_1 38-1	1	4D
	BC_1 615-3	BC_1F_1 42-2	1	4D
	BC_1 616-2	BC_1F_1 35-1	1	4D
	BC_1 616-2	BC_1F_1 35-2	1	4D
	BC_1 616-3	BC_1F_1 36-1	1	4D
	BC_1 617-1	BC_1F_1 37-1	1	4D
	BC_1 618-3	BC_1F_1 39-1	1	7A
	BC_1 615-4	BC_1F_1 43-2	1	7A
	BC_1 615-4	BC_1F_1 43-3	1	4D
	BC_1 615-6	BC_1F_1 121-3	1	7A
	BC_1 615-1	BC_1F_1 120-3	1	7A

5.5.4 Genotyping Malawian wheat/*T. urartu* BC_1 and BC_1F_1 plants with chromosome-specific KASP markers

Wheat/*T. urartu*, DH-254, three *T. urartu* accessions and the three Malawian varieties were genotyped using 144 chromosome-specific KASP markers polymorphic between wheat and *T. urartu.* Markers were selected for linkage group 5 based on their availability and results from the previous work (Grewal et al., 2018a). KASP analysis showed that only nine of the 144 markers failed to score the genotypes. Genotyping of

35 BC_1 plants with the group 5 markers within the region of the 76.40 Mbps segment (WRC605 and WRC608) gave heterozygous calls for the *T. urartu* segment on wheat Chr 5A in 31 lines and a homozygous wheat call on the remaining lines. The marker detecting the $5A^u$ smaller segment (28.7 Mbps) was unable to detect the DH-254 controls, and thus none of the BC_1 plants could be scored for the small segment. Subsequent characterisation of 81 BC_1F_1 plants for the larger $5A^u$ segment detected 14 homozygous lines, 50 heterozygous lines and 21 lines with no segment (Table 5.4). Among the 14 homozygous lines, only 11 grew to maturity, produced seed and could be carried forward to the next generation.

Table 5.4: A list of Malawian wheat/*Am. muticum* BC_1 and BC_1F_1 lines showing number of *T. urartu* segments detected by KASP markers and their location on the wheat genome.

Cross combination	BC_1 code	BC_1F_1 code	No of A^U segments	Location on wheat chromosome
DHF1 254 x Kenya nyati	BC_1 642-5	BC_1F_1 91-3	1	5A
	BC_1 642-2	BC_1F_1 89-2	1	5A
	BC_1 640-4	BC_1F_1 82-1	1	5A
	BC_1 640-5	BC_1F_1 83-1	1	5A
	BC_1 642-5	BC_1F_1 91-1	1	5A
	BC_1 640-2	BC_1F_1 81-1	1	5A
	BC_1 640-2	BC_1F_1 81-2	1	5A
	BC_1 644-4	BC_1F_1 97-2	1	5A
DHF1 254 x Nduna	BC_1 647-5	BC_1F_1 105-1	1	5A
	BC_1 647-1	BC_1F_1 102-2	1	5A
	BC_1 647-5	BC_1F_1 105-3	1	5A
Kenya Nyati x DHF1 254	BC_1 649-4	BC_1F_1 108-1	1	5A
	BC_1 649-1	BC_1F_1 87-2	1	5A
	BC_1 648-2	BC_1F_1 107-4	1	5A

5.5.5 Cytogenetic characterisation

5.5.5.1 Multi-colour GISH of Malawian wheat/*Am. muticum* BC_1 and BC_1F_1 root metaphase spreads

To validate the genotyping results, metaphase spreads from roots of Malawian wheat/*Am. muticum* BC_1 were analysed using multi-colour (mc) GISH. From the 47

BC_1 plants heterozygous for the *Am. muticum* segments, mc-GISH was performed on 40 plants. Results from BC_1 KASP analysis (Figure 5.6) revealed the presence of both the 4T and 7T heterozygous segments on wheat Chr 4D and Chr 7A in 19 introgression lines. GISH analysis of these lines validated the presence of a heterozygous segment on the distal end of the short arm of Chr 4D. Similar to the analysis of DH-348, mc-GISH did not validate the presence of the 7T segment because of the small size. KASP analysis of the BC_1 population also revealed the presence of 4T segment in 15 introgression lines, and GISH validated this.

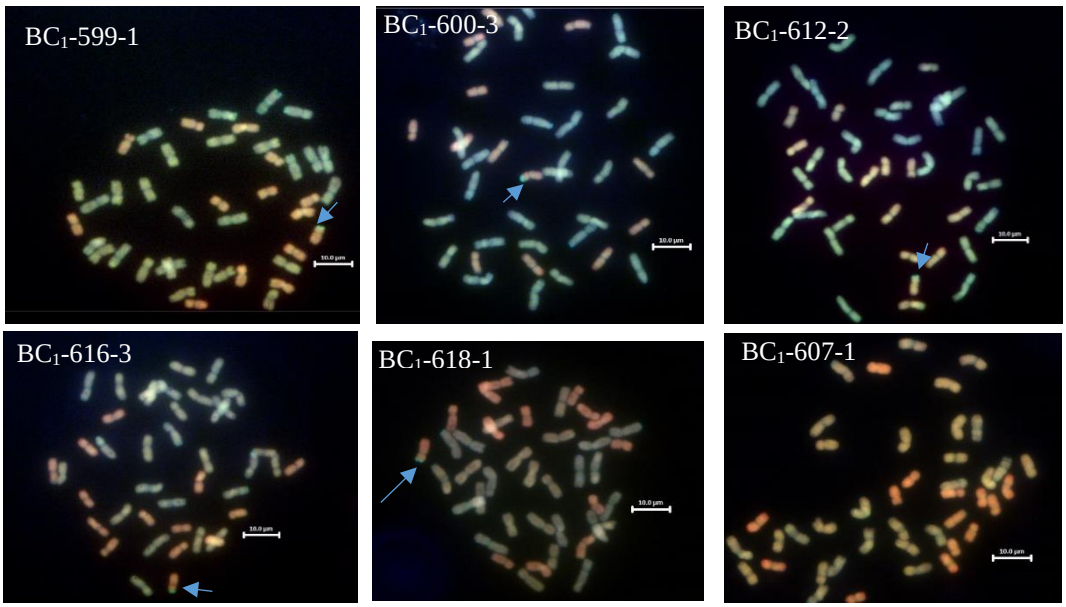


Figure 5.6: GISH images of BC_1 root metaphase spreads showing the A, B, D and T genomes (A genome - green, B genome - blue, D genome – red, T genome - gold). The blue arrows indicate the site of *Am. muticum* (T) introgressions into Chr 4D of wheat. GISH image for line BC_1-607-1 shows a plant where KASP showed a segment on wheat chromosome 7A, and GISH showed no segment present

Mc-GISH was also used to validate 31 of the 41 BC_1F_1 plants homozygous for the *Am. muticum* segments. KASP analysis of the BC_1F_1 plants revealed the presence of both the 4T and 7T homozygous segments on wheat Chr 4D and Chr 7A in introgression lines BC_1F_1 64-2 and BC_1F_1 61-2. Mc-GISH analysis (Figure 5.6) validated the results in BC_1F_1 64-2 as it showed a pair of 4T segments on wheat Chr 4D. BC_1F_1 61-2 could not be validated, because the roots obtained did not give good metaphase spreads. The presence of a pair of 4T segments in the lines observed with only a 4T/4D recombination was also validated in 23 of the 25 lines. GISH did not detect any

segment in seven of the thirteen lines with the 7T/4D recombination. GISH also showed that 25 of the 31 BC_1F_1 lines analysed had maintained the euploid chromosome condition, while five lines had a missing D chromosome, likely inherited from the monosomic deletion observed in the sequence of the parental line DH-348 (Table 5.7). Line BC_1F_1 58-1 showed the entire chromosome set, plus an extra B chromosome.

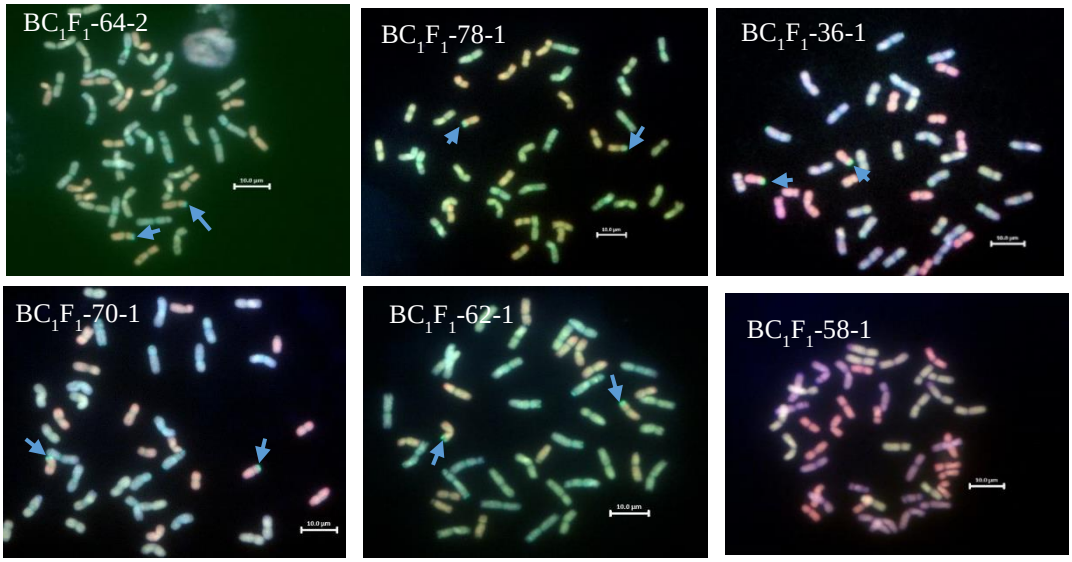


Figure 5.7: GISH images of BC_1F_1 root metaphase spreads showing the A, B, D and T genomes (A genome - green, B genome - blue, D genome – red, T genome - gold). The blue arrows indicate the site of *Am. muticum* (T) introgressions into Chr 4D of wheat. BC_1F_1-64-2 shows only a pair of 4T segments on wheat Chr 4D, GISH did not capture the other set of 7T segments revealed by KASP. BC_1F_1-78-1, BC_1F_1-36-1 and BC_1F_1-70-1 shows euploid sets of chromosomes each with a pair of 4T segments on wheat Chr 4D. BC_1F_1-62-1 shows an anueploid metaphase spread with a pair of 4T segments on Chr 4D. BC_1F_1-58 has a 7A segment undetectable by GISH; however, it shows an aneuploid set of chromosomes (43 with an extra B chromosome)

Table 5.5: A list of Malawian wheat/*Am. muticum* BC_1F_1 (homozygous) lines showing number of *Am. muticum* segments detected by KASP, validated by mc-GISH and total number of chromosomes

Cross combination	BC_1F_1 code	No. of segments KASP	No. of segments GISH	No. of chromosomes	Missing/ extra
DHF1 348 x Nduna	BC_1F_1 64-2	2	1	42	0
	BC_1F_1 62-1	1	1	41	D
	BC_1F_1 67-4	1	0	42	0
	BC_1F_1 67-2	1	1	42	0

DHF1 348 x Kadzibonga	BC_1F_1 78-1	1	1	42	0
	BC_1F_1 72-2	1	1	42	0
	BC_1F_1 73-2	1	1	42	0
	BC_1F_1 70-1	1	1	42	0
	BC_1F_1 71-1	1	1	42	0
Nduna x DHF1 348	BC_1F_1 60-1	1	1	42	0
	BC_1F_1 60-2	1	1	42	0
	BC_1F_1 56-2	1	1	41	D
	BC_1F_1 59-2	1	1	42	0
	BC_1F_1 58-1	1	1	43	B
	BC_1F_1 61-2	1	1	42	0
	BC_1F_1 113-2	1	1	42	0
	BC_1F_1 116-2	1	1	42	0
	BC_1F_1 62-1	1	1	42	0
	BC_1F_1 62-3	1	1	42	0
Kadzibonga x DHF1 348	BC_1F_1 50-1	1	1	42	0
	BC_1F_1 51-1	1	1	42	0
	BC_1F_1 49-1	1	1	42	0
	BC_1F_1 50-2	1	1	42	0
Kenya Nyati x DHF1 348	BC_1F_1 38-1	1	1	42	0
	BC_1F_1 42-2	1	1	41	D
	BC_1F_1 35-1	1	1	42	0
	BC_1F_1 35-2	1	1	41	D
	BC_1F_1 36-1	1	1	42	0
	BC_1F_1 37-1	1	1	41	D
	BC_1F_1 39-1	1	1	42	0
	BC_1F_1 43-2	1	1	42	0

5.5.5.2 Multi-colour GISH of Malawian wheat/T. *urartu* BC1 and BC_1F_1 root metaphase spreads

GISH analysis of selected BC_1 and BC_1F_1 did not validate the presence of the $5A^u$ segment recombined with the 5A chromosome of wheat. However, GISH detected the presence of the 5A-5D translocation initially observed in both the sequence visualisation and GISH image of the parental line (DH-254). GISH analysis also showed the number of chromosomes of each line as shown in Table 5.6.

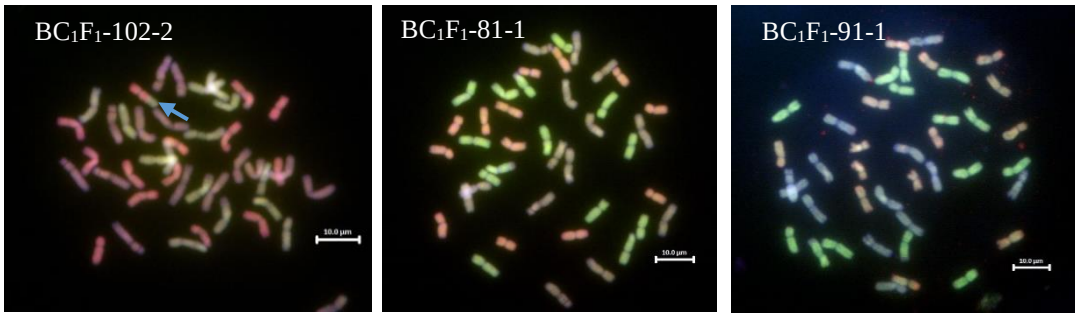


Figure 5.8: GISH images of metaphase spreads from the BC_1F_1 roots of Malawian wheat/*T. urartu* introgression lines showing the A, B and D genomes (A genome - green, B genome - blue, D genome – red. The blue arrow on BC_1F_1-102-2 shows the 5A-5D translocation.

Table 5.6: A list of Malawian wheat/*T. urartu* BC_1F_1 lines showing number of *Am. muticum* segments detected by KASP, and total number of chromosomes by GISH

Cross combinations	BC_1F_1 code	No. of segments KASP	Number of chromosomes
DHF1 254 x Kenya nyati	BC_1F_1 91-3	1	42
	BC_1F_1 82-1	1	13A,14B,13D+1A/D
	BC_1F_1 83-1	1	42
	BC_1F_1 91-1	1	42
	BC_1F_1 81-1	1	41
	BC_1F_1 81-2	1	41
DHF1 254 x Nduna	BC_1F_1 105-1	1	42
	BC_1F_1 102-2	1	14A ,14B, 13D+1A/D

5.6 Discussion

A number of *Am. muticum* and *T. urartu* introgression lines developed for trait analysis (King et al., 2017, King et al., 2019a, Grewal et al., 2018a, Grewal et al., 2021) were shown to have potential for increased grain Zn, Fe and Ca concentration above their recurrent parents (see Chapter 3 and 4). Previously, *T. urartu*, DH-254 and *Am. muticum*, DH-348 showed grain Zn concentrations above 100 mg kg^{-1} under glasshouse conditions (Khokar *et al.*, unpublished). The two lines were therefore selected for this study based on their increased Zn concentration above the other DH lines, and above their recurrent parents. This study focused on developing two segregating populations by hybridising the high grain Zn lines (DH-348 and DH-254)

with low grain Zn Malawian hexaploid wheat cvs *Kenya nyati, Nduna and Kadzibonga* (see Chapter 3). Whole genome sequencing of DH-348 and DH-254 showed that the two lines carry 4T and 7T, and two $5A^u$ introgressions respectively, potentially increasing grain Zn concentration in the hexaploid wheat background. These findings were verified by both KASP and GISH analysis. Sequencing the parental lines was very useful for identifying the small wild relative segments, which were previously undetectable by both SNP and GISH analysis.

From the 49 DH-348/Malawian wheat crosses, 263 F_1 seeds were obtained. Crosses between *Nduna*, *Kenya nyati* and DH-348 produced more F_1 seeds compared to DH-348/*Kadzibonga* crosses mainly because *Kadzibonga* had very few plants due to germination problems. Similarly, DH-254 was successfully crossed to *Kenya nyati* and *Nduna*, but not *Kadzibonga*. In comparison to DH-348, the heading date of DH-254 was considerably later (although the flowering period was longer), resulting in a failure to coincide heading and flowering period of most plants with two of the Malawian wheat varieties *Nduna and Kadzibonga*. This subsequently affected the number of crosses and the number of F_1 seeds obtained (only 20 crosses were done, and 120 seeds obtained). F_1 seeds from each combination in both the DH-348 and DH-254 crosses were used to generate a BC_1 population by crossing to their recurrent parents. Among the Malawian wheat/DH-348 cross combinations, crosses from the F_1 seeds of DH-348 × *Nduna* produced the most BC_1 seeds, while among the Malawian wheat/DH-254 combinations crosses from the F_1 seeds of DH-254 × *Kenya nyati* produced the most BC_1 seeds. This result is also attributed to the heading and flowering duration of the DH lines and the Malawian wheats, in that *Nduna's* flowering dates coincided well with DH-348 while *Kenya nyati's* flowering dates coincided well with DH-254. Crossing the parental lines in both directions (as male and female) did not have an effect on the success of the crosses, number of F_1 seeds obtained and the transfer of the introgressions into the F_1 progenies, as observed by the BC_1 genotyping results. Although several backcrosses (at least 4 or 5) are required to recover a good percentage of the genome of the recurrent parents, only one backcross was done in this study due to limited time. The introgression lines were generated in this study for the purpose of phenotyping for grain Zn and other mineral elements under field conditions in Malawi. Thus, it was necessary to self-fertilise them at an early stage, in order to bulk enough seeds for the field experiment.

KASP genotyping analysis of the wheat/*Am. muticum* and wheat/*T. urartu* BC_1 plants showed that 58% and 89% of the lines carried heterozygous *Am. muticum* and *T. urartu* introgressions, respectively. Following self-fertilisation of the selected BC_1 plants, KASP analysis of the BC_1F_1 plants showed that 42% of the wheat/*Am. muticum* lines were homozygous for the *Am. muticum* segments, while only 17% of the wheat/*T. urartu* lines were homozygous for the larger $5A^u$ segment. Using the DH technique to generate homozygous wheat/*Am. muticum* lines and wheat/*T. urartu* lines, 56% of the BC_3 lines with the *Am. muticum* segment and 41% of the BC_3 lines with *T. urartu* segments were able to produce doubled haploids (King et al., 2019b, Grewal et al., 2021). Although both the self-fertilisation and DH techniques generally show a low rate of homozygous lines generated, the percentage is a higher for both species using the DH technique.

Genomic *in-situ* hybridisation of the wheat/*Am. muticum* BC_1 and BC_1F_1 did not completely validate the genotyping results, as only the larger *Am. muticum* segment (4T) was detected, while the smaller segment (size) was not. Similar results were shown in the parental line, DH-348. Currently, the smallest segment that can be detected with GISH is about 18 Mbp (Grewal et al., 2021). Although GISH analysis was carried out for both the BC_1 and BC_1F_1 wheat/*T. urartu* plants, it was not possible to validate the presence of the $5A^u$ segments translocated to the 5A chromosome of wheat. Generally, the probe used for detecting the A genome of wheat is prepared from the wheat progenitor, *T. urartu* and thus in wheat/*T. urartu* introgression lines, the probe detects both the A and A^u genomes (Grewal et al., 2021). Therefore, the GISH images from root metaphase spreads show both genomes in the same colour (in this study, green). GISH is only able to detect A genome segments translocated to either the B or D genome. However, depending on the crossing system used, there can be a high probability of inter-genomic recombination within wheat, as well as recombination between the wheat and *T. urartu* chromosomes, making it impossible to determine which A genome (A or A^u) was involved in the recombination event (Grewal et al., 2021). For example, the original DH-254 introgression line was initially generated via a cross between *T. urartu* and Chinese Spring with the *Ph1* gene deleted. The *Ph1* gene in wheat has been shown to prevent homoeologous pairing of chromosomes at meiosis and hence its removal could have resulted in inter-genomic translocations. In this study, wheat/*T. urartu* GISH was useful for detecting the 5A-

5D inter-genomic recombination initially detected in the *T. urartu* DH-254 sequence. GISH was also useful for checking abnormalities/changes in the chromosome numbers. It should be noted that, none of the methods used to characterise the introgression lines were able to give the 'whole picture' by themselves. However, by combining the KASPs, GISH and sequencing, it was possible to understand the genomic composition of the introgression lines generated

This study has shown that *Am. muticum* and *T. urartu* introgressions from doubled haploid lines can be successfully transferred to different hexaploid wheats. The study also shows that self-fertilisation of the BC_1 population can generate a substantial number of homozygous lines, particularly in the wheat/*Am. muticum* lines. Both chromosome-specific KASP markers and GISH have been useful in detecting and validating the introgressions and the exact location on the wheat chromosome where the wild segment has been translocated and the number of chromosomes in each introgression line.

CHAPTER 6

6 Effects of the 4T and 7T introgressions from *Amblyopyrum muticum* and the 5A^{u} introgression from *Triticum urartu* on grain and straw zinc, iron, selenium and calcium concentrations of three Malawian wheat varieties

6.1 Abstract

Mineral nutrient deficiencies (MNDs) particularly zinc (Zn), iron (Fe), selenium (Se) and calcium (Ca) remain widespread in low-income countries of sub-Saharan Africa (SSA) due to low dietary intake. Wheat is an important source of energy globally, although cultivated wheat is inherently low in mineral micronutrients. Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* BC_1F_3 lines, developed by crossing three Malawian wheat varieties (*Kenya nyati*, *Nduna* and *Kadzibonga*) with DH-348 (wheat/*Am. muticum*) and DH-254 (Wheat/*T. urartu*), were phenotyped for grain and straw Zn, Fe, Ca, Se, and associated agronomic traits in Zn-deficient soils, under field conditions, in Malawi. 98% (47) of the BC_1F_3 introgression lines showed higher Zn above the checks Paragon, Chinese Spring, *Kadzibonga*, *Kenya Nyati* and *Nduna*. 23% (11) of the introgression lines showed high yields and an increase in grain Zn by 16-30 mg kg $^{-1}$ above *Nduna* and *Kadzibonga*, and 11-25 mg kg $^{-1}$ above *Kenya nyati*, Paragon and Chinese Spring. Among the 23%, 64% (7) also showed 8-12 mg kg $^{-1}$ improvement in grain Fe than *Nduna* and *Kenya nyati*. Four lines showed 6-10 µg kg $^{-1}$ Se concentration above Paragon and the three Malawian checks, although the introgressions were not associated with an increase in grain Ca. Grain Zn concentration showed a significant positive correlation with grain Fe, whilst grain Zn and Fe negatively and significantly correlated with TKW and grain yield. Grain Se and grain Ca showed moderate positive correlations with both grain Zn and Fe. Grain Se also negatively and insignificantly correlated with grain yield and TKW. There was also a low and significant association between straw and grain Se concentration, and a low but insignificant association between grain and straw Zn, Fe and Ca concentration. This work will contribute to the efforts of increasing mineral nutrient density in wheat, specifically targeting countries in the SSA.

6.2 Introduction

Mineral nutrient deficiencies (MNDs) remains a global challenge, affecting an approximated 2 billion people (World Health, 2009, White and Broadley, 2009). Zinc (Zn) and iron (Fe) deficiencies are widespread in low-income countries, particularly Sub-Saharan Africa (SSA) and South-east Asia (Gupta et al., 2020). Inadequate intake and bioavailability of these elements in diets remain the major reasons for increased deficiencies risks (Caulfield and Black, 2002, Maret and Sandstead, 2006, Prasad et al., 2014). A high dependence on cereal diets and inability to afford foods that are rich in essential micronutrients for a majority of people in SSA has resulted in risk deficiencies of up to 96%, with a number of countries falling above 50% (Kumssa et al., 2015). In Malawi for instance, zinc deficiency risk is at ~60% with most households having deficiency risks in the range of 50-75% (Likoswe et al., 2020, Joy et al., 2014, NSO et al., 2016). It is estimated that malnutrition results in an annual economic burden of 10.3% of Malawi's gross domestic product (UNICEF, 2019). In Africa, the estimated risk of calcium (Ca) deficiency also remain prevalent, particularly in the southern (99%), eastern (69%) and northern (62%) regions, whilst the mean estimated risk of Se deficiency is 28%, and is highly prevalent in the eastern region (Joy et al., 2014). Food based approaches, particularly food fortification, agronomic biofortification and genetic biofortification of staple crops, were identified as strategies to combat Zn deficiencies globally (Gibson and Ferguson, 1998, Graham et al., 1999, Bouis, 2003, Velu et al., 2014). However, food fortification and agronomic biofortification programs are more feasible in developed countries than in the least-developed, low-income countries, owing to low accessibility and cost of industrially processed food and micronutrient rich fertilisers (Horton, 2006, Gomez-Galera et al., 2010, Cakmak, 2008). In contrast, genetic biofortification, which aims at enhancing grain micronutrient concentration and substances that promote nutrient bioavailability through plant breeding (Velu et al., 2014, Bouis and Saltzman, 2017), is a viable and cost-effective approach for delivering essential micronutrients to low-income countries, particularly in SSA.

Wheat is an important staple crop providing more than 20-25% daily calorie intake in Africa (FAO, 2019). In recent years, demand for wheat and wheat products in SSA has substantially increased and is projected to increase further in the immediate future (Shiferaw et al., 2011a, Mason et al., 2015). Previous work has shown that genetic

variation for grain Zn and Fe in the majority of cultivated wheat is not enough to meet the estimated average requirement (EAR) for both children and adults of reproductive and non-reproductive age. In contrast, wheat progenitor species and other wild relatives in the wheat secondary and tertiary gene pools have revealed a substantial genetic variation for grain Zn, Fe and other essential minerals (Neelam et al., 2011, Rawat et al., 2011a, Tiwari et al., 2015, Rawat et al., 2009, Chhuneja et al., 2006). Thus, the transfer of genetic variation from wheat wild relatives to cultivated wheat through introgression of chromosome segments from wheat wild relatives offers an alternative approach for improving nutritional quality of wheat to the target levels required for improving human nutrition. Breeding micronutrient dense crops also helps to increase crop yield and improve disease tolerance and resistance (Welch, 2002, Bouis, 2003, Genc et al., 2005, Velu et al., 2019, Thapa et al., 2022). The breeding target for grain Zn and Fe in wheat was set at an additional 12 and 22 mg/kg respectively from the baselines (Bouis et al., 2011, Bouis and Saltzman, 2017). These targets were set to meet 60-80% of EAR for preschool children (4–6 years old) and for non-pregnant and non-lactating women of reproductive age (Bouis and Saltzman, 2017).

Pre-breeding efforts have resulted in the successful transfer of a number of progenitor and wild relative chromosomes from the genus, *Triticum*, *Aegilops*, *Amblyopyrum* and *Thinopyrum* (Grewal et al., 2021, King et al., 2018, King et al., 2019a, Grewal et al., 2018b). Mineral analysis of some of the pre-breeding materials have shown substantial variation in grain Zn, Fe and Ca (see chapters 3 and 4), and these are useful for transferring the introgressions into other adapted wheat backgrounds. Previously, rye translocations in a Pavon 76 wheat background significantly increased grain zinc concentration above the recurrent parent (Velu et al., 2019). In CIMMYT, the use of *Triticum aestivum* ssp. spelta- and *Triticum turgidum* ssp. dicoccum-based synthetics have resulted in the release of varieties with 20-40% higher Zn levels compared to local varieties (Singh et al., 2017, Velu et al., 2019, Guzman et al., 2019). Similarly, HarvestPlus Yield Trials (HPYT) of CIMMYT biofortified wheat varieties released in Nepal showed a combination of high yields and high grain Zn and Fe concentration above the local checks (Thapa et al., 2022).

The specific aims of this study were:

(i) To phenotype *T. urartu/Am. muticum*-Malawian introgression lines for grain and straw Zn, Fe, Ca, Se and associated agronomic traits under field conditions in Malawi.

(ii) To understand the relationship between grain and straw Zn, Fe, Se and Ca and the relationship between agronomic traits and grain and straw mineral concentration.

6.3 Materials and methods

6.3.1 Germplasm

The introgression lines used in this study (Table 6.1 and 6.2) were developed by crossing DH-348 and DH-254 with the three Malawian varieties (*Kadzibonga*, *Nduna* and *Kenya nyati*). Chapter 5 describes the crossing program and the number of seeds obtained at each round of crossing and self-fertilisation.

Table 6.1: Description of the Malawian wheat-*Am. muticum* introgression lines

Cross combinations	BC_1F_1 code	BC_1F_3 code	Segment
DHF1 348 x Kadzibonga	BC_1F_1-78-1	BC_1F_3-1	4D
	BC_1F_1-73-2	BC_1F_3-2	4D
	BC_1F_1-72-2	BC_1F_3-3	4D
	BC_1F_1-71-1	BC_1F_3-5	7A
	BC_1F_1-71-3	BC_1F_3-31	7A
	BC_1F_1-70-1	BC_1F_3-6	4D
	BC_1F_1-123-3	BC_1F_3-32	7A
DHF1 348 x Nduna	BC_1F_1-67-2	BC_1F_3-7	4D
	BC_1F_1-63-2	BC_1F_3-11	4D
	BC_1F_1-64-2	BC_1F_3-30	4D, 7A
	BC_1F_1-67-4	BC_1F_3-13	7A
Nduna x DHF1 348	BC_1F_1-62-3	BC_1F_3-15	7A
	BC_1F_1-61-2	BC_1F_3-16	7A
	BC_1F_1-60-2	BC_1F_3-17	4D
	BC_1F_1-60-1	BC_1F_3-18	4D
	BC_1F_1-59-2	BC_1F_3-19	4D
	BC_1F_1-56-1	BC_1F_3-20	4D
	BC_1F_1-56-2	BC_1F_3-21	4D
	BC_1F_1-113-2	BC_1F_3-9	4D
	BC_1F_1-116-2	BC_1F_3-57	7A

	BC_1F_1-120-1	BC_1F_1-60	7A
	BC_1F_1-62-1	BC_1F_3-10	4D
	BC_1F_1-54-1	BC_1F_3-23	7A
Kadzibonga x DHF1 348	BC_1F_1-53-1	BC_1F_3-26	7A
	BC_1F_1-49-1	BC_1F_3-27	4D
	BC_1F_1-50-1	BC_1F_3-28	4D
	BC_1F_1-50-2	BC_1F_3-29	4D
Kenya Nyati x DHF1 348	BC_1F_1-42-2	BC_1F_3-33	4D
	BC_1F_1-43-2	BC_1F_3-34	7A
	BC_1F_1-43-3	BC_1F_3-35	4D
	BC_1F_1-121-3	BC_1F_3-36	7A
	BC_1F_1-35-1	BC_1F_3-37	4D
	BC_1F_1-35-2	BC_1F_3-38	4D
	BC_1F_1-36-1	BC_1F_3-39	4D
	BC_1F_1-37-1	BC_1F_3-40	4D
	BC_1F_1-38-1	BC_1F_3-41	4D
	BC_1F_1-39-1	BC_1F_3-42	7A

The Nottingham *BBSRC* Wheat Research Centre (WRC), at the University of Nottingham, UK developed DH-348 and DH-254, and these have been described in the previous chapters. Pavon 76, Chinese spring and Paragon were also obtained from WRC, and the three Malawian checks *Kenya Nyati*, *Kadzibonga* and *Nduna* were obtained from Lilongwe University of Agriculture and Natural Resources (LUANAR) Malawi.

Table 6.2: Description of the Malawian wheat-*T. urartu* introgression lines

Cross combination	**BC_1F_1 code**	**BC_1F_3 code**	**Segment**
DHF1 254 x Kenya nyati	BC_1F_1-81-1	BC_1F_3-44	5A
	BC_1F_1-82-1	BC_1F_3-45	5A
	BC_1F_1-83-2	BC_1F_3-46	5A
	BC_1F_1-89-2	BC_1F_3-47	5A
	BC_1F_1-91-1	BC_1F_3-48	5A
	BC_1F_1-97-2	BC_1F_3-49	5A
DHF1 254 x Nduna	BC_1F_1-102	BC_1F_3-50	5A
	BC_1F_1-105	BC_1F_3-51	5A
	BC_1F_1-105	BC_1F_3-52	5A
	BC_1F_1-101	BC_1F_3-53	5A
Kenya Nyati x DHF1 254	BC_1F_1-87-2	BC_1F_3-54	5A

6.3.2 Soil sampling, preparation and analysis

A composite soil sample was collected on each block at the trial site (see below paragraph for a description of the trial site and layout). Samples were collected, prepared and analysed using the methodologies described in Chapter 3. Briefly, Soil pH was determined following suspension of 5g of soil sample into 12.5mls Milli-Q water (18.2 MΩ cm; 1:2.5 m/v). Total nitrogen (N) was measured using the Kjeldahl method following digestion of 0.2g of soil samples in a solution of hydrogen peroxide, lithium sulphate and sulphuric acid (Kjeldahl, 1883). Multi-element analysis using inductively coupled mass spectrometry (ICP-MS; Thermo-Fisher Scientific^{TM} iCAP Q) was used to measure Ca and Se following aqua-regia nitric acid (HNO3) digestion (Crosland et al., 1995). Organic matter was determined using the Walkley and Black method (Walkley and Black, 1934). Extractable soil Zn and Fe were determined by the diethylene triamine penta-acetic acid (DTPA) extraction method (Lindsay and Norvell, 1978) followed by multi-element analysis with ICP-MS. Available phosphorus (P) was measured using the Mehlich- 3 extraction (Mehlich, 1984) and concentration was read using a UV spectrophometer at a wavelength of 680. Phosphorus (K) was also measured using the Mehlich 3 extraction method (Mehlich, 1984), with an atomic absorption spectrometer (AAS) used to determine the concentration.

6.3.3 Experimental design and trial management

The experiment was conducted in the winter of 2022 (May to October). Wheat lines were grown under field conditions (Figure 6.1) in an optimally irrigated environment at Lilongwe University of Agriculture and Natural Resources (14.18'S 33.76' E) in Lilongwe, Malawi. Forty eight BC_1F_3 introgression lines (11 Malawian wheat/*T. urartu* and 37 Malawian wheat/*Am. muticum*) were planted alongside three Malawian wheat varieties (*Kadzibonga*, *Kenya nyati* and *Nduna*), two DH lines (DH-348 and DH-254) and three UK checks (Paragon, Pavon 76 and Chinese spring) in a randomised complete block design (RCBD) with three replicates (Figure 1). Plots were $2m^2$ each, with six rows spaced at 0.15 m. Plot spacing was 0.30 m and block spacing was 1.0 m. Basal dressing fertiliser 23 (10:5 +6S +1Zn (SuperFert Fertilisers, Harare, Zimbabwe) was applied 14 days after planting at a rate of 200kg N/ha. Urea (46% N) was applied as top dressing at a rate of 100kg N/ha 3 weeks later, and basal and top dressing were applied according to the Malawi guide to agriculture production

(GAP, 2020) guidelines. First weeding was done 4 weeks after planting and subsequent weeding as soon as weeds appeared. Insect pests were controlled by applying Profex Super (Profencfos 40% + Cypermenthrin 4% EC –Kewalram Chanrai group). Irrigation was done from sowing to maturity at which point it was withdrawn to allow plants to dry for harvesting.

Figure 6.1: Field layout of 37 Malawian wheat/*Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in 2022 winter season

6.3.4 Data collection

Data was collected from the four middle rows leaving the two outer rows as boarders. The following data was collected; Days to heading (DH), days to flowering (DF), days to maturity (DM), plant height, thousand kernel weight (TKW) and grain yield. Grain yield was converted from g/m^2 to kg/ha. Plant height and number of tillers were collected from five randomly selected plants in the net plot, to get an average of both.

6.3.5 Sample preparation

Grain and straw samples were prepared in November 2022. Sample preparation and digestion was done as described in Chapter 3. Briefly, grain samples were digested in 2022 using a hot block acid digestion system (Anton Paar Gmbh, Graz, Austria). Approximately 0.4 g of each of the grain samples along with certified reference material (wheat flour 1567b-CRM) and laboratory reference material (Paragon wheat-LRM) were digested using a Multicube 48 digestion block (Anton Paar Gmbh, Graz, Austria). Two operational blanks were added in each run. The digestion block was set as described in Chapter 3.

Straw samples were digested in 2022 using a microwave digestion platform described in Chapter 3. 0.2 g of each finely ground sample was weighed in pressure-activated venting vessels (56-ml 'SMART VENT', Anton Paar) along with three reference materials (CRM-Tom-1573a, BCR-Hay 129 and LRM-Cabbage) and two operational

blanks in each run. The samples were digested in a Multiwave PRO microwave with a 41-vessel digestion rotor (41HVT56).

6.3.6 Multi-elemental analysis

Grain and straw multi-element analysis was undertaken using inductively coupled plasma mass spectrometry as described in Chapter 3. Briefly, 33 elements, Zn, Fe, Ca, Ag, Al, As, B, Ba, Be, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Sr, Ti, Tl, U, V and Zn, were analysed. A total of 189 grain and 192 straw samples (three replicates for each sample, as described in the field layout) including blanks, CRMs and LRMs were analysed. The Zn, Fe, Se and Ca specific recovery from CRMs from grain samples was 99, 97, 102 and 101% respectively. The Limit of detection (LOD) values for grain Zn, Fe, Ca and Se were 0.7, 2.2, 19.0 and 0.002 respectively. The Zn, Fe, Se and Ca specific recovery from CRMs from straw samples was 106, 108, 118 and 149% respectively. The LOD values for grain Zn, Fe, Ca and Se were 0.6, 1.2, 7.1 and 0.0024 respectively

6.4 Statistical analysis

One-way analysis of variance (ANOVA) was performed using Genstat for windows statistical package, version 21 (VSN, 2022). Correlation and regression analysis were performed in XLSTAT 2022.3.1 (Addinsoft, 2022). The statistical linear model considered the response Y_{ij} of the jth treatment in the ith replication expressed as:

$$Y_{ij}=\mu+\beta_{i}+\tau_{j}+e_{ij}$$

where μ is the grand mean of all genotypes, β_i is the block effect, τ_j is the effect of the jth treatment (genotype) and e_{ij} is the average experimental error. Fishers protected least significant difference (LSD, $P < 0.05$) was used to separate means. Correlation analysis was performed using Pearson correlation coefficient

6.5 Results

6.5.1 Soil analysis

Table 6.3 describes the soil physio-chemical properties of the soils at the experimental site. The soils were classified as clay loam, with an average soil pH of 6.7. DTPA-Zn and Fe were 0.3 and 7.7 mg kg^{-1} respectively, while soil Ca and Se were 3585 and 0.2 mg kg^{-1} respectively. Soil analysis also showed that the soil samples had an average of 0.2% total nitrogen, 20.6 mg kg^{-1} available P and 67.4 mg kg^{-1} K.

Table 6.3: Physio-chemical properties of soil samples collected from the three replicates of the experimental site

Parameter	Block 1	Block 2	Block 3	Average
Soil pH	6.6	6.7	6.7	6.7
Organic matter (%)	2.0	2.9	2.3	2.4
DTPA-Zn (mg/kg)	0.2	0.4	0.3	0.3
DTPA-Fe (mg/kg)	7.2	8.3	7.7	7.7
Se (mg/kg)	0.2	0.2	0.3	0.2
Ca (mg/kg)	3572	3590	3594	3585
Total N (%)	0.3	0.2	0.2	0.2
Available P (mg/kg)	20.3	20.6	21	20.6
K (mg/kg)	62.1	56.9	83.2	67.4
Silt (%)	16	14	16	15.3
Clay (%)	44	44	44	44.0
Sand (%)	42	40	40	40.7
Textural class	Clay loam	Clay loam	Clay loam	Clay loam

6.5.2 Grain zinc

Analysis of grain samples showed a significant variation in grain Zn concentration ($P < 0.0001$) among the 55 genotypes (Table 6.4). Grain Zn concentration varied from 35.5-108.6 mg kg^{-1} with an overall mean of 57.9 mg kg^{-1}. DH-348 had the highest grain Zn concentration of all the genotypes analysed with 108.6 mg kg^{-1}, while BC_1F_3-30 had the highest grain Zn concentration of the BC_1F_3 introgression lines with 84.9 mg kg^{-1}. Overall, 13% of the BC_1F_3 lines had Zn concentrations between 70-85 mg kg^{-1}, 25% between 60-68 mg kg^{-1} and 43% between 50-59 mg kg^{-1}. The three Malawian checks, *Kenya nyati*, *Kadzibonga* and *Nduna* had grain zinc concentrations of 42.0, 35.8 and 35.3 mg kg^{-1} respectively, and these were the lowest among all the genotypes, with the exception of BC_1F_3-44, which had a concentration of 38.6 mg kg^{-1}. Mineral analysis also showed a significant variation among the UK checks, with Pavon 76

having the highest concentration. Mineral analysis for DH-254 was not conducted because the plants did not produce any seed.

Table 6.4: Variation in grain zinc, iron, calcium and selenium of 37 Malawian wheat/*Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in the 2022 winter season. The introgression lines have been ordered according to grain Zn (highest to lowest)

Genotype	Grain Zn (mg kg^{-1})	Grain Fe (mg kg^{-1})	Grain Ca (mg kg^{-1})	Grain Se (µg kg^{-1})
DH 348	108.6 ^{a}	95.8 ^{a}	785 ^{efghijklmno}	18.0 ^{ab}
BC_1F_3-30	84.9 ^{b}	87.3 ^{a}	1001 ^{a}	12.5 ^{cdefg}
BC_1F_3-10	76.9 ^{bc}	55.0 ^{fghijklmnopq}	791 ^{efghijklmno}	11.7 ^{cdefg}
BC_1F_3-39	76.6 ^{bcd}	59.2 ^{defghi}	728 ^{klmnopu}	10.4 ^{cdefg}
BC_1F_3-13	75.9 ^{bcde}	76.7 ^{b}	621 ^{stuvwxy}	11.8 ^{cdefg}
BC_1F_3-11	73.2 ^{bcdef}	70.7 ^{bc}	827 ^{bcdefghijklm}	14.5 ^{abcde}
BC_1F_3-28	73.1 ^{bcdef}	66.0 ^{cde}	851 ^{bcdefghij}	15.1 ^{abcd}
BC_1F_3-27	68.2 ^{cdefg}	47.3 ^{klmnopqrstuv}	650 ^{qrstuvy}	8.9 ^{fgh}
BC_1F_3-34	67.1 ^{cdefgh}	48.1 ^{jklmnopqrstuv}	776 ^{fghijklmnop}	11.9 ^{cdefgh}
BC_1F_3-36	66.7 ^{cdefghi}	52.0 ^{ghijklmnopqr}	763 ^{ghijklmnopq}	11.2 ^{cdefgh}
BC_1F_3-38	65.7 ^{cdefghij}	49.9 ^{hijklmnopqrst}	831 ^{bcdefghijkl}	10.9 ^{cdefgh}
BC_1F_3-29	64.2 ^{cdefghijk}	64.0 ^{cdef}	924 ^{abcd}	13.2 ^{bcdefgh}
Pavon-76	63.9 ^{cdefghijk}	61.4 ^{cdefg}	716 ^{lmnopqrstu}	11.2 ^{cdefgh}
BC_1F_3-20	63.8 ^{cdefghik}	60.3 ^{defg}	829 ^{bcdefghijkl}	12.3 ^{cdefgh}
BC_1F_3-32	63.8 ^{cdefghijk}	48.9 ^{ghijklmnopqrs}	569 ^{xy}	11.1 ^{cdefgh}
BC_1F_3-42	63.3 ^{cdefghijkl}	48.9 ^{ghijklmnopqrs}	789 ^{efghijklmno}	13.1 ^{bcdefgh}
BC_1F_3-47	61.0 ^{cdefghijklm}	52.6 ^{ghijklmnopqr}	942 ^{ab}	10.9 ^{cdefgh}
BC_1F_3-40	60.8 ^{cdefghijklmn}	47.7 ^{jklmnopqrstuv}	852 ^{bcdefghij}	9.2 ^{defgh}
BC_1F_3-31	60.6 ^{cdefghijklmn}	51.5 ^{ghijklmnopqrs}	593 ^{vwxy}	12.1 ^{cdefgh}
BC_1F_3-37	60.4 ^{cdefghijklmn}	51.5 ^{ghijklmnopqrs}	864 ^{bcdefgh}	9.7 ^{defgh}
BC_1F_3-15	60.3 ^{defghijklmn}	55.8 ^{efghijklmnop}	929 ^{abc}	13.1 ^{bcdefgh}
BC_1F_3-35	59.9 ^{efghijklmn}	52.1 ^{ghijklmnopqr}	900 ^{abcde}	9.3 ^{defgh}
BC_1F_3-18	59.9 ^{efghijklmn}	51.2 ^{ghijklmnopqr}	857 ^{bcdefghi}	10.2 ^{cdefgh}
BC_1F_3-16	59.7 ^{efghijklmn}	53.4 ^{ghijklmnopqr}	744 ^{hijklmnopqr}	11.1 ^{cdefgh}
BC_1F_3-46	58.9 ^{efghijklmn}	45.1 ^{qrstuv}	704 ^{nopqrstuvw}	11.2 ^{cdefgh}
BC_1F_3-53	58.2 ^{fghijklmnop}	53.5 ^{ghijklmnopq}	872 ^{bcdefg}	15.5 ^{abc}
BC_1F_3-57	57.7 ^{fghijklmnop}	56.1 ^{defghijklm}	852 ^{bcdefghij}	11.1 ^{cdefgh}
BC_1F_3-41	57.2 ^{fghijklmnop}	61.2 ^{cdefg}	858 ^{bcdefghi}	9.9 ^{defgh}
BC_1F_3-45	57.0 ^{fghijklmnop}	56.6 ^{defghijkl}	704 ^{nopqrstuvw}	12.1 ^{cdefgh}
BC_1F_3-49	56.8 ^{fghijklmnop}	45.6 ^{qrstuv}	838 ^{bcdefghijkl}	10.1 ^{cdefgh}
BC_1F_3-19	55.8 ^{fghijklmnop}	54.8 ^{fghijklmnopq}	924 ^{abcd}	11.1 ^{cdefgh}
BC_1F_3-9	55.7 ^{fghijklmnop}	51.4 ^{ghijklmnopqrs}	802 ^{defghijkl}	12.6 ^{cdefgh}
BC_1F_3-2	55.4 ^{fghijklmnop}	59.3 ^{defgh}	681 ^{opqrstuvwx}	18.9 ^{a}
BC_1F_3-33	53.5 ^{ghijklmnopqr}	56.3 ^{defghijklm}	841 ^{bcdefghijk}	11.1 ^{cdefgh}
BC_1F_3-6	52.5 ^{ghijklmnopqr}	38.8 ^{uv}	582 ^{wxy}	13.5 ^{bcdefgh}
BC_1F_3-3	52.4 ^{ghijklmnopqr}	52.7 ^{ghijklmnopqr}	553 ^{y}	15.3 ^{abc}

BC_1F_3-60	52.0 ghijklmnopqr	56.0 defghijklmn	865 bcdefgh	12.6 cdefgh
BC_1F_3-1	51.9 ghijklmnopqr	57.2 defghijk	695 nopqrstuv	14.0 abcde
BC_1F_3-26	51.9 ghijklmnopqr	66.2 cd	801 efghijklmn	11.8 cdefgh
BC_1F_3-48	50.3 ijklmnopqr	45.7 opqrstuv	1010 a	10.4 cdefgh
BC_1F_3-52	50.0 ijklmnopqr	45.5 pqrstuv	724 klmnopqrstu	8.0 h
BC_1F_3-7	49.5 jklmnopqr	54.7 fjhiklmnopq	656 pqrstuvwx	13.1 bcdefgh
BC_1F_3-51	49.2 jklmnopqr	38.3 v	635 rstuvwxy	9.9 defgh
BC_1F_3-23	48.4 klmnopqr	46.2 mnopqrtuv	863 bcdefgh	10.8 cdefgh
Chinese spring	48.2 klmnopqr	60.4 cdefg	611 tuvwxy	10.6 cdefgh
BC_1F_3-21	47.4 lmnopqr	53.4 ghijklmnopqr	896 abcdef	8.8 fgh
BC_1F_3-50	45.0 mnopqr	49.0 pqrstuv	701 nopqrstuvw	10.2 cdefgh
BC_1F_3-43	44.9 mnopqr	41.6 stuv	778 efghijklmnop	8.8 fgh
BC_1F_3-5	44.4 nopqr	51.7 ghijklmnopqrs	727 klmnopqrstu	11.4 cdefhg
BC_1F_3-17	43.2 opqr	40.1 tuv	732 jklmnopqrst	9.5 efgh
BC_1F_3-54	43.19 opqr	51.7 ghijklmnopqrs	807 cdefghijklmn	8.1 h
Paragon	43.0 pqr	46.6 lmnopqrstuv	629 rstuvwxy	8.2 gh
Kenya Nyati	42.0 pqr	43.1 rstuv	705 nopqrstuvw	8.6 gh
BC_1F_3-44	38.6 qr	45.9 qrstuv	738 ijklmnopqrs	9.3 efgh
Kadzibonga	35.8 r	53.2 ghijklmnopprs	624 rstuvwxy	7.8 h
Nduna	35.3 r	41.3 stuv	608 uvwxy	8.2 h
Grand mean	57.9	54.4	772	11.4
F. probability	<0.0001	<0.0001	<0.0001	0.055
LSD (5%)	16.4	9.8	122	5.4
CV%	17.4	11.1	9.6	28.9

Degrees of freedom (df) for replicates = 2, df for genotypes = 54

For each variable, means with different superscript letters are significantly different at P<0.05, following ANOVA and Fishers protected LSD tests

6.5.3 Grain iron

Significant variation (P <0.0001) was observed in the grain Fe concentrations of the 55 genotypes (Table 6.4). The Fe concentration varied from 38.3-95.8 mg kg $^{-1}$ with an overall mean of 54.2 mg kg^{-1}. DH-348 showed the highest grain Fe concentrations (95.8 mg kg $^{-1}$) followed by BC_1F_3-30 (87.4 mg kg $^{-1}$), BC_1F_3-13 (76.6 mg kg^{-1}) and BC_1F_3-11 (70.7 mg kg^{-1}) respectively. Overall, 6% of the BC_1F_3 introgression lines had Fe concentrations between 70-87 mg kg $^{-1}$, 10 % between 60-66 mg kg^{-1}, 54% between 50-59 mg kg $^{-1}$, and the remaining had Fe concentrations above 40 mg kg^{-1}, with the exceptions of BC_1F_3-6 and BC_1F_3-51, which had 38.8 and 38.3 mg kg^{-1}. The Fe concentrations of the Malawian checks, *Kadzibonga*, *Kenya nyati* and *Nduna* were 53.2, 42.0 and 35.3 mg kg^{-1} while Pavon 76, Chinese spring and Paragon had Fe concentrations of 61.4, 60.4 and 46.6 mg kg^{-1} respectively.

6.5.4 Grain selenium

There was significant variation in the grain Se concentration (P = 0.043) among the 55 genotypes, varying from 7.8- 18.9 µg kg $^{-1}$, with an overall mean of 11.4 µg kg $^{-1}$ (Table 6.4). BC_1F_3-2 had the highest Se concentration (18.9 µg kg $^{-1}$) followed by DH-348 which had a concentration of 18.3 µg kg $^{-1}$ and three further BC_1F_3 introgression lines, BC_1F_3-3, BC_1F_3-11 and BC_1F_3-28 had Se concentrations above 15 µg kg $^{-1}$. All the other introgression lines had Se concentrations between 10-11 µg kg $^{-1}$. The Se concentrations of the Malawian checks *Kenya nyati*, *Nduna* and *Kadzibonga* was 8.6, 8.1 and 7.8 µg kg $^{-1}$ respectively while Pavon 76, Chinese Spring and Paragon had Se concentrations of 11.2, 10.6 and 8.2 µg kg^{-1} respectively.

6.5.5 Grain calcium

Significant variation (P <0.0001) was observed in the grain Ca concentration (P <0.0001) among the 55 genotypes. Grain Ca varied from 553- 1010 mg kg^{-1}, with an overall mean of 773 mg kg^{-1} (Table 6.4). BC_1F_3-48 had the highest Ca concentration (1010 mg kg $^{-1}$) followed by BC_1F_3- 30 which had a concentration of 1001 mg kg $^{-1}$. Overall, 14% of the BC_1F_3 introgression lines had grain Ca concentrations between 900-1010 mg kg $^{-1}$, 37% between 800-896 mg kg $^{-1}$, 27% between 700-791 mg kg $^{-1}$, 13% between 600-695 mg kg $^{-1}$ and the rest had Ca concentrations above 500 mg kg $^{-1}$. The Ca concentration for DH-348 was 785 mg kg $^{-1}$, and this was lower than 56% (27) of the BC_1F_3 introgression lines. Ca concentration of the Malawian checks *Kenya nyati*, *Kadzibonga* and *Nduna* were 705, 624 and 607 µg kg $^{-1}$ respectively. Pavon 76, Chinese spring and Paragon had Ca concentration of 716, 629 and 611 mg kg^{-1} respectively.

6.5.6 Agronomic data

6.5.6.1 Grain yield

Grain yields among the genotypes varied significantly (P< 0.0001), ranging from 300-5741 kg ha^{-1}, with an overall mean of 2448 kg ha^{-1} (Table 6.5). BC_1F_3-49, BC_1F_3-34 BC_1F_3-46, BC_1F_3-37, BC_1F_3-36, BC_1F_3-40, BC_1F_3-17, BC_1F_3-19, BC_1F_3-9 and BC_1F_3-6 yielded 4741, 4630, 4556, 4186, 4148, 3741, 3667, 3481, 3333 and 3259 kg ha^{-1} respectively. The yields of these 10 lines were higher than the highest yielding Malawian check *Nduna*, which had a yield of 3185 kg ha^{-1}. Although *Nduna* showed

the highest yield among the Malawian checks, *Kadzibonga* and *Kenya nyati*, and introgression lines BC_1F_3-38, BC_1F_3-18, BC_1F_3-51, BC_1F_3-15 and BC_1F_3-31 had statistically similar yields. Paragon had the lowest grain yield (890 kg ha^{-1}) out of the three UK checks, while Pavon 76 and Chinese Spring had 1222 and 2148 kg ha^{-1} respectively. Grain yields for BC_1F_3-30 and DH-348 were 729 and 724 kg ha^{-1}, and these were the lowest yields among all the genotypes.

Table 6.5: Grain yield, a thousand kernel weight, number of tillers, days to flowering, days to heading, days to maturity and awn type of 37 Malawian wheat/*Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in 2022 winter season. The introgression lines have been ordered according to grain yield (highest to lowest)

Genotypes	Grain yield (kg/ha)	Thousand kernel weight (g)	Number of tillers	Plant Height (cm)	Days to heading	Days to flowering	Days to maturity	Spike type
BC_1F_3-49	4741 a	55 a	8 cde	61 ghijklmnopqr	68 mn	71 lm	98 mno	Awned
BC_1F_3-34	4630 ab	50 abcd	6 efghijklm	59 hijklmnopqrst	90 cdefghij	94 cdefghi	120 cdefghij	Awned
BC_1F_3-46	4556 abc	50 abcd	6 efghijklm	68 defghijkl	72 ijklmn	76 jklm	103 ijklmno	Awned
BC_1F_3-37	4186 abcd	50 abcd	7 cdefghi	53 nopqrstu	69 lmno	73 jklm	99 lmno	Awned
BC_1F_3-36	4148 abcd	50 abcd	7 cdefghi	62 ghijklmnopq	67 no	71 lm	97 no	Awned
BC_1F_3-40	3741 bcde	50 abcd	7 cdefghi	58 jklmnopqrstu	73 ijklmn	76 hijklm	103 ijklmno	Awned
BC_1F_3-17	3667 bcdef	50 abcd	4 hijklmn	50 qrstu	65 o	69 m	95 o	Awnless
BC_1F_3-19	3481 abcdef	50 abcd	5 defghijklm	46 tu	81 hijklmno	84 fghijklm	111 hijklmno	Awned
BC_1F_3-9	3333 abcdefgh	52 ab	5 defghijklm	58 ijklmnopqrstu	77 ijklmno	81 ghijklm	107 ijklmno	Awned
BC_1F_3-6	3259 abcdefgh	50 abcd	6 defghijkl	62 ghijklmnopqr	86 fghijklmn	90 efghijkl	116 fghijklmn	Awnless
Nduna	3185 bcdefghi	50 abcd	6 defghijkl	55 lmnopqrstu	68 mno	72 lm	98 mno	Awned
BC_1F_3-38	3148 abcdefghi	50 abcd	7 cdefghi	51 opqrstu	74 ijklmno	78 ghijklm	104 ijklmno	Awned
Kadzibonga	3111 abcdefghi	49 abcd	3 lmn	57 jklmnopqrstu	81 hijklmno	85 fghijklm	111 hijklmno	Awnless
BC_1F_3-18	3111 abcdefghi	50 abcd	3 lmn	47 stu	65 o	68 m	95 o	Awned
BC_1F_3-51	3074 bcdefghi	50 abcd	6 defghijklm	58 ijklmnopqrstu	65 o	69 m	95 o	Awned
Kenya Nyati	3037 bcdefghi	50 abcd	6 defghijklm	56 klmnopqrtu	69 lmno	72 lm	99 lmno	Awned
BC_1F_3-15	3037 bcdefghi	50 abcd	6 defghijklm	51 opqrstu	68 mno	73 klm	98 lmno	Awned
BC_1F_3-31	3037 bcdefghi	50 abcd	7 cdefghi	48 rstu	78 ijklmno	72 klm	108 ijklmno	Awned
BC_1F_3-5	2962 cdefghij	50 abcd	6 efghijklm	63 fghijklmnopq	89 defghijkl	93 defghijk	119 defghijkl	Awnless
BC_1F_3-10	2889 cdefghijk	50 abcd	5 fghijklmno	55 lmnopqrstu	67 no	71 lm	97 mno	Awned
BC_1F_3-44	2852 defghijkl	51 abcd	7 cdefghi	67 efghijklm	72 ijklmno	66 m	103 ijklmno	Awned
BC_1F_3-33	2844 defghijkl	50 abcd	9 bcde	61 ghijklmnopqrs	70 klmno	74 ijklm	100 klmno	Awned
BC_1F_3-57	2815 defghijkl	50 abcd	5 fghijklmno	67 defghijklm	68 mno	72 lm	98 lmno	Awned
BC_1F_3-7	2778 efghijklm	50 abcd	6 efghijklm	68 defghijklm	89 defghijk	93 defghi	119 defghijkl	Awned

BC_1F_3-39	2778 efghijklm	50 abcd	6 efghijklm	62 ghijklmnopq	72 ijklmno	76 hijklm	102 ijklmno	Awnless
BC_1F_3-52	2741 defghijklm	50 abcd	3 lmn	49 qrstu	65 o	68 m	95 o	Awned
BC_1F_3-47	2630 defghijklmn	42 de	8 cde	74 cdefg	119 a	123 a	149 a	Awned
BC_1F_3-26	2617 efghijklmn	50 abcd	10 bc	43 u	123 a	128 a	153 a	Awned
BC_1F_3-27	2407 efghijklmno	50 abcd	5 fghijklmno	54 mnopqrstu	65 o	68 m	95 o	Awnless
BC_1F_3-20	2370 efghijklmnop	50 abcd	4 hijklmn	45 u	66 o	70 lm	96 no	Awned
BC_1F_3-3	2333 efghijklmnop	50 abcd	6 defghijklm	74 cdefg	81 hijklmno	85 fghijklm	113 hijklmno	Awnless
BC_1F_3-32	2333 efghijklmnop	50 abcd	6 defghijklm	62 ghijklmnopqr	67 no	71 lm	97 no	Awnless
BC_1F_3-35	2326 efghijklmnop	49 abcd	7 cdefghi	64 fghijklmnop	72 ijklmno	76 hijklm	102 ijklmno	Awned
BC_1F_3-16	2259 efghijklmnop	49 abcd	4 hijklmn	53 mnopqrstu	67 no	72 lm	97 no	Awnless
Pavon	2148 efghijklmnop	50 abcd	6 defghijklm	66 efghijklmn	73 ijklmno	77 ghijklm	103 ijklmno	Awned
BC_1F_3-1	2148 efghijklmnop	48 abcde	5 fghijklmno	52 opqrstu	72 ijklmno	76 ijklmno	102 ijklmno	Awned
BC_1F_3-21	2074 fghijklmnop	43 bcde	8 cde	51 pqrstu	70 klmno	73 lm	100 klmno	Awned
BC_1F_3-23	1815 ghijklmnop	42 de	3 lmn	53 nopqrstu	70 klmno	77 ghijklm	103 ijklmno	Awned
BC_1F_3-53	1815 ghijklmnop	43 bcde	9 bc	73 cdefg	119 a	123 a	149 a	Awned
BC_1F_3-41	1804 ghikjlmnop	51 abcd	7 cdefghi	70 defghijk	73 ijklmno	77 ghijklm	103 ijklmno	Awned
BC_1F_3-48	1688 ghijklmnop	50 abcd	5 fghijklmno	82 bcd	67 no	72 lm	97 no	Awned
BC_1F_3-13	1630 hijklmnop	43 bcde	8 cde	54 mnopqrstu	71 ijklmno	75 ijklm	101 jklmno	Awnless
BC_1F_3-50	1556 ijklmnop	50 abcd	9 bc	72 cdefghi	83 ghijklmno	97 cdefg	113 ghijklmno	Awned
BC_1F_3-42	1378 jklmnop	40 e	8 cde	89 ab	114 ab	117 abc	144 ab	Awned
BC_1F_3-45	1259 klmnop	49 abcd	8 cde	80 bcde	119 a	122 ab	149 a	Awned
BC_1F_3-28	1222 lmnop	42 de	8 cde	85 bc	110 abc	114 abc	140 abc	Awnless
Chinese spring	1222 lmnop	48 abcde	13 a	100 a	87 efghijklmn	91 defghijkl	117 efghijklm	Awnless
BC_1F_3-2	1148 mnop	55 a	5 ghijklmno	77 bcdef	72 ijklmno	76 hijklm	102 ijklmno	Awnless
BC_1F_3-11	1091 nop	40 e	7 cdefghi	80 bcde	92 cdefghi	96 cdefgh	122 cdefghi	Awnless
BC_1F_3-29	1014 nop	40 e	7 cdefghi	71 cdefghij	98 bcdefgh	102 bcdef	129 bcdefgh	Awnless
BC_1F_3-60	1000 nop	44 bcde	7 cdefghi	73 cdefghij	94 bcdefg	98 cdefg	132 abcdefg	Awned
Paragon	890 op	18 fg	8 cde	64 fghijklmnop	106 abcde	110 abcde	136 abcde	Awnless
BC_1F_3-54	815 op	45 bcde	12 ab	72 cdefghij	75 ijklmno	79 ghijklm	104 ijklmno	Awned
DH 348	729 p	35 e	7 cdefghi	54 mnopqrstu	105 abcdef	109 abcde	135 abcdef	Awnless

BC_1F_3-30	724 [p]	25 [f]	8 [cde]	60 [ghijklmnopqrs]	119 [a]	123 [a]	149 [a]	Awned
Grand mean	2448	47	6	63	81	85	111	
P- Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
LSD (5%)	1455	8.5	3.2	14.1	19.7	20.3	19.7	
CV%	20.2	11.1	31.0	13.9	15.0	14.7	10.9	

Degrees of freedom (df) for replicates = 2, df for genotypes = 54

For each variable, means with different superscript letters are significantly different at P<0.05, following ANOVA and Fishers protected LSD tests

6.5.6.2 Thousand-kernel weight (TKW)

There was significant variation (P<0.0001) in the thousand kernel weight of the 55 genotypes (Table 6.5). TKW varied from 18-55 g, with an overall mean of 47 g. BC_1F_3-49 and BC_1F_3-2 had the highest kernel weight (55 g each), followed by BC_1F_3-9 and BC_1F_3-26, both with 52 g. TKW for the majority of the BC_1F_3 introgression lines was 50 g and this was statistically comparable to all three Malawian checks, Pavon 76, and Chinese Spring. TKW for DH-348, BC_1F_3-30 and Paragon was 35, 25 and 18 g respectively, and these were the lowest among all the genotypes.

6.5.6.3 Days to flowering, days to heading and days to maturity

Significant variation (P <0.0001) was observed in the days to flowering, days to heading and days to maturity (Table 6.5). Days to heading varied from 65-123, days to flowering 69-128 while days to maturity varied from 95-153. BC_1F_3- 26 BC_1F_3- 55 BC_1F_3-30, BC_1F_3-53, BC_1F_3-47 and BC_1F_3-45 had the longest time to heading, flowering and maturing, while BC_1F_3-18, BC_1F_3-52, BC_1F_3- 17, BC_1F_3-27 and BC_1F_3-51 took the shortest time. The majority of the BC_1F_3 introgression lines took 65-80 days to heading, 69-85 days to flowering, and 95-108 days to maturing. Among the check genotypes, DH-348 and Paragon took the longest days to heading, flowering and maturing, while *Kenya Nyati* and *Nduna* took fewer days to heading, flowering and maturity.

6.5.6.4 Number of tillers, plant height and awn type

The number of tillers were significantly variable (P < 0.0001) among the 55 genotypes (Table 6.5). Tiller number varied from 3-13 with an overall mean of six. Chinese Spring had the highest number of tillers (13), followed by BC_1F_3-54 with 12 tillers. Among the introgression lines, 22, 20, 18 and 16 % had seven, six, eight and five tillers respectively. A small number of some of the BC_1F_3 lines had nine, four and three tillers each. Among the Malawian checks, Kenya and *Nduna* had six tillers each, while *Kadzibonga* had three tillers. Paragon and Pavon 76 had eight and six tillers respectively. Plant height of the 55 genotypes was also highly significant

Table 6.6: Correlation coefficients for grain mineral-elements and phenotypic and phenological data of 37 Malawian wheat/ *Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in 2022 winter season

Variables	G Zn	G Fe	G Ca	G Se	DH	NT	DF	DM	PH	TKW	GY
G Zn	**1**										
G Fe	**0.716**	**1**									
G Ca	**0.232**	**0.259**	**1**								
G Se	**0.324**	**0.349**	0.034	**1**							
DH	**0.197**	**0.340**	0.126	**0.222**	**1**						
NT	0.022	**0.187**	0.026	-0.034	**0.290**	**1**					
DF	**0.212**	**0.339**	0.125	**0.205**	**0.968**	**0.290**	**1**				
DM	**0.194**	**0.337**	0.127	**0.218**	**0.999**	**0.286**	**0.969**	**1**			
PH	-0.071	0.045	-0.021	0.123	**0.298**	**0.334**	**0.299**	**0.297**	**1**		
TKW	**-0.317**	**-0.381**	-0.153	-0.027	**-0.473**	-0.095	**-0.467**	**-0.474**	-0.016	**1**	
GY	**-0.205**	**-0.454**	-0.053	-0.150	**-0.358**	**-0.169**	**-0.374**	**-0.360**	**-0.214**	**0.524**	**1**

Values in bold are significantly different at alpha level =0.05

(P <0.0001), ranging from 33-100 cm. Plant height for the majority of the BC_1F_3 lines varied from 50-70 cm, with a few lines between 71-89 cm. Chinese Spring grew to 100 cm, while Paragon and Pavon 76 were 64 and 66 cm tall respectively. *Nduna*, *Kenya nyati* and *Kadzibonga* had heights of 55, 56 and 57 cm respectively. It was observed that among the BC_1F_3 introgression lines, 72% of the spikes had awns while 28% were awnless. Among the checks, Paragon, Chinese Spring, DH-348 and *Kadzibonga* had awnless spikes, while Pavon 76, *Kenya nyati* and *Nduna* showed awned spikes.

6.5.7 Correlation analysis

Table 6.6 shows that grain Zn concentration positively and significantly correlated with grain Fe (r = 0.716, P = <0.0001), grain Se (r = 0.324, P <0.0001), and moderately correlated with grain Ca (r = 0.232, P = 0.003). Grain Zn was also positively correlated with days to heading (r = 0.197, P = 0.012), days to flowering (r = 0.212, P = 0.007) and days to maturity (r = 0.194, P = 0.013). However, grain Zn negatively and significantly correlated with both TKW(r = -0.317, P <0.0001) and Grain yield (r = -0.205, P = 0.009). Grain Fe showed a positive and moderate correlation with grain Ca (r = 0.259, P = 0.001) and grain Se (r = 0.349, P <0.0001). Grain Fe also positively correlated with days to heading (r = 0.340, P < 0.0001), days to flowering (r = 0.339, P <0.0001), days to maturity (r = 0.337, P < 0.0001) and number of tillers (r = 0.187, P = 0.017). Correlation analysis also showed that grain Fe negatively and significantly correlated with both TKW (r = -0.381, P <0.0001), and grain yield (r = 0.454, P <0.0001). Grain Se showed a significant, but not strong, positive correlation with days to heading (r = 0.222, P = 0.004), days to flowering (r = 0.205, P = 0.009), days to maturity (r = 0.218, P = 0.005). TKW positively correlated with Grain yield (r = 0.530, P <0.0001), and there were a strong positive correlations between days to heading, flowering and maturity.

6.5.8 Straw Zinc

There was significant variation (P <0.0001) in straw Zn concentrations among the genotypes phenotyped (Table 6.7). Straw Zn varied from 12.2 to 37.1 mg kg^{-1} with an overall mean of 23.1 mg kg^{-1}. BC_1F_3-15 and BC_1F_3-10 had significantly higher straw Zn with 37.1 and 35.1 mg kg^{-1} respectively. Six introgression lines had higher straw

Zn concentrations compared to DH-348. Overall, 15% of the introgression lines had straw Zn concentrations between 30.9-37.1 mg kg^{-1}, 62 % between 20.1-29.8 mg kg^{-1} and 23% between 12.2-19.2 mg kg^{-1}. Paragon, Pavon 76 and Chinese Spring had straw Zn concentrations of 28.3, 21.5 and 17.8 mg kg^{-1} respectively. *Nduna*, *Kenya nyati* and *Kadzibonga* had 16.1, 15.9 and 13.1 mg kg^{-1} respectively.

Table 6.7: Variation in straw mineral concentration of 37 Malawian wheat/ *Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in the 2022 winter season. The introgression lines have been ordered according to straw Zn (highest to lowest)

Genotypes	Straw Zn (mg kg^{-1})	Straw Fe (mg kg^{-1})	Straw Ca (mg kg^{-1})	Straw Se (mg kg^{-1})
BC_1F_3-15	37.1 ^{a}	384 ^{bcdefghijkl}	7611 ^{abcdef}	14.8 ^{cdefg}
BC_1F_3-10	35.1 ^{ab}	406 ^{bcdefghijk}	6616 ^{def}	14.3 ^{defgh}
BC_1F_3-27	33.7 ^{abc}	311 ^{fghijkl}	8680 ^{ab}	13.6 ^{defgh}
BC_1F_3-60	32.8 ^{abcd}	528 ^{abc}	6554 ^{def}	16.1 ^{bcdefg}
BC_1F_3-16	31.4 ^{abcdef}	296 ^{hijkl}	8507 ^{abc}	13.8 ^{defgh}
BC_1F_3-26	31.2 ^{abcde}	530 ^{ab}	6811 ^{cdef}	20.3 ^{abc}
BC_1F_3-32	30.9 ^{abcdefg}	288 ^{hijkl}	6493 ^{def}	13.3 ^{defgh}
DH 348	30.3 ^{abcdefg}	533 ^{ab}	6247 ^{ef}	18.2 ^{abcde}
BC_1F_3-17	29.8 ^{abcdefghl}	348 ^{bcdefghijkl}	4952 ^{gh}	14.9 ^{cdefg}
BC_1F_3-47	29.1 ^{abcdefghi}	360 ^{bcdefghijkl}	7732 ^{abcde}	12.5 ^{efgh}
Paragon	28.3 ^{abcdefghijk}	658 ^{a}	4418 ^{gh}	22.1 ^{ab}
BC_1F_3-18	27.1 ^{abcdefghijkl}	284 ^{hijkl}	9221 ^{a}	14.9 ^{cdefg}
BC_1F_3-31	27.0 ^{abcdefghijkl}	440 ^{bcdefghij}	4239 ^{gh}	15.5 ^{cdefg}
BC_1F_3-50	26.7 ^{abcdefghijkl}	391 ^{bcdefghijkl}	4704 ^{gh}	14.0 ^{defgh}
BC_1F_3-19	25.6 ^{abcdefghijklm}	320 ^{efghijkl}	6091 ^{ef}	14.4 ^{cdefg}
BC_1F_3-29	25.5 ^{abcdefghijklm}	448 ^{bcdefghi}	4892 ^{gh}	16.5 ^{bcdef}
BC_1F_3-38	25.3 ^{abcdefghijklm}	516 ^{abcd}	3805 ^{h}	14.7 ^{cdefg}
BC_1F_3-41	25.1 ^{abcdefghijklm}	506 ^{abcde}	7757 ^{abcde}	18.7 ^{abcd}
BC_1F_3-52	25.0 ^{abcdefghijklm}	311 ^{fghijkl}	5658 ^{gh}	12.5 ^{efgh}
BC_1F_3-20	24.7 ^{abcdefghijklmn}	433 ^{bcdefghij}	4755 ^{gh}	17.3 ^{bcdef}
BC_1F_3-1	24.1 ^{abcdefghijklmn}	364 ^{bcdefghijkl}	7835 ^{abcde}	13.8 ^{defgh}
BC_1F_3-3	23.3 ^{bcdefghijklmn}	401 ^{bcdefghijkl}	5934 ^{fg}	14.5 ^{cdefg}
BC_1F_3-42	23.1 ^{bcdefghijklmn}	381 ^{bcdefghijkl}	4919 ^{gh}	15.5 ^{cdefg}
BC_1F_3-30	22.8 ^{bcdefghijklmn}	296 ^{hijkl}	6548 ^{def}	12.5 ^{efgh}
BC_1F_3-54	22.7 ^{bcdefghijklmn}	496 ^{abcdef}	6459 ^{def}	23.1 ^{a}
BC_1F_3-35	22.7 ^{bcdefghijklmn}	422 ^{bcdefghijkl}	6959 ^{bcdef}	15.2 ^{cdefg}
BC_1F_3-13	22.3 ^{cdefghijklmn}	374 ^{bcdefghijkl}	6475 ^{defg}	8.6 ^{h}
BC_1F_3-53	22.2 ^{cdefghijklmn}	299 ^{hijkl}	7442 ^{abcdef}	12.8 ^{efgh}
BC_1F_3-45	22.2 ^{bcdefghijklmn}	490 ^{abcdefg}	6260 ^{def}	15.1 ^{cdefg}
BC_1F_3-48	21.6 ^{cdefghijklmn}	434 ^{bcdefghij}	6368 ^{cdef}	12.0 ^{fgh}
BC_1F_3-23	21.6 ^{cdefghijklmn}	212 ^{l}	6179 ^{ef}	14.8 ^{cdefg}
Pavon	21.5 ^{cdefghijklmn}	371 ^{bcdefghijkl}	6035 ^{ef}	15.8 ^{cdefg}

BC_1F_3-33	21.4 defghijklmn	346 defghijkl	7266 bcdef	13.7 defgh
BC_1F_3-11	21.4 defghijklmn	233 kl	6510 def	10.5 gh
BC_1F_3-6	21.2 defghijklmn	356 bcdefghijkl	6300 def	15.6 cdefg
BC_1F_3-7	21.2 defghijklmn	270 ijkl	7017 bcdef	13.5 defgh
BC_1F_3-5	20.7 defghijklmn	361 bcdefghijkl	7594 abcdef	11.8 fgh
BC_1F_3-2	20.7 defghijklmn	304 fghijkl	4255 gh	12.7 efgh
BC_1F_3-21	20.4 defghijklmn	341 defghijkl	7066 bcdef	12.0 fgh
BC_1F_3-9	20.1 efghijklmn	391 bcdefghijkl	7119 bcdef	12.3 fgh
BC_1F_3-49	19.8 efghijklmn	340 defghijkl	5668 gh	12.7 efgh
BC_1F_3-39	19.2 efghijklmn	357 bcdefghijkl	5623 gh	11.7 fgh
BC_1F_3-37	18.8 fghijklmn	376 bcdefghijkl	6933 bcdef	13.2 defgh
BC_1F_3-51	18.4 fghijklmn	271 ijkl	6300 def	14.8 cdefg
BC_1F_3-40	18.0 ijklmn	390 bcdefghijkl	8022 abcdef	13.3 defgh
Chinese spring	17.8 ijklmn	386 bcdefghijkl	6761 cdef	14.0 defgh
BC_1F_3-34	17.2 ijklmn	386 bcdefghijkl	4229 gh	12.9 efgh
BC_1F_3-46	17.1 ijklmn	348 bcdefghijkl	6768 cdef	13.5 defgh
BC_1F_3-57	16.2 lmn	461 bcdefgh	7243 bcdef	13.6 defgh
Nduna	16.1 klmn	376 bcdefghijkl	5528 gh	14.2 defgh
Kenya Nyati	15.9 lmn	343 defghijkl	6628 def	12.7 efgh
BC_1F_3-36	13.3 mn	367 bcdefghijk	6784 cdef	12.7 efgh
Kadzibonga	13.1 mn	460 bcdefghi	5845 gh	14.7 cdefg
BC_1F_3-44	12.3 mn	331 efghijk	5366 gh	11.8 fgh
BC_1F_3-28	12.2 n	370 bcdefghijkl	4666 gh	13.7 defgh
Grand mean	23.1	386	6334	14.3
p- value	<0.0001	<0.0001	<0.0001	0.018
LSD (5%)	10.2	153	1498	5.4
CV%	25.4	21.7	13.6	20.5

Degrees of freedom (df) for replicates = 2, df for genotypes = 54

For each variable, means with different superscript letters are significantly different at P<0.05, following ANOVA and Fishers protected LSD tests

6.5.9 Straw Fe

Straw Fe varied from 212 to 685 mg kg^{-1} with an overall mean of 386 mg kg^{-1} (Table 6.7). Paragon, DH-348 and BC_1F_3-26 had the highest straw Fe concentration with 658, 533 and 530 mg kg^{-1} respectively. Overall, 8% of the introgression lines had straw Fe concentration between 506-530 mg kg^{-1}, 21 % between 401-496 mg kg^{-1}, 52% between 404-391 mg kg^{-1} and 19% between 212-299 mg kg^{-1}. Chinese Spring and Pavon 76 had straw Fe concentration of 386 and 371 mg kg^{-1} respectively, whilst Malawian checks *Kadzibong*a, *Nduna* and *Kenya nyati* had 460, 376 and 343 mg kg^{-1} respectively.

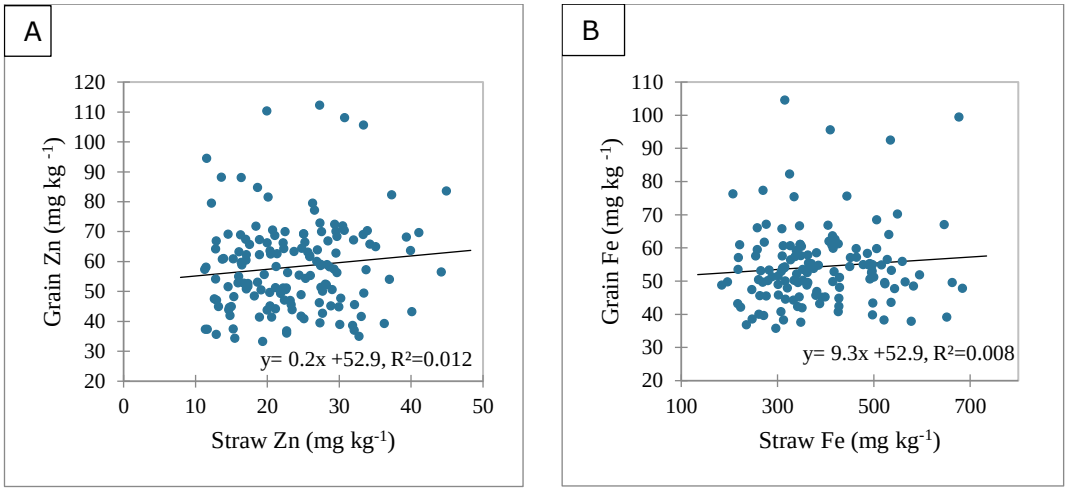
6.5.10 Straw Se

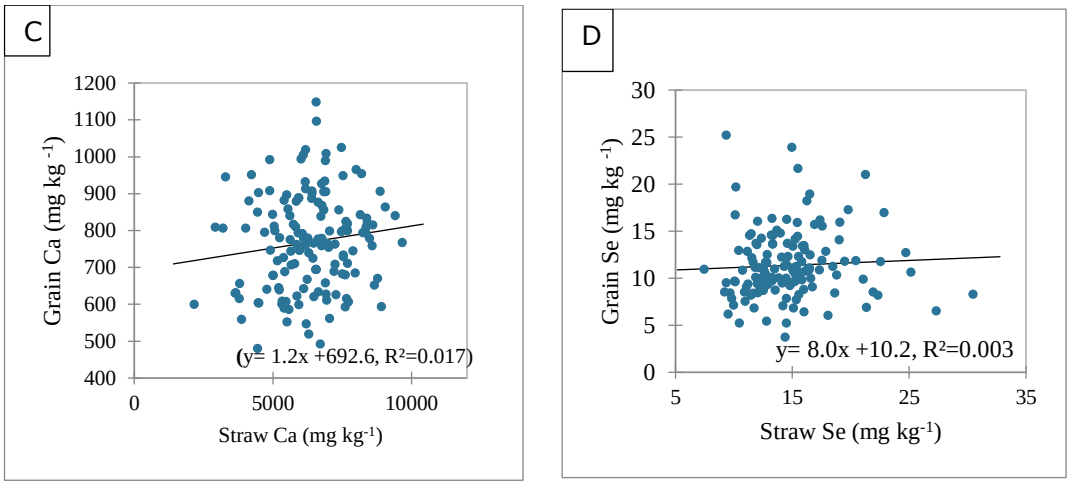
There was significant variation (P = 0.018) in straw Se concentration among the genotypes phenotyped (Table 6.7). Straw Se varied from 8.6 to 23.1 to mg kg^{-1} with an overall mean of 14.3 mg kg^{-1}. Only three lines (BC_1F3-15 and BC_1F3-10) had Se concentration above 20 mg kg^{-1}. The majority of the BC_1F_3 introgression lines had straw Se concentration between 12-15 mg kg^{-1}. Checks DH-348, Chinese Spring, Pavon 76 and Paragon had straw Ca concentration of 14.3, 13.8, 12.8 and 14.8 mg kg^{-1} respectively, whilst Malawian checks Nduna, *Kadzibong*a and *Kenya nyati* had 15.5, 12.5 and 12.0 mg kg^{-1} respectively.

6.5.11 Straw Ca

Straw Ca varied from 9221 to 8680 mg kg^{-1} with an overall mean of 6334 mg kg^{-1} (Table 6.7). BC_1F_3-18 and BC_1F_3-27 had the highest straw Ca concentration with 9221 and 8680 mg kg^{-1} respectively. Overall, 4% of the introgression lines had a straw Ca concentration above 8000 mg kg^{-1}, 23 % between 7017-7835 mg kg^{-1}, 40% between 6035-6959 mg kg^{-1}, 10% between 5366-5934 and 21% between 3805-4705 mg kg^{-1}. Checks DH-348, Chinese Spring, Pavon 76 and Paragon had straw Ca concentrations of 6247, 6761, 6035 and 4418 mg kg^{-1} respectively, whilst Malawian checks *Kenya nyati*, *Kadzibong*a and *Nduna* had 6622, 5845 and 5522 mg kg^{-1} respectively.

6.5.12 Relationship between grain and straw Zn, Fe, Ca and Se





Fitted and observed relationship with 95% confidence limits

Figure 6.2: Regression analysis between grain and straw Zn concentration (A) grain and straw Fe concentrations (B) grain and straw Ca concentrations (C) grain and straw Se concentration (D) 37 Malawian wheat/*Am. muticum* and 11 Malawian wheat/*T. urartu* BC_1F_3 introgression lines grown in the 2022 winter season.

There was a low but significant association (Figure 6.2) between straw and grain Se concentration (R^2 = 0.03, P = 0.05). Regression analysis also showed a low but insignificant association between grain and straw Zn concentration (R^2 = 0.012, P = 0.178), grain and straw Fe (R^2 = 0.008, P = 0.302) and grain and straw Ca (R^2 = 0.017, P = 0.116).

6.6 Discussion

The use of chromosome introgressions from distantly related or unrelated species that carry genetic variation for high mineral concentration of essential elements is one of the approaches that can be utilised to increase micronutrient concentration in crops (Velu et al., 2019). Recently, high Zn wheat varieties were developed from crossing the wheat progenitor *Ae. tauschii* with *T. durum*/wild tetraploid *T. dicoccum* via synthetic wheat, and these were released in Pakistan and India (Singh et al., 2017, Velu et al., 2019). Screening of rye translocation lines in a wheat backgrounds also showed significantly higher Zn and Fe concentration above their recurrent parents (Velu et al., 2019). In this study, BC_1F_3 introgression lines carrying *Am. muticum* and *T. urartu* chromosome segments in three Malawian wheat genetic backgrounds were phenotyped for grain and straw Zn, Fe, Ca, Se and related agronomic traits under field

conditions in Malawi. Soil samples collected at the trial site showed that the soils can be classified as Zn-deficient (Noulas et al., 2018, De Groote et al., 2021).

Grain Zn concentration varied widely among the introgression lines, with 98% (47)of the lines showing a grain Zn concentration above Chinese Spring, Paragon (the wheats in the background of DH-348), and the three recurrent parents/Malawian checks (*Kenya nyati*, *Kadzibonga* and *Nduna*), and 80% (38)of these improving in grain Zn concentration up to 50% above *Kadzibonga* and *Nduna*. Although 10% of the BC_1F_3 introgression lines had grain Zn between 70-80 mg kg^{-1}, all of them had lower yields than the potential yield of the three Malawian checks (~3000 kg ha^{-1}). However, one line (BC_1F_3-10) had a grain yield slightly lower (2889 kg ha^{-1}) than the Malawian checks, with a good combination of grain Zn and Fe concentrations. The number of crosses could have affected total grain yield of the introgression lines. Due to limited time, the two DH lines had only been crossed twice to the Malawian genotypes. Therefore, a quarter of the background of the introgression lines was still Chinese spring/Paragon, which are not adapted to Malawian conditions. Crossing the interesting lines a few more times with the Malawian wheat varieties is likely going to improve their yields/ agronomic performance. 23% of the introgression lines (BC_1F_3-34, BC_1F_3-36, BC_1F_3-38, BC_1F_3-40, BC_1F_3-31, BC_1F_3-37, BC_1F_3-15, BC_1F_3-46, BC_1F_3-19, BC_1F_3-9 and BC_1F_3-6) showed a good combination of grain Zn and grain yield. Grain yield of these lines was similar or exceeded most of the local checks, ranging from 3037 to 4630 kg ha^{-1}, with Zn concentration ranging from 53-67 mg kg^{-1}, which represents a 16-30 mg kg^{-1} improvement in grain Zn from *Nduna* and *Kadzibonga* and 11-25 mg kg^{-1} from *Kenya nyati*, Paragon and Chinese Spring. Interestingly, 10 of the 11 lines were awned, with a maturity periods between 97-120 days, making them more suited to the SSA environments. Ten of the 11 lines carry either the 4T or 7T segments from *Am. muticum*, and only one carries the $5A^u$ segment from *T. urartu*. Although most of the lines with the *T. urartu* had increased grain Zn concentrations, most of them were long duration with yields much lower than the Malawian checks. This could be an effect of the size of the $5A^u$ segment, carrying along genes that negatively affect the performance of the introgression lines. In the previous chapter, the *T. urartu* donor parent (DH-254) was shown to have longer days to heading and flowering, which affected the number of crosses made, as the heading and flowering did not coincide with that of the early maturing recurrent parents.

Among the 23% (11) high Zn, high yield introgression lines, 64% (7) lines also had an 8-12 mg kg $^{-1}$ higher Fe concentration than the recurrent parents *Nduna* and *Kenya nyati,* although they did not hit the target for Fe biofortification in wheat (60 mg kg $^{-1}$). Of the 48 BC_1F_3 introgression lines, only nine lines reached ~60 mg kg $^{-1}$. However, the yields of the lines were much lower (< 2000 kg ha^{-1}) than the yields of the Malawian checks. Grain Zn showed a significant positive correlation with grain Fe concentration implying that the two can be improved simultaneously. Similar findings were previously reported (Crespo-Herrera et al., 2016, Velu et al., 2019, Thapa et al., 2022, Velu et al., 2022, Velu et al., 2011). The significant negative correlation between grain Zn and TKW/yield and Fe and TKW/yield implies that an increase in Zn and Fe concentration decreases TKW and yields. Similar results were reported previously (Velu et al., 2011, Liu et al., 2014, Velu et al., 2019, Thapa et al., 2022, Velu et al., 2022). Liu *et al*. (2014) showed that for every 1000 kg ha^{-1} increase in grain yield, Fe concentration decreased by 2.1 mg kg^{-1} for spring wheat, and Zn concentration decreased by 0.9 mg kg^{-1} due to dilution effect. Straw Zn and Fe did not show a complete similar pattern with grain Zn and Fe. Generally, Zn and Fe were higher in the grain samples as compared to the straw samples, although a few lines with low grain Zn and Fe also showed low straw Zn and Fe and a few high Zn and Fe lines also showed high straw Zn and Fe. Thus, the regression analysis only showed a low and insignificant association between these variables.

Although there was a significant variation in grain Se, none of the genotypes had a sufficiently high Se concentration to make a substantial contribution to human nutrition (50-100 µg kg $^{-1}$). High Se concentration was shown in the donor parent DH-348, and only BC_1F_3-2 had a similar concentration. Only 8% of the BC_1F_3 lines showed a higher Se in the range of 6-10 µg kg $^{-1}$ above Paragon and the three Malawian checks, which are also the recurrent parents of the introgression lines. The results in this study are a reflection of the status of soil Se as shown in the soil analysis results. Previous studies have shown that soils with high pH and high Se concentration contribute to high grain Se concentration and those with low pH and low Se results in low grain Se concentration (Chilimba et al., 2011). Grain Se showed a moderate positive correlation with both grain Zn and Fe, and this could mean that accumulation of the two elements could have an impact on the accumulation of Se. Grain Se also negatively and insignificantly correlated with grain yield and TKW. Mean straw Se was higher than

mean grain Se, and regression analysis showed a low but significant association between two.

The majority of the BC_1F_3 introgression lines showed a higher Ca concentration above both the donor and recurrent parents. 56% (27) of the introgression lines had higher grain Ca than DH-348. BC_1F_3-48 and BC_1F_3-30 had a significantly higher Ca than all the other lines. The result shows that variation in Ca concentration could be attributed to the soil Ca concentration, rather than the effect of the introgression segments. In chapter 4, the interaction between soil type and genotype showed a significant effect on grain Ca concentration particularly in Chitedze soils. Grain Ca moderately correlated with grain Fe and Zn. Similar findings were reported previously (Pandey et al., 2016, Bhatta et al., 2018). The insignificant negative correlation between grain Ca and TKW and grain yield showed that an increase in grain Ca concentration had a negligible effect on grain yield.

6.7 Conclusion

98% of the BC_1F_3 introgression lines showed higher grain Zn above the checks Paragon, Chinese Spring, *Kadzibonga*, *Kenya Nyati* and *Nduna*. 23% (11) of the introgression lines showed high yield (3037 to 4630 kg ha^{-1}) and an increase in grain Zn by 16-30 mg kg^{-1} above *Nduna* and *Kadzibonga* and 11-25 mg kg^{-1} above *Kenya nyati,* Paragon and Chinese Spring. Among the eleven lines, seven lines also showed an 8-12 mg kg^{-1} improvement in grain Fe compared to *Nduna* and *Kenya nyati*. 8% (4) of the introgression lines showed a 6-10 µg kg^{-1} Se concentration above both Paragon and the three Malawian checks. These results show the possible significant impact of the 4T and 7T introgressions from *Am. muticum* and the $5A^u$ introgression from *T. urartu* on the genetic biofortification of Malawian wheat varieties particularly with grain Zn and Fe. Mapping quantitative trait loci (QTLs)/identifying candidate genes associated with the high accumulation of grain Zn and Fe will be useful for future work. Currently, sequencing of *Am. muticum* accessions and hexaploid wheat/*Am. muticum* introgressions lines are being undertaken at the Nottingham BBSRC Wheat Research Centre. These are likely going to play a major role in gene identification in the future. Further testing of introgression lines in replicated and multi-location trials will also be useful to measure stability, heritability and yields of the introgression lines.

CHAPTER 7

7 General discussion

Biofortification of food crops with essential mineral nutrients can be a sustainable and cost-effective strategy to combat dietary mineral deficiencies, which are prevalent in resource-poor countries. Wheat is one of the major sources of dietary energy for billions of people globally. Cultivated wheat is inherently low in grain micronutrients and the genotypic variability is relatively narrow (Monasterio and Graham, 2000, Calderini and Ortiz-Monasterio, 2003, Guzman et al., 2014). Availability of substantial and useful genetic variation in crops is a prerequisite for increasing grain micronutrient concentration through breeding (Cakmak et al., 2004). Therefore, the first objective of the present thesis was to identify new sources of genetic variability for grain Zn, Fe, Se and Ca in wheat progenitors and wild relatives. Wheat wild relatives generally provides a vast reservoir of genetic variation that remains untapped (Friebe et al., 1996), and in wheat, some diploid *Aegilops* species, einkorn and wild emmer wheat were previously identified as potential sources of variation for grain Zn and Fe (Velu et al., 2011, Singh et al., 2017, Velu et al., 2019).

To determine the Zn, Fe, Ca and Se natural variation in the wild relatives (Chapter 3), inductively coupled mass spectrophometry (ICP-MS) was undertaken to screen 31 different wild relative accessions in the genus *Triticum*, *Aegilops*, *Thinopyrum*, *Ambryopylum* and *Secale*. The wild relatives were screened alongside wheat varieties that are commonly grown in Malawi (*Kadzibonga*, *Nduna* and *Kenya nyati*), and Chinese Spring and Paragon. ICP-MS results showed a wide variation in grain Zn, Fe and Ca. Unfortunately, variation for Se was very narrow, and none of the wheat wild relative accessions showed a sufficiently high Se concentration to make a substantial contribution to wheat improvement for human nutrition. These results suggest that the screening of wheat progenitors and wild relatives has a high potential to facilitate the discovery of novel sources of genetic variability for improvement of some of the essential mineral elements in wheat. By comparing the variation in mineral concentrations of the 31 wild species accessions, this thesis has demonstrated that *Am. muticum*, *T. urartu* and *Ae. speltoides* accessions could be potential sources of novel genetic variability for grain Zn, *T. tauschii* and *Am. muticum* for grain Fe whilst *Thinopyrum* species for grain Ca. Grain size and weight analysis showed that *Am.*

muticum, *T. urartu*, *Ae. speltoides* and two of the *Thinopyrum* species (*T. ponticum* and *T. bessarabicum*) have the lowest grain weight and smallest grain size parameters (area, length and width). A significant negative correlation of grain Zn with grain weight and grain size parameters, indicate that grain Zn concentration is affected by seed size. Since there was a narrow variation for grain Se among the wild relatives, identification of novel sources of high grain Se concentration would require screening more species grown in variable soil conditions. Lyons *et al.* (2004) found significant variation in grain Se of diploid wild wheat, ancestral wheat, wheat landrace accessions and commercial wheat. However, much of the variation was associated with spatial variation in soil Se. Although further screening for novel sources of Se in wheat wild species can be recommended for future biofortification programs, Lyons *et al.* (2004) suggested that agronomic biofortification may be a more practical and productive way for increasing grain Se for human nutrition.

Identification of novel sources of variation in wild relatives and transferring this variation into an intermediate set of materials is a step forward to their utilisation for crop improvement. King *et al*. (2017) and Grewal *et al*. (2018) describe the generation of interspecific hexaploid wheat/*Am. muticum* and hexaploid wheat/*T. urartu* hybrids for trait analysis. To generate stable homozygous lines, several BC_3 lines generated from these interspecific lines, were used to develop doubled haloid (DH) lines for further use in breeding programs (King et al., 2019a, Grewal et al., 2021). In the present study, a preliminary field-based phenotyping of 48 randomly selected wheat/*Am. muticum* and wheat/*T. urartu* DH lines was undertaken in Malawi. The main objective of the study was to determine if the observed variation in Zn, Fe, Ca and Se in the wild relatives could be tracked from any of the chromosome segments introgressed in the DH lines. Mineral analysis revealed that 25 (12) and 44% (21) of the lines had high grain Zn above their recurrent parent (hexaploid wheat cv. Paragon). The results showed that 95% of the lines had higher grain Ca compared to Paragon. For Se, little variation was observed in the DH lines, and this is in line with the results obtained for the progenitors and wild relatives. The higher mineral concentration of the DH lines compared to their recurrent parents shows that some of the wild segments in the wheat/*Am. muticum* and wheat/*T. urartu* DH lines potentially harbour some genetic sources of mineral improvement. This result agrees with the earlier findings that *T. urartu* and *Am. muticum* accessions are potential sources of useful genetic

variability for Zn, Fe, Ca but not Se. Comparing the findings in this thesis with previous work, particularly on quantitative trait loci (QTLs) associated with grain Zn, Fe and Ca, it was discovered that the majority of the previously mapped QTLs were located on similar chromosomes as the *Am. muticum* and *T. urartu* introgressions in the DH lines with higher Zn, Fe and Ca concentration. This finding suggests that the wild chromosome segments from *T. urartu* and *Am. muticum* could potentially harbour QTLs associated with grain Zn, Fe and Ca.

Another important aspect in improving the mineral concentration of crops through breeding is to understand the soil physio-chemical properties, plant mineral uptake and mineral mobilisation and remobilisation dynamics. Previous work clearly shows that concentration of mineral nutrients in the grains, is associated with their bioavailabilities as regulated by various soil physio-chemical properties (Elrashidi et al., 1989, Broadley et al., 2006, Hawkesford and Zhao, 2007, Alloway, 2009, Flis, 2019, Rengel, 2015). To investigate the effects of soil physio-chemical properties on grain Zn, Fe, Se and Ca concentration (Chapter 4), 12 wheat/*Am. muticum* and 4 wheat/*T. urartu* DH lines were phenotyped in two different soil types along with *Kenya nyati*, *Nduna*, *Kadzibonga*, Paragon and Chinese Spring. The two soil types were collected from Chitedze and Ngabu Agriculture Research Stations, in Malawi. Chitedze soils were characterised by higher Zn, Fe, Se and organic matter, and lower pH and Ca. Ngabu soils were characterised by lower Zn, Fe, Se and organic matter, and higher pH and Ca.

In both soil types, phenotyping results revealed a clear relationship between soil type and grain mineral concentration, which were likely associated with soil pH, DTPA-Zn, DTPA-Fe and organic matter. For example, grain Zn and Fe concentrations were largely influenced by soil type, although genotype and the interaction between genotype and soil type also affected the concentrations. A two-fold and 1.3-fold increase in grain Zn and Fe in plants grown in Chitedze soils compared to plants grown in Ngabu soils is likely attributed to the higher soil bioavailability of soil Zn, Fe and soil pH. As with grain Zn and Fe, a clear relationship between soil type and grain Se was also showed. Previous work on grain Se showed that grain Se is highly influenced availability of Se in the soil, soil pH and organic matter (Chilimba et al., 2011, Chilimba et al., 2019a, Stroud et al., 2010). The selenium in soil also depends, to a large extent on the parent rocks (Pan et al., 2023). In the present thesis, mean Se

concentration was higher in plants grown in Ngabu soils, and lower in plants grown in Chitedze soils. Statistical analysis showed that differences in grain Se were greatly associated with soil type and partially by genotypes and the interaction between the two. The higher soil pH in Ngabu soils likely increased the bioavailability of Se for plant uptake, while the low pH in Chitedze soils might have reduced the bioavailability. Genotypes showing high mineral concentration in both soils suggests the efficiency of the genotype in soil mineral uptake, mobilisation and re-mobilisation into the grain. Unlike grain Zn, Fe and Se, grain Ca concentration was not clearly influenced by soil type but genotype. However, the interaction between genotypes and not soil type showed a low significant impact on grain Ca concentration. Soil analysis showed a high Ca concentration in Chitedze soils, and a lower Ca concentration in Ngabu soils, although mean grain concentration of plants grown in the two soils were not very different, explaining why soil type alone did not have an effect on grain Ca. It is therefore important that any breeding program for high mineral concentration particularly, Zn, Fe and Se, should consider both availability of substantial and useful genetic variation in relation to soil physio-chemical properties.

To transfer the *Am. muticum* (TT) and *T. urartu (*A^uA^u*)* introgressions potentially increasing mineral nutrients in the DH lines into Malawian wheat varieties (Chapter 5), hexaploid wheat/A*m. muticum* DH-348 and hexaploid wheat/*T. urartu* DH-254 were crossed with the three Malawian wheat varieties (*Kadzibonga*, *Nduna* and *Kennya nyati*). A combination of whole genome sequencing, KASP analysis and genomic *in situ* hybridisation (GISH) revealed a 4T and a 7T segment of *Am. muticum* on wheat chromosome 4D and 7A of DH-348. Whole genome sequencing and KASP analysis also revealed the presence of two $5A^u$ segments on wheat chromosome 5A of DH-254.

A crossing program for DH-348 and DH-254 with *Kadzibonga*, *Nduna* and *Kenya nyati* resulted in the generation of forty-one Malawian wheat/*Am. muticum* BC_1F_3 introgression lines with both the 4T and 7T segments, 4T segments only, and 7T segments only. Eleven Malawian wheat/*T. urartu* BC_1F_3 introgression lines with the $5A^u$ segment were also generated. The availability of high-throughput genotyping technologies has enabled the process of tracking wild chromosome segments in a wheat genetic background easier. Through a combination of whole genome sequencing, KASP genotyping with chromosome specific markers and GISH, a clear

picture of the genetic make-up of the donor parents was revealed. This made it easier to track the chromosome segments though the breeding pedigree.

To evaluate the effects of the 4T, 7T and $5A^u$ introgression on grain and straw mineral nutrients, and associated agronomic traits of the Malawian wheat varieties (Chapter 6), a field based phenotyping study of the 11 Malawian wheat/*T. urartu* and the 37 Malawian wheat/*Am. muticum* was undertaken in Malawi. The study was designed in a randomised complete block design (RCBD) with three replicates. *Kadzibonga*, *Kenya nyati*, *Nduna*, DH-348 and DH-254, Paragon, Pavon 76 and Chinese spring were used as checks. Mineral analysis showed high yields and 11-30 mg kg^{-1} improvement in grain Zn in 11 introgression lines, above the three Malawian wheat varieties and Chinese Spring and Paragon. These lines also showed 8-12 mg kg^{-1} improvement in grain Fe than *Nduna* and *Kenya nyati*. Four lines showed a 6-10 µg kg^{-1} Se concentration improvement above Paragon and the three Malawian checks and no notable improvement was shown on Ca concentration. These findings show clear effects of the 4T, 7T and $5A^u$ segments on improvement of mineral nutrients, particularly Zn, Fe and Se. A number of studies have shown a strong negative correlation between TKW, grain yield and grain Zn and TKW, grain yield and grain Fe (Velu et al., 2022, Thapa et al., 2022). In the present study, correlation analysis between grain Zn, Fe and TKW/yield also showed a strong negative correlation. This result implies than an increase in grain yield, decreases Fe and Zn concentration.

Across the four experiments undertaken for this thesis, grain Zn showed strong and significant positive correlations with grain Fe concentration, clearly showing that the two mineral nutrients can be improved simultaneously. Previous studies have showed a link between some QTLs for grain Zn and Fe, which explains the strong positive correlation between the two mineral elements. For example, a single QTL on chromosome 7A for grain Zn concentration was identified and mapped on the same interval as that of grain iron concentration (Tiwari et al., 2009a). Another QTL for grain Zn, which was mapped on chromosome 2B of wheat was also co-localised with that of grain Fe (Velu et al., 2017b).

Overall, grain Ca moderately and significantly correlated with grain Zn and Fe, except in the wheat lines grown in Chitedze soils. These findings are comparable with findings from previous studies (Pandey et al., 2016, Bhatta et al., 2018).

To evaluate the association of grain and straw mineral concentration, and understand the partitioning of Zn, Fe, Se and Ca in wheat plants, straw samples were also analysed for Zn, Fe, Se and Ca. Correlation and regression analysis generally showed a positive and low significance or a positive but insignificant association between the two. The absence of a hypothesised strong and significant correlation between the mineral elements in the grain and the straw samples could be associated with the time the straw samples were collected. To further research on this objective, it would be good to consider collecting and analysing leaf and stem samples for mineral nutrients before grain filling or immediately after grain filling.

CHAPTER 8

8 Perspectives and future work

- Although a wide variation and very high concentrations of grain mineral nutrients in the wheat wild species accessions were shown, field-based phenotyping of these accessions in a uniform environment should be considered. This thesis (Chapter 4) and previous work have demonstrated that concentrations of mineral micronutrients, particularly Zn (Cakmak, 2008, Manzeke et al., 2019), Fe (Ramzani et al., 2016) and Se (Chilimba et al., 2019a, Stroud et al., 2010), are associated with soil physio-chemical properties that affect their bioavailabilities. The wild relatives studied in this thesis were previously multiplied under glasshouse conditions with a high supply of nutrients likely to increase mineral concentrations compared to field conditions.
- The breeding target for grain Zn and Fe in wheat was set at an additional 12 and 22 mg kg^{-1} from the baseline respectively (Bouis et al., 2011, Bouis and Saltzman, 2017). These targets were set to meet 60-80% of estimated average requirement (EAR) for women and children. A field-based phenotyping of the 48 *Am. muticum* and *T. urartu* DH lines showed higher grain Zn in 77% (37) of the genotypes above the set target, and 25% (12) of the genotypes showed higher grain Zn above Paragon, which is the genetic background of the DH lines. Phenotyping results also showed that 44% (21) of the genotypes had Fe concentrations above the set target, and above Paragon. The major limitation of this study was the limited number of seed available for a replicated trial. Therefore, an ear-row un-replicated trial was undertaken. Unfortunately, due to unavailability of trial replicates, the results from the experiment must be considered as preliminary. To further exploit these materials for future breeding programs for mineral micronutrients, a replicated trial should be considered.
- Field phenotyping of the 48 BC_1F_3 lines showed high grain Zn in 98% (47) of the Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* lines above the three Malawian wheats and Paragon and Chinese Spring. However, only 22% (11) showed a combination of high yield and high grain Zn. This is likely because the two DH lines were crossed only twice with the Malawian

genotypes, due to the time limit of the PhD program. This implies that 25% of the genetic background of the introgression lines was still Chinese Spring/Paragon, which are not adapted to Malawian conditions. To improve the agronomic performance of the introgression lines, further crossing of some of the high-Zn lines with the Malawian wheat, and phenotyping the progenies under field conditions should be considered.

- Lines showing high yields and high grain Zn and Fe can also be phenotyped in different locations with different environmental conditions to test genotpye×environment (G×E) effects, measure heritability, yields and evaluate effects of different soil type on mineral accumulation. Previous studies have shown significant effects of G×E interactions on stability and genetic variation for micronutrients in cultivated wheat (Oury et al., 2006), ancient wheats (Peleg et al., 2009, Gomez-Becerra et al., 2010b) and biofortified spring wheat (Velu et al., 2012, Srinivasa et al., 2014a). For easy implementation and monitoring, the Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* introgression lines were evaluated in a wheat non-traditional growing area in Malawi. Future work must include evaluating these lines in more traditional wheat growing areas.
- To test the effect of the *Am. muticum* and *T. urartu* segments in the introgression lines. It will be necessary to cross a few interesting lines with the Malawian wheat parents and generate a new heterozygous population. Self fertilisation of the heterozygous population will generate homozygous lines, lines with no segments and lines maintaining the heterozygous condition. Selecting and multiplying seeds of the homozygous lines and lines with no segment and phenotyping them under field conditions will confirm the effects of the segments on the Malawian wheat varieties.
- Chromosome-specific KASP markers, GISH and whole genome sequencing has facillitated the identificatation and characterisation of chromosome segments that show potential for improving grain mineral nutrients in a hexaploid wheat background. Identification of specific QTLs/genomic regions underlying the concentration of grain Zn, Fe, Ca and Se in the wild chromosomes should also be considered. One way of achieving this is by reducing the size of the introgression, to provide a reduced number of genes to

work with. The introgression lines can therefore be sequenced and then compared to the whole genome.

- Randomised contolled trials can be used to evaluate stable lines for bioavailabity and digestibility through the use of available biomarkers of Zn status. Further work might also consider identifying where in the seed the minerals are stored, ie seed coat, endosperm, etc. This would be particularly important where white flour was the requirement and most of the minerals were in the seed coat and therefore lost on milling

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10 Appendices

10.1 Appendix 1: Buffers and reagents

SS DNA-140µg/ml (for 50mls)

5mls 50X TAE (pH 6.3)

0.7 ml SS DNA (10mg/ml)

44.3mls water

Store at 4°C

Template preparation solution (for 400mls)

40mls 1M Tris (pH 9.5)

8mls 0.5M EDTA

30g KCl

Water (Make up to 400 mls)

3M Sodium Acetate (for 100mls)

40.8 g Sodium acetate

100ml water

Adjust pH to 5.2

Sterilise the solution by 0.22 µm membrane filter

6M ammonium acetate (for 50mls)

23.124g 6M ammonium acetate

50mls water

Store solution at 4°C

5% Sodium hypochlorite (ClNaO)

5 ml ClNaO

95 ml water

1 drop Tween 20

Extraction buffer (for 100mls)

10mls 0.1M Tris-HCl (pH 7.5)

10mls 0.05M EDTA (pH 8.0)

12.5mls 10% SDS

67.5mls water

Cell digestion enzyme for (100mls)

0.1 g Pectolyase Y-23 (1% w/w)

0.2 g Cellulase Onozuka R-10 (2% w/w)

9.7 g 1X Citric Buffer (pH 5.5)

Enzyme solution made on ice and stored at -20^{0}C

5x Citric Buffer (for 50mls)

50mls water

0.735g Sodium Citrate (50 mM)

5mls 0.5M EDTA (50mM)

Adjust to pH 5.5 using Citric acid monohydrate powder

1x Citric Buffer (for 100 mls)

20mls 5x citric Buffer

80mls water

2xSSC+1×TE solution (for 20 ml)

10mls 20×SSC

2mls 10×TE

16mls Water

2× SSC (for 100ml)

10mls 20x SSC

90mls water

10 × T.E

100mM Tris

10mM EDTA

pH7.5

1 × T.E

10mls 10XT.E

90mls water

DTPA-TEA solution (for 1 litre)

1.97 g DTPA

1.1 g $CaCl_2$

13.38mls TEA

900mls Milli Q water.

Adjust the pH to exactly 7.3 with 6N hydrochloric acid

The final extractant solution comprises of 0.005 M DTPA, 0.1 M TEA, and 0.01 M $CaCl_2$

MEHLICH III solution (for 1 litre)

Ammonium fluoride- EDTA stock reagent

138.9 g NH_4F

73.5 g EDTA

1-litre water

Extracting reagent

80 g ammonium nitrate into 3000 ml distilled water

16 ml ammonium fluoride-EDTA stock reagent

46 ml Acetic acid

3.28 ml concentrated nitric acid

Adjust the pH to 2.0^{+-} 0.1

4-litres water.

The final extractant solution comprises of 0.2 M Acetic acid, 0.25 M Ammonium nitrate

0.015 M Ammonium fluoride, 0.013 M Nitric acid and 0.001 M EDTA

1N Potassium dichromate

49.04 g AR potassium dichromate

Distilled H_2O

0.5 N Ferrous ammonium sulfate

196 g Ferrous ammonium sulfate

5mls concentrated Sulphuric acid

Make up to 1 litre with distilled water

Diphenylamine indicator solution

0.5 g diphenylamine

100 ml conc. sulphuric acid

20 ml distilled water

85-90% Conc. phosphoric acid

N1 solution (for 1500mls)

68 g Sodium salicylate

50 g Sodium citrate (Tri-Sodium Citrate)

50 g Sodium tartrate

0.24 g Sodium nitroprusside

Dilute to 2000 ml

N2 solution (for 1500mls)

60 g NaOH

28.5 ml of 3.5 % sodium hypochlorite solution

1500 ml water.

Calgon or dispersal solution (for 1-litre)

20g Sodium hexametaphosphate

8g Sodium hydroxide

1-litre water

P working solution-Murphy Riley Solution

0.291g Antimony potassium tartrate

12g ammonium molybdate

140mls concentrated sulphuric

1-litre water

Murphy- Riley working solution

100 ml Murphy Riley solution

500ml water

0.526 g ascorbic Acid

10.2 Appendix 2: KASP markers primer sequences

Marker list	Primer_AlleleFAM	Primer_AlleleHEX	Primer_Common
WRC0010	GGTGCACTGACACTAACCCACT	GTGCACTGACACTAACCCACC	TGATTCACTTTGCAGACTAAATTCCTCAC
WRC0013	GGATGCAACTCTTCTAGCAAATCCAA	GATGCAACTCTTCTAGCAAATCCAG	TAGGCAATTATGTGGATTATGAAGACAAAA
WRC0016	GGATCAGTTTATTCACATGCTTGCT	GGATCAGTTTATTCACATGCTTGCC	GATTAGAGCTTGCCATTGTCAAAAGACAT
WRC0022	AACATAACATGATAGATCAACCTGGGA	CATAACATGATAGATCAACCTGGGC	CGTTGCAACTTGCAGGACTCTTGTA
WRC0023	TCGTCGAACACTATTTGTCGTTC	CTTCGTCGAACACTATTTGTCGTTG	TGGTACCCTGTTTACAGCCCACTT
WRC0024	GAYGCACCAGTCTCACACTTT	GAYGCACCAGTCTCACACTTC	CCATGGCCGAGGCGACTTGG
WRC0028	GTACACCAGCTGAAGGCAAGG	GGTACACCAGCTGAAGGCAAGA	CTCTTCCTGTCTTGGGCTCTTGG
WRC0032	CTAATTCAGTTGCAATACAAGTGACATA	CTAATTCAGTTGCAATACAAGTGACATG	AACATATTAGCAACCCTCGGCTTTAAAKAA
WRC0040	ACAAGATGAACCTGTATCGGTTACG	AACAAGATGAACCTGTATCGGTTACA	ATTGCACCACTGTCACAACCTGCTT
WRC0042	GGAGTGACTTTCGTCTTGAAGTG	GGAGTGACTTTCGTCTTGAAGTC	GCACATACCTTGTYGCTGCACAAAA
WRC0045	AATCGAGATCTGTTCGAATCCGAC	AATCGAGATCTGTTCGAATCCGAG	CTTGTCGCGGGAGAGTGCTTCA
WRC0047	CTATGAAGGCATACAAGTTCTTCCAA	CTATGAAGGCATACAAGTTCTTCCAG	GATGGGTTTGATATAAAGATGTGTGGGTAT
WRC0051	TAAGTGGAGCGGTGTGTAGC	ACTTAAGTGGAGCGGTGTGTAGT	CTCCAGTTATGTGTACAGTAATCCATCAT
WRC0090	GGTTAACCTGTATTGAATTTAAACAATTGG	GGGTTAACCTGTATTGAATTTAAACAATTGA	GCGCCCCAAAGATGGCATGAATTTA
WRC0095	GATACACCCAGTTTTATCCACTCATAAA	ATACACCCAGTTTTATCCACTCATAAG	TTGGGATGTGAGGTATTAAAACCATGAGT
WRC0122	CGAGGAGAACGAGATGCTACAC	ACGAGGAGAACGAGATGCTACAT	ATTTTCTGCTCGTATTCTAGCCAC
WRC0139	GGATCCAGCAAGCACGCG	CTGGATCCAGCAAGCACGCA	CGCTCTCTTCCATGGCGACAAC
WRC0142	CCGATGGCAGTACAGAGAGATCT	CGATGGCAGTACAGAGAGATCC	CCGCTTGTAATCCSTGCTTGCC
WRC0143	AGTAGCCATAGTATTGATGCTAGTTTC	CAGTAGCCATAGTATTGATGCTAGTTTT	GCGCCAGGAGGTGGCCCAA
WRC0145	CAGAGGTGATCCCGCGTTAAATC	AACAGAGGTGATCCCGCGTTAAATT	GTGGATCATTTTGGTGGAGAGRGTTAA
WRC0152	GCCCGTCCAAGCTTTGTACTCA	CCCGTCCAAGCTTTGTACTCG	CAGGAACTCCATGACCGATGCAG
WRC0153	AGAAATGAAACCGCAGGATGTGTC	CAGAAATGAAACCGCAGGATGTGTT	CAAGGCTGACCTAGCACAAACCAAT
WRC0156	CAGCTTTCTCGAGTAGCTTGGC	CCAGCTTTCTCGAGTAGCTTGGT	CCATGGCGGACTGCAACACCAT
WRC0161	CGCTGCTTCTTCCCCGTTTSAA	CGCTGCTTCTTCCCCGTTTSAT	CGGGGTTCGGCGCGCAGA
WRC0163	ATTACCAGGTAGGGATACTGCCTT	ACCAGGTAGGGATACTGCCTC	GTCCGATGGACTTTGCCAACTACTA
WRC0164	CGYCACATGTAGGTGTCAGC	CTCGYCACATGTAGGTGTCAGT	GSTACATAACCGAACACAGGGTGAA

WRC0168	GTACGATAGGCCGGTCCTCTC	AGTACGATAGGCCGGTCCTCTT	GGGCCTTCGTCCGTGGGCT
WRC0175	GAAATCCAGGAGGCTTGAAACG	GAAATCCAGGAGGCTTGAAACT	CGCATGTAGAGCTTTAAGCTCCTTG
WRC0178	GGTGAACTCTTGAATCCCACACT	GGTGAACTCTTGAATCCCACACA	CATGGAACATTAGCCRCTGATGGATT
WRC0180	GCCTGATGTTGGAGAAGAGTCC	ATGCCTGATGTTGGAGAAGAGTCT	CTTAGGTGAAACCTTGACCTCCTTCA A
WRC0181	CTGTATCCTCAGCTCCTCACG	GTCTGTATCCTCAGCTCCTCACA	CGAGGTCAAGCAGTCTCTGCCTTT
WRC0183	STCGGATTGGAGAGATCGATTC	GTSTCGGATTGGAGAGATCGATTT	GGAGACCATGCACCAGCTGCA
WRC0187	CACCTGGCATCCTTTTGTTGGCA	ACCTGGCATCCTTTTGTTGGCG	AACTGCATCAATAGCTTAACTAGCTAGCA
WRC0188	CATTGCTGATTGTATAATTGCTGGTAC	CCATTGCTGATTGTATAATTGCTGGTAA	ATTTCCTACCTGAGTTTGAGGTTCTGTAT
WRC0191	ACACATGTTCTGTAAAATACTCACCG	CACACATGTTCTGTAAAATACTCACCA	GATGGCCAATGGTGYGCTAAGGAA
WRC0193	GTATTTCCTCTCAGTCCATGTCTG	GTATTTCCTCTCAGTCCATGTCTC	GTACGTACATGTAGCACACAAAGGAG
WRC0196	TCGTCTAACATGCATGTTGTATATTATTG	CTTCGTCTAACATGCATGTTGTATATTATTT	TCAATGTTAACTGTTATCACGGGTCAGAA
WRC0222	ATTTCTATTTGGGCCAAAGTAACACAC	CAATTTCTATTTGGGCCAAAGTAACACAA	CTGGAGCTTTTGTGAACTGATGAGG
WRC0236	GATCMAGCAGAGCAAGAACTCG	GATCMAGCAGAGCAAGAACTCC	GACGGCGAAGGGTTGCAGCAA
WRC0240	CATCAATATCTCCGGCATGGTCAA	ATCAATATCTCCGGCATGGTCAC	TGATAAACCTCCATCATTACTGGGGTATT
WRC0253	GCACATGCGATGATCCAGC	GCTGCACATGCGATGATCCAGT	CAGAGTGCCTGAATTTCAGCATCTTT C
WRC0256	GCGCACAGGGAAACCAACCC	GCGCACAGGGAAACCAACCG	GAGAAACATGCCAAATGCGTATACATCT
WRC0264	GCATCCAGTTCTCCGGTTCAAC	GCATCCAGTTCTCCGGTTCAAG	CSAAAATACTTGCAGGGCAAGAGCTT
WRC0276	AACTCAGGTGAATTTGCCGAGTTCT	CTCAGGTGAATTTGCCGAGTTCC	CATGGCCAGAGGGCAATCACC
WRC0278	GCACAGAGCCTCCGCGGT	GCACAGAGCCTCCGCGGC	GGACGAAGACGAGGCTGGCAT
WRC0281	GCACCAAAGACTTCCATCCACG	GCACCAAAGACTTCCATCCACT	ATGAGGTTTCATGCTGCCAGAGCAA
WRC0290	TGTTGGACACTGAAAATTTGATCTG	CTTGTTGGACACTGAAAATTTGATCTC	AGTGGCAGGACACTGAGTAGGG
WRC0292	GGTGCACTAAGTTGAGGACACTG	GGTGCACTAAGTTGAGGACACTA	CAAGTGAGAGGATCAACAGGGATCT
WRC0295	ATAGCTTGGGCTCTCGGTCTGT	GCTTGGGCTCTCGGTCTGC	CGGTTACGGTGACGCAAACCCA
WRC0301	CCTTTCTTGTTTCGCTTGAACTTAGA	CCTTTCTTGTTTCGCTTGAACTTAGT	AAGGCAGGACARCTCAAGCTCCA
WRC0302	TCTCCTGGAGCACAGTGGCA	CTCCTGGAGCACAGTGGCG	AGCAACACTGAGGTGCAGATCACAA
WRC0307	CCTCGGATGCTTAATCTTGGTAATTC	CCTCGGATGCTTAATCTTGGTAATTT	CTAAATTGTTTGAGTTGATTAGCACTGGC
WRC0308	CCTCGGATGCTTAATCTTGGTAATTC	CCTCGGATGCTTAATCTTGGTAATTT	CTAAATTGTTTGAGTTGATTAGCACTGGC
WRC0312	GRCTTCTTTATCCTGTGAAATTGATCA	CTTCTTTATCCTGTGAAATTGATCC	CCAGTCAGCACTACAATGTCACGAA

WRC0314	TGAGATTGTGCAGCTCCAACAC	GTTGAGATTGTGCAGCTCCAACAT	CGGCTTCTCCACCGCAACCA
WRC0317	GTAAGGATTCAAATTCCCAGGGAGA	AAGGATTCAAATTCCCAGGGAGC	CACCTGTGACATCTTCACAAATCACAC
WRC0318	AGCCCGTACTGATCCTGAATATTAC	CAGCCCGTACTGATCCTGAATATTAT	TTTATTCAGCCTAGACTAATACWGTACATG
WRC0326	CTCGCTCAAGTACTCCTGATAG	ACTCTCGCTCAAGTACTCCTGATAA	GATACAAGTACCTGAACCTCTGGG
WRC0327	ACAAAATTCCCTGGCAACCAACAATT	CAAAATTCCCTGGCAACCAACAATC	GTGGGCGATGTTTTGTTTCTGCGTT
WRC0332	CAAGGTCACTGTTTCTACAGAGTG	CTCAAGGTCACTGTTTCTACAGAGTA	TGTGTGTAGATGCCACCAACCTCTA
WRC0333	AACGTCATGCTGTTTGGTGTACTC	GAACGTCATGCTGTTTGGTGTACTT	GGTGGTAGTAATGGCCAGCTAAATC
WRC0350	GAGGACCTGAAGCCGAAACTC	CGAGGACCTGAAGCCGAAACTT	GCGAACCAGAGTTTCAGCAGYGA
WRC0352	GTGGGGAGGGTGCCATCTAC	GGTGGGGAGGGTGCCATCTAT	AAAGTCGTCTGAACAGTTATCTGGTAGT
WRC0360	GTGCGAACCTCAAGGAGATAACC	GTGCGAACCTCAAGGAGATAACT	CCTCTGCGTCCTCAGTGCCT
WRC0364	GCGGCGGCAGTGCGCTC	CGCGGCGGCAGTGCGCTT	GATGAGGACGAGCTCCTGGATG
WRC0367	AAGGGTACATTGCCCTTCACAAGA	GGGTACATTGCCCTTCACAAGG	CCAAATTGGAGACAATAGYATTAGACGAG
WRC0378	GGAAGCGGATATGGCTGGTGG	GGAAGCGGATATGGCTGGTGA	ATGACCTGGAAGGAGGACCGGT
WRC0381	ATAATGTCAATCTGGACTGTGTACTTG	CATAATGTCAATCTGGACTGTGTACTTA	TACTAGGTAGGTTTCAGAGAATCCAAATAT
WRC0384	GCCTTTGCACGGRTCATTGGTG	GCCTTTGCACGGRTCATTGGTA	AAGTTTGCTGTTATGACACAACCTSTGA
WRC0399	GCAGCTTCCTTCATCATGTCCG	GCAGCTTCCTTCATCATGTCCC	AAGGGGCTCTTCTTGGATAAAWCATCATA
WRC0405	CAAAAGTACTGCGTTTTACCCTCCAT	AAAAGTACTGCGTTTTACCCTCCAC	GACARGACAGTCTCGGTCAAAGCAT
WRC0433	CCTCTCTTCCTCCATCACCAG	CTCCTCTCTTCCTCCATCACCAA	CGCTCGACCTCATAATTGTTTTSAAAGTA
WRC0438	GTCACAAAAATCCCCATGTCTGATGA	CACAAAAATCCCCATGTCTGATGC	ACCGACAATGGCAGGTTCTACGTTT
WRC0440	CAAGCAGGCAGAGCCCCGAT	AAGCAGGCAGAGCCCCGAC	ACAGAACACTGGGAACATCGGTTAG
WRC0441	CGAGAGGCAAAAACAGGAACACG	ACGAGAGGCAAAAACAGGAACACA	TGTTGTGCTGAAAATAAATTCTTAGTTCAT
WRC0447	AAGGTTGATCGGTCTGAAGTCTCTA	GGTTGATCGGTCTGAAGTCTCTG	CAACGAGTCCATCCTGACGCTATTA
WRC0449	CCTCGGAGAAACTTTGCGACTG	GCCTCGGAGAAACTTTGCGACTT	CAGGCTCTAAGCTTGAAAAWTACATCTTC
WRC0453	ATGTCTCGTGCATGACACCAGG	ATGTCTCGTGCATGACACCAGC	CGCACAAGTGGAGGCCGATGAA
WRC0456	CAACACCAAAGCTCAATATTTATGTGC	GTCAACACCAAAGCTCAATATTTATGTGT	CCGACACTATGAAGCCCCCACT
WRC0461	CAGTGCAAGGCTACAAGCA	CTCAGTGCAAGGCTACAAGCG	CATGCCSCATCGACTCCCGAT
WRC0463	CAAGACTCCCTTCTCCTATAATGGT	AAGACTCCCTTCTCCTATAATGGC	GGTACTGTACGGATTCATCAGAGGA
WRC0472	AGAGCGAGATAAACCCGGCG	GTAGAGCGAGATAAACCCGGCA	GGCGCTTCAACAGAAGGTTYCACAA
WRC0473	CATCTCGGGACCTTGTTTTCCC	ATCATCTCGGGACCTTGTTTTCCT	CGTAGTAGCATCATACCTAAAACTAGAAAA
WRC0474	GCCCCGGATCCTGATTTTGCC	GCCCCGGATCCTGATTTTGCA	CATCGTTAACTTTGCAGGTGGACGTT

WRC0523	GGTCCATCAGCAGCCCTGG	GGTCCATCAGCAGCCCTGA	GTTTCAGTTTTGAGAGACGAGGTTAACAA
WRC0531	GAAACTGATTACGCGACTGACTACAA	AAACTGATTACGCGACTGACTACAG	GTCATGTCTTGAACTCTRGCTAGATCTT
WRC0560	GAAGATCCTGTGCAGAACCCG	GTGAAGATCCTGTGCAGAACCCT	AGCTTACTCCCGCAAAACTTGGGTT
WRC0566	CCGGATTGACTGAAGAAGCATGTTTT	GACTGAAGAAGCATGTTTC	GAGAGGTGCCKGGGATTTATTCC
WRC0569	GAAGGTGTACACTTCTTGGTATCCT	AAGGTGTACACTTCTTGGTATCCC	CCAGGGATTGAAAAACTGGTCCTYTTT
WRC0574	CATATGCGCGTTCGAGAACTTCAT	CATATGCGCGTTCGAGAACTTCAA	CAACTGCAGGATGATTGTGCATATGATAT
WRC0580	AACCATTCGGACTTCCAGAGATC	GTAACCATTCGGACTTCCAGAGATT	TATCTTGAAGTGCATTCCGGTTAAATCG
WRC0581	CATGTTGGCCATTAGTTATTAGGAARC	CCATGTTGGCCATTAGTTATTAGGAART	ATCATCCATGGATCCAAGATCATACCAT
WRC0586	ATGCAGTAAAGCAAGACTAAGCTGAT	GCAGTAAAGCAAGACTAAGCTGAC	GGCGGTTCGTCTCACTGTACTATTTT
WRC0597	TCGTACTGCAACCGGAATTCTCAT	CGTACTGCAACCGGAATTCTCAC	ACACCTTTGGGAGCTTGACATGGAA
WRC0599	ACGAGGACTCGATTGCATTTTAGC	GACGAGGACTCGATTGCATTTTAGT	CGGTCCACACACTTTGCAAACATGAT
WRC0605	AACTGAAGAAGTTGAATTGTTGTTG	GCTAACTGAAGAAGTTGAATTGTTGTTA	CACCTGGAAAGCTTGTAGGTTCCAA
WRC0607	GAGGCTACGGGAGGAAGCCA	AGGCTACGGGAGGAAGCCG	CTCTCCTCGAAGAATTGATTCTGTGC
WRC0608	CCTTGAAGAATTGACTCTGTGCCT	CTTGAAGAATTGACTCTGTGCCG	GCACAGAATCAACTCTTYGAGGAGA
WRC0614	CCCGTAAGTTTGTTTGTGGAAATGAC	CCCGTAAGTTTGTTTGTGGAAATGAT	TGAATGGCATTCCACGAAATTCAGAGAAT
WRC0638	GTGTATCCAGAGGATTATACCATTGTT	GTGTATCCAGAGGATTATACCATTGTA	CTTSACCAATCCATTGCCATATCAAATCTT
WRC0662	TACGTTGATATATGCTGGCTGCTTT	ACGTTGATATATGCTGGCTGCTTA	CATTGTTGTGTGTACAAGAGAATGCRGT
WRC0664	CCATCAGTATACTACACCCACCC	CCATCAGTATACTACACCCACCG	CTATGCTTATTTGCTGGSCTTTTCAATTG
WRC0665	TCGGCCGCTCCTGCGGA	CGGCCGCTCCTGCGGG	GCGGAGGAGGTGGTTCTCAACAA
WRC0672	AAGGGATAGATGTTGTATACTTGCCAT	AAGGGATAGATGTTGTATACTTGCCAA	ATGTGATCAAGATAGATGGGTACTCAAG
WRC0673	CAACGCAGACGGAGCTGTTCATA	AACGCAGACGGAGCTGTTCATG	TGCCGGCCGTCGTCRTCC
WRC0679	CACTGCACTCCCCAATCTCTG	CACTGCACTCCCCAATCTCTA	TAGACAGTGCWCATATTATAAATGAACAAA
WRC0688	GTCGTTTACTTGGCTTATCAAACTA	GTCGTTTACTTGGCTTATCAAACTC	TGGGCCACGCGCCTCTTTCAT
WRC0689	TTGGGCGCGTGAGAGACAAA	TGGGCGCGTGAGAGACAAG	TCAACTCTGKAGTTTGATAAGCCAAGTAAA
WRC0711	CAGGTGAAGCTTTCCATCATGGAA	AGGTGAAGCTTTCCATCATGGAG	GTCGTGCAACTYGTTGGCGAGA
WRC0713	GTAACTAGATGCCTTGAAATAGCACTA	AACTAGATGCCTTGAAATAGCACTG	GTTCYGGTTCTTGTTGCCTGCACT
WRC0720	GTGGAAGGCGCAGATTACAGTTA	GTGGAAGGCGCAGATTACAGTTT	CTTTTCCGTAAAAAAGGGGGGAAAGGT
WRC0721	CCGATCAGAGTCACTTCCCATAC	AACCGATCAGAGTCACTTCCCATAT	GTTAAGTCTCGAGCATGTCGCYGTA
WRC0725	CAAGGCCCAACCCTAGGCC	CCAAGGCCCAACCCTAGGCT	GTTCGTCTAAATGTTGCGATCACAC
WRC0730	GTTGCCATCTGAGCTTATATGC	GCTGTTGCCATCTGAGCTTATATGT	CCCGCCAGAAGCATAAACCAAACAA

WRC0731	TCCCTACCGTGTGCC	CATGCTTCCCTACCGTGTGCT	GCTAGGTAGTCAAGGACAAGGTGAT
WRC0745	GAACATGACTAATTAAGACAACCCAC	CGAACATGACTAATTAAGACAACCCAA	TACTAATTTGGTCTAATATGTAATTTCGAT
WRC0747	AGGTAGAATCGAATCTCTTTGCTTGTA	AGGTAGAATCGAATCTCTTTGCTTGTT	TCTGGAAGCCACGCGACGCAAT
WRC0750	AACAGTGTGCCTGGTTTGCCC	GTAACAGTGTGCCTGGTTTGCCT	GTCCAGGATCCTGATCATGATGAGAT
WRC0754	AGCAGCAGCAGCCTTCTTGATTTTA	CAGCAGCAGCCTTCTTGATTTTG	TGCCYGACACGTACAACACACGTA
WRC0755	GAAAAGGCACGATGTATTGTGAACG	AAGAAAAGGCACGATGTATTGTGAACT	CACCCTAGCAACGATCYGATTTACTT T
WRC0762	ACCTTTGCCAGTCTCTCCTCCA	ACCTTTGCCAGTCTCTCCTCCT	CTTTTGTCTCCCCGTAAATTCARAGCA
WRC0769	AACACCTGTAGTCCTAGATCACC	AACACCTGTAGTCCTAGATCACG	AGGTTTYAGGTCGAATATGAAGGAACAAT
WRC0789	GGAACATGTCGATCGCAAAGTATGAT	GAACATGTCGATCGCAAAGTATGAC	CCGCTCCCTATGGCAATGGCT
WRC0801	CCTCCTCCGACGGCTTG	GCTCCTCCTCCGACGGCTTA	ATCGCATTCTACATCTTTGGCCCGA
WRC0804	ATATGCCCATTAAAGCACAAGCCAG	GATATGCCCATTAAAGCACAAGCCAA	GCGGAGCCTAAATGCAAAATGTTYA C
WRC0811	GGATGGATCRAAATCTTGGCAATCG	AGGATGGATCRAAATCTTGGCAATCA	GGCAACATCTTCCAGGACTGYGAA
WRC0814	GGAAGCACAGCGATGTCCGA	GGAAGCACAGCGATGTCCGG	GCTCCCGCACTTTGGGCTCG
WRC0816	GCATCCACATCATCAGTTTTATCACTT	CATCCACATCATCAGTTTTATCACTC	AACTGTCCAGAAGATGAAGATGTTGAGAT
WRC0820	CAATCACTATTAAAAATCATGGAATCGCG	CAATCACTATTAAAAATCATGGAATCGCA	TGACATTGGTGGAGGCGCATGATAT
WRC0823	GGTGAGTGMTATTTGTATGCTTGATT	GGTGAGTGMTATTTGTATGCTTGATC	CATTAGTATTTGCACTGTTCATATTCTATT
WRC0825	TTCGCAAAACAATCTTTCWGGTCAA	TCGCAAAACAATCTTTCWGGTCAC	AGTTTATTATAGTCTAGACGAAGGGTCATT
WRC0827	CCGCTCCATCTCCGAGACG	CCCGCTCCATCTCCGAGACA	CTCATACAACCGCATCCTGGCG
WRC0844	CCACCGTCAGCACCTCCTG	CACCACCGTCAGCACCTCCTA	TGCTGCCGGACGCGCTGGA
WRC0852	CAGACTGGATTCTTGGCCTGC	GCAGACTGGATTCTTGGCCTGT	TGAGGGTCTGAGRAAAGGCTCCAA
WRC0866	CATCTAAGCTCGCGTCTTTGCC	CCATCTAAGCTCGCGTCTTTGCA	CGCCGTTCAACGTGGCCTTGAT
WRC0869	GTTCTAAGTTGTACGTTAGCTTCACTA	CTAAGTTGTACGTTAGCTTCACTC	CTTAACACTCCCAAGCYAGGCCAT
WRC0871	ATGTGCTGAAGAATGCAAAGGAACAA	GTGCTGAAGAATGCAAAGGAACAG	CTTGCTCCRTTTTGTGTAATATGGACCAA
WRC0879	ATCCCTTTTGGCGCTCTACGTG	CATCCCTTTTGGCGCTCTACGTA	GAGGAAGTGCCTGTTGCTCAGAGA
WRC0880	GGAGGTCCCTGGGGGGGA	GAGGTCCCTGGGGGGGG	CGAGCCCGTTGGCRTCTGGTT
WRC0884	GGGATTGTGCACCACTTG	CGCTGGGATTGTGCACCACTTA	AAGATGCAACACAGTGGAGGAGATATT
WRC0886	GATAAAATGTCCTGCCAAGCCGTTT	ATAAAATGTCCTGCCAAGCCGTTC	CGACTGGACTGATTTTGTGTATCCG
WRC0892	TCTACGGACAATCTCGTTTTCTCC	GTTCTACGGACAATCTCGTTTTCTCT	CACCAGCAGAGCCGCCTGCT

WRC0893	CAGCTCGTCTGTTCCTTCCAGAT	AGCTCGTCTGTTCCTTCCAGAC	CTCATTCCGGGACAAGGCCGTT
WRC0895	GAGTTGCGGCTGCCAGTCTAG	GAGTTGCGGCTGCCAGTCTAC	CACAACAATGGGTACAGTGTAGCAATAAAT
WRC0899	GCATTGCGCAAGATGAAGGAACG	ATGCATTGCGCAAGATGAAGGAACA	MTTCAGTTTGCCCCAACTTCTCTTCA
WRC0913	CCTTCCCCCTCCTCCGCG	CCTTCCCCCTCCTCCGCC	GACRAAGAGGCAACTGGCACTGAT
WRC0918	GCAGCCCGGTCAATGGCATG	GCAGCCCGGTCAATGGCATA	GGTGCAGCATTGGCAGTCTCAGAA
WRC0949	CACTTGCTAAGAAAGTCTTCATGTCATA	ACTTGCTAAGAAAGTCTTCATGTCATG	ATTTTTGAGATACAGTAACCCAAGCAGTTT
WRC0953	CGTGAACTACGCGCCTGGA	CTCGTGAACTACGCGCCTGGT	TATCGCCTATGTAGTGTGGGCCATT
WRC0954	CGGCAAAAGCAGTCCCCG	ACTCGGCAAAAGCAGTCCCCA	CGCCTGGATCAACTAATGACAGGAT A
WRC0957	CCGACGGAACTAGCCATCGAG	CCGACGGAACTAGCCATCGAA	GAGATGCTTCTGAGGCTTGAAAAGGAA
WRC0972	GTCCAGTCACAGCTCAAGACTTTA	CCAGTCACAGCTCAAGACTTTG	GATGCCCTTGTAGCTCRCGGAAAA
WRC0978	GAGCCTCCTCGTGTCGGTC	GAGCCTCCTCGTGTCGGTG	GTGAACGAGACGACGTCGACG
WRC0984	CGGTTATAGGAACCTCTGATCTTG	CGGTTATAGGAACCTCTGATCTTC	CAGAGTAAAGATGGCAAGAAAGAGAACAT
WRC0992	ATTGCGAGTTGTGCCATGAAAAATCT	GCGAGTTGTGCCATGAAAAATCC	GCTCCTCTGGCGGTGACTATTCAT
WRC1002	ATTCTGGTGTTCTCTTCAGTCCAA	ATTCTGGTGTTCTCTTCAGTCCAT	ACTCCCTGATCAAAGAAGTTG
WRC1010	CGCCATCGCTATGAGGCTAT	CGCCATCGCTATGAGGCTAC	GCCATCCAGTACTCCTCC
WRC1016	ACACATCAAGTGCAAGTTCCT	ACACATCAAGTGCAAGTTCCC	CTAGTGGAACCTCTCACGC
WRC1019	CCAACTAAGCTGAGAAGTGGC	CCAACTAAGCTGAGAAGTGGG	CTCCTCGATCTTCTCTTGAA
WRC1020	CTATCGCATTCGTGGATCCTT	CTATCGCATTCGTGGATCCTG	GTCGAACTTGAGATGGTCTT
WRC1021	GGAAGTTCTCGACCTCTCTAT	GGAAGTTCTCGACCTCTCTAA	GCTGCTCGTTGGACTTGT
WRC1023	CCTGTCATAATCAAATCACTA	CCTGTCATAATCAAATCACTG	GGGAATGTTAACTGAAGCCT
WRC1024	GCTGATGTTGCAGTCAATGTA	GCTGATGTTGCAGTCAATGTC	ATTGTTCGTAGGAGCATTTG
WRC1026	TGGTAAATTGGTGGATGAGAG	TGGTAAATTGGTGGATGAGAC	TGACATTGTCTTCAGTCACAG
WRC1055	CGTTTGAAGGGTTCCTGAAC	CGTTTGAAGGGTTCCTGAAT	TTGAGACTGCCAAAACTAGC
WRC1064	CCGTCACATTCATATGGCAAAAT	CCGTCACATTCATATGGCAAAAC	CACCTCCATTCATGCACGAAC
WRC1066	AACCAGAGCTAACACTTTTAATGC	AACCAGAGCTAACACTTTTAATGT	AATCGCTCATAGAATTCAGAACA
WRC1067	ATATTAGACGCTCGATGGCAGA	ATATTAGACGCTCGATGGCAGT	AAGCAGGCACCTTCKGCA
WRC1091	GAAGAACAAGTGGAGACAGGGG	GAAGAACAAGTGGAGACAGGGA	GTAATAGAGCCTCTTCTCTGGCAGAT
WRC1117	GCCTGAGAAACAGCAGATCTGC	GGCCTGAGAAACAGCAGATCTGT	GCAGAAACAAGAAACTCGCAGCCAT
WRC1132	GAGTCATCGTTAATTTGCTGCCATCA	AGTCATCGTTAATTTGCTGCCATCG	TCTTAAGCTACATGACAGACGAAACGAAA

WRC1135	GAGTATATGAAGAATAAACAGATCATGGT	GAGTATATGAAGAATAAACAGATCATGGA	CATCGCATTACGCTTTGAAATCACGAATT
WRC1136	CACCCGTAGGCAATCCCAATTAT	CACCCGTAGGCAATCCCAATTAC	GTAGATGGACCATGGGCAATTGTGA A
WRC1138	ATAAAACAAACAACTACTACCAACGAGAA T	AAACAAACAACTACTACCAACGAGAAC	TTTGTACACGGTAAATGTTAGCTCTCTAAT
WRC1155	CGCTACCAATCAGGACTCCTG	CGCTACCAATCAGGACTCCTC	AGGAAATTCCCATCACAGTTCAAATGTTAT
WRC1158	AGAAGGAAGAAGGTGGAGATCCT	GAAGGAAGAAGGTGGAGATCCC	CGCTAGTCTGCCTTTTTGCTCTGAT
WRC1159	GCAGCACATTTCTTGTGGTGTCG	AGCAGCACATTTCTTGTGGTGTCA	AGATTTGCTATTGAGTCTTTGAAACCAGAA
WRC1160	GCGAATATTATATGGCGACTGGC	AGGCGAATATTATATGGCGACTGGT	CAACATGTGAGATCTGAACTCCACCTA
WRC1161	AGTATGTTCCCGAGCTCAACCA	AGTATGTTCCCGAGCTCAACCT	GGTAGTCCTGATCAGAGACACCAAA
WRC1162	CGGGAGTGCTGGTGCACTCA	GGGAGTGCTGGTGCACTCG	AGCAAAGAGATTCGATTCTACCTAGTGTA
WRC1205	CAAACCTCCCGAATGCGTCG	GTCAAACCTCCCGAATGCGTCA	GCCGAGCTTGGCAGCGGCAA
WRC1206	AGGAAGCAGCTAATAAGGTGGGTT	GGAAGCAGCTAATAAGGTGGGTC	TGATGGGCCGTGGCCTCTTCAT
WRC1207	AGCTAATGGATACATGTGCCGTC	ACAGCTAATGGATACATGTGCCGTT	CGACCCTCAGCCCTTCTCTGAA
WRC1233	ATTCTAAAGTAAACCTTACACAGATGTTGT	CTAAAGTAAACCTTACACAGATGTTGC	CTAGAGTTAAGCATGTAAGGTTACACAGTT
WRC1249	AGTTTCTAGAAAATATGTTGTCTCCTTTC	AATAGTTTCTAGAAAATATGTTGTCTCCTTT T	GAACTAACTTTTTAATGCTGGACATGACAA
WRC1264	ATAAGAATGTTGTTGTGGCAATGCACA	AAGAATGTTGTTGTGGCAATGCACG	CTAAGTATTGGCGAGGATCGCTCTA
WRC1291	AGTCGCTCACTCGCCTCGC	AGTCGCTCACTCGCCTCGG	CCTTTCTTCGCTTGATCGACACCAA
WRC1314	GACCCGTCAACCCTCCTCAC	GACCCGTCAACCCTCCTCAT	GAGATGGAGAACACGCCC
WRC1315	CAGAGCCTTCCTTGCTCGGC	CAGAGCCTTCCTTGCTCGGT	GAGAGGAAAGTTGAGAAGGG
WRC1316	GCATGTATGCATGACCATTGAAT	GCATGTATGCATGACCATTGAAC	CATCCCAAGACACACCATC
WRC1329	TTGCATTCCAACACAGTCT	TTGCATTCCAACACAGTCC	ACAGAAAAGATTGTGTCAGGA
WRC1330	TTTATTTCCACGGGAAACCACAT	TTTATTTCCACGGGAAACCACAC	ACGAAATGCGGGAATCTG
WRC1380	ACATTTTACTGGCTTAGGTAGGTC	ACATTTTACTGGCTTAGGTAGGTG	CGCATCGCATGAGCAGAATC
WRC1382	CACAGGAGCTGAGGTTATTTTCC	CACAGGAGCTGAGGTTATTTTCA	AATCAAATCAATCGGCGGCG
WRC1399	TCTCTACACGCATAAGTCAACAA	TCTCTACACGCATAAGTCAACAG	CAAACTGACCCCGGGCTAG
WRC1402	GACAATCTTTAGTCACTGCATTGT	GACAATCTTTAGTCACTGCATTGC	GTGACCACAGCCTCGACTG
WRC1403	GTCCCCCACGGATCTGCC	GTCCCCCACGGATCTGCA	GTGCGTGTCCAATGGCAATG
WRC1406	CGTACCGTGAATGGAGCATC	CGTACCGTGAATGGAGCATA	TGGACCCACGATCATTTAGGT

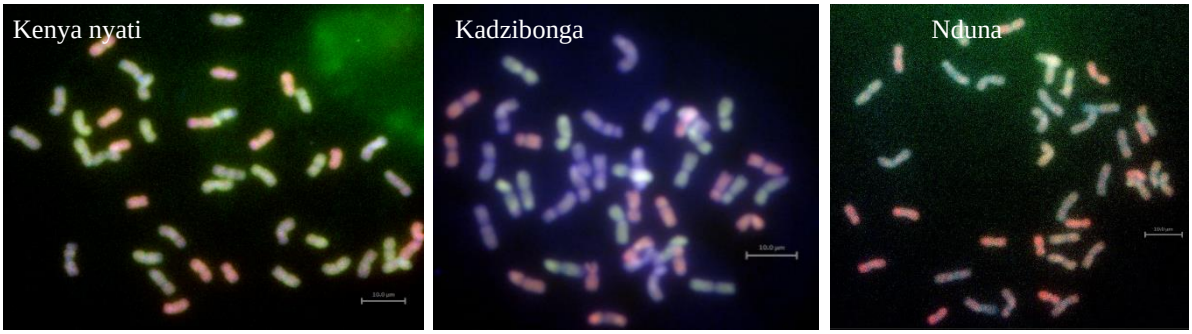
WRC1408	GGAAGGCCACCACCATAGTAT	GGAAGGCCACCACCATAGTAC	ATTGCTCGTCCTCCTTGTCG
WRC1417	GGTCAAGCCTAAATTTTCTATGACC	GGTCAAGCCTAAATTTTCTATGACA	TGCCAACAAGGGGTGTAAGA
WRC1418	ATAGAAACAGGGAGGGAGGT	ATAGAAACAGGGAGGGAGGC	GCCGGAGAAAACGATGGAGA
WRC1425	CCTTGTGTCTTGTGAATGCAAAA	CCTTGTGTCTTGTGAATGCAAAT	TGATGTCGCCATGTTTGCTT
WRC1427	CACCAAACCTACACAGCACAA	CACCAAACCTACACAGCACAG	GCCATTAGCTTTGGTGGTGC
WRC1429	ACTCCCAACCACATCACCAAA	ACTCCCAACCACATCACCAAC	GTGAAGTGCACAAGGGCAAG
WRC1432	CGATCTAGCGCCGGATAACC	CGATCTAGCGCCGGATAACT	CACAGTCAGTTCCAGCTCCA
WRC1441	GGGGTTTTTCCATGTCTATCAAGAA	GGGGTTTTTCCATGTCTATCAAGAG	AAGTGGTTCAAGCGGTGTTG
WRC1443	CTCTCGGGCCAGGATGACA	CTCTCGGGCCAGGATGACG	CCAAGCATGGCCTTACTAGATG
WRC1446	TGAGATCGCTGGTTCGAACT	TGAGATCGCTGGTTCGAACC	AAGGAGTGTGCGAAAAGTGC
WRC1453	GCTTGAGCATATTTACTTGGAGCA	GCTTGAGCATATTTACTTGGAGCG	GCCATGTTTGTGTCTTAAGCAGA
WRC1455	CTTAAAACCCCACTAAATTGGCC	CTTAAAACCCCACTAAATTGGCT	CATGCCAAGTGTCGCATCTC
WRC1460	AGGGTCGGACCAGTTCTAAA	AGGGTCGGACCAGTTCTAAC	GGGAGTGGTGAACTTCGACC
WRC1461	CGAACGACTCCCAGTAACTCTA	CGAACGACTCCCAGTAACTCTG	TGTGTGAGTAATAGTGAGGCCA
WRC1464	ACTTGAACATGTGTAGCGGT	ACTTGAACATGTGTAGCGGC	TCCGAGTGTGATGAGGACAT
WRC1465	TCCACAAGCAAATGCCAGCT	TCCACAAGCAAATGCCAGCA	GTGCATCCGCTCTCGTAGTT
WRC1472	ACATGTAGCAATGAAACCTCACT	ACATGTAGCAATGAAACCTCACC	TGTTGGCATCGAGGCTTCTT
WRC1477	GTGTTCAGAGTGATGTTGCCT	GTGTTCAGAGTGATGTTGCCA	AGGCAAACTAGGCAAACAGC
WRC1482	GCACGTGCGAGAAATGCA	GCACGTGCGAGAAATGCG	ACAATGCCCACCAGGAATCA
WRC1487	GCGGGAGGAGGCCGATAA	GCGGGAGGAGGCCGATAG	GTGCCTACGCTATCTCTGCA
WRC1489	CCCAAGCATAGGGGAACTGT	CCCAAGCATAGGGGAACTGC	TCGAGGGAGTGGCTTTACCT
WRC1491	TGGATTTCTTTGCTCTCCCTTTC	TGGATTTCTTTGCTCTCCCTTTG	TTGCAGCATCGAGGTTGTCT
WRC1493	GCTCACCCAGCCATACACT	GCTCACCCAGCCATACACA	GGCTCTCTACAATGACGCTGA
WRC1499	TCAAAGAGCGGCAGAGAACC	TCAAAGAGCGGCAGAGAACA	GGAAGAGCTCTGAACGGCAT
WRC1507	GGATCCGCTCTCTTTCCTTTTC	GGATCCGCTCTCTTTCCTTTTT	CCCTAGTTTTCAAGAGAATTGTGTG
WRC1508	CCCGTCGTCGAAAGCCTA	CCCGTCGTCGAAAGCCTG	CGGGATGCCCAGATATGTGA
WRC1514	TTATTTGCCCCCACTCCACC	TTATTTGCCCCCACTCCACA	ACCATGGGAATCCAGGAGGA
WRC1515	GCCCCCTCTCCTTCAACCTT	GCCCCCTCTCCTTCAACCTC	TTTCTAGCCGAGGATGCTCC
WRC1519	TTGCGAAGGAGATCGATGGT	TTGCGAAGGAGATCGATGGC	TGTAACCCAGCCTGCTTCAG
WRC1526	ACTATTGGTAGGTAGGCGGA	ACTATTGGTAGGTAGGCGGG	CTCATCAATAGCCACCCCCA

WRC1530	GTGGAGATCCTGGACACCAT	GTGGAGATCCTGGACACCAC	GCAGCTTTTGGGCTTCTCAC
WRC1532	GTTGGAATCTCCTGGCAGGA	GTTGGAATCTCCTGGCAGGG	TGCTGCTGTTTCACTGATGT
WRC1534	TGTGAAACTGGGCTTGAAGT	TGTGAAACTGGGCTTGAAGC	TTGGCGCAATGAACAAGTGG
WRC1542	GCATGCATCGTCACCATTTCAC	GCATGCATCGTCACCATTTCAT	CGTTCATGGATTCAGCCTCCT
WRC1543	CATTGGATATCAATTCTGGTGACA	CATTGGATATCAATTCTGGTGACG	ACCAAGGATCGCATGTCCAA
WRC1545	CAGCATGCGACGAGAAATTC	CAGCATGCGACGAGAAATTT	TGTCTGCGTAAACTTGCCCT
WRC1547	CTGCGTCGTCTCATCGGA	CTGCGTCGTCTCATCGGG	CGCGAGAAGAACCACAAGATG
WRC1549	CCTCTCTTGTAAGCTCTTAACCCC	CCTCTCTTGTAAGCTCTTAACCCT	GAGCTGGAACCTGTGAGACA
WRC1556	GGCTAACGAACGCATCATAGATT	GGCTAACGAACGCATCATAGATC	ACCAGTCCCCAGTTACTCCA
WRC1580	TACAGATTCACGGCGTTTGT	TACAGATTCACGGCGTTTGC	CGCTGTTGGACGCTGAAAAT
WRC1582	ACCCAAGACATCAAATGCCAT	ACCCAAGACATCAAATGCCAG	CGGGGGTGGTAATCTGTCTC
WRC1586	ACAAAATCTTGTTTTCCTTACGGT	ACAAAATCTTGTTTTCCTTACGGG	AGAGACTGAATGGTTGCTGCT
WRC1590	CTTACTGGGAGCCGAGGAGT	CTTACTGGGAGCCGAGGAGC	GAGGCGTCGAGTCTTCTCAG
WRC1593	TGGGACAAAAAGGACACTGG	TGGGACAAAAAGGACACTGA	TGGCACAATGAAACTTGGGA
WRC1600	TCTTTGACATCGTGTTTTTGCG	TCTTTGACATCGTGTTTTTGCC	AGACTGATGACCACCACACG
WRC1602	GTATATGTCCCCTGAACCTAAATTG	GTATATGTCCCCTGAACCTAAATTA	GGCCTCCTGCAATCTGGTAT
WRC1607	TGCATGATCCATCCATTCCTCT	TGCATGATCCATCCATTCCTCC	CGGAGGAATGGAAAAGGAAGGA
WRC1624	AGAAACAATCCTGCCGCTGG	AGAAACAATCCTGCCGCTGA	CGGGGGAACTGTGAAGAACA
WRC1632	CGGCGATTGGAGGCTTCTTT	CGGCGATTGGAGGCTTCTTG	TCCGAGAAGCAACACCCAC
WRC1633	TGACCATGGCTAATGTCTTCA	TGACCATGGCTAATGTCTTCC	TCATCCAAGATTGCGTTCCG
WRC1647	TTAAGTGGATCAGCAGAGTTGC	TTAAGTGGATCAGCAGAGTTGT	TGGGGCAACTGCTATATTTAACA
WRC1661	CCATCTTGGCTTCCGTCCAA	CCATCTTGGCTTCCGTCCAG	GGGGAGCAAACAATCTGACC
WRC1667	ACCAAAACCTCTAGTTGCAAGAG	ACCAAAACCTCTAGTTGCAAGAA	TCTGGTGTGGGCTTCTGAAT
WRC1670	GTTAGCGGTCAGGTCACTCC	GTTAGCGGTCAGGTCACTCT	CCTTGGTTAACGCCAGTGTG
WRC1677	CCAAACCGGCCACTGAGG	CCAAACCGGCCACTGAGA	TGCAGAGAAGCCTCGTCTAC
WRC1684	CCCCACAAGGCCACAACT	CCCCACAAGGCCACAACC	AGCTCAAACCACTGCTCCTC
WRC1689	AAATGAACTACCATCGACACCT	AAATGAACTACCATCGACACCA	TGTGGGCTTCACTTTTGCTT
WRC1696	GTTCGGACGGTGGTACTACTT	GTTCGGACGGTGGTACTACTG	TGTTCATTGCTTGCAACGGC
WRC1703	TTGACGTCCCTGAATGCTTT	TTGACGTCCCTGAATGCTTG	CCATCGCACCGTAGAATGGT
WRC1708	GAGTCCCCGCCATTCCTTAG	GAGTCCCCGCCATTCCTTAA	CGTGGCTCACTCGTTTCCTA

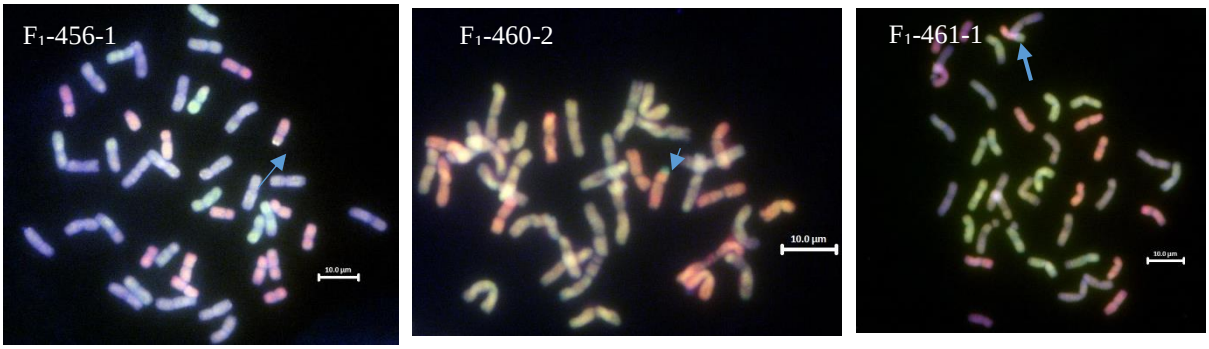
WRC1711	AAACAGCTAACTTGCTCCCT	AAACAGCTAACTTGCTCCCC	GCACTCTCTGGAAGTCCTGA
WRC1855	CATTTCATTTCATTGAAGCACTGTT	CATTTCATTTCATTGAAGCACTGTG	GCACCTGCAGAAAAGCACAG
WRC1857	TGCGTTAATCCATTCTGATGTATGT	TGCGTTAATCCATTCTGATGTATGG	CCACTGCAGACAACACTGGA
WRC1861	CATCGGTTGATGTGCACAA	CATCGGTTGATGTGCACAG	GCCGATTTGTTCAGCATCGA
WRC1871	GGAGTCGTCGGATCGGGAC	GGAGTCGTCGGATCGGGAA	CATCGGGAGCGGTCATTCC
WRC1875	CTTGGCTGTCTCGGTGCA	CTTGGCTGTCTCGGTGCC	AGAATGAACGCGTGGCTGTA
WRC1899	TCTCAAAGGCATCACCGTCA	TCTCAAAGGCATCACCGTCG	AAGGTGGTGATAGCCGTGC
WRC2034	TGAATGCATTGGTTACGCAGA	TGAATGCATTGGTTACGCAGG	CCTACAACCTTATGTCATTTGGGT
WRC2035	GCCTGATCAGTAGTCGATGAGTAT	GCCTGATCAGTAGTCGATGAGTAG	AAGGGGAGACATCACGTCCT
WRC2048	CGGCAGCCCATTTTCTCCA	CGGCAGCCCATTTTCTCCG	ATTTTTGTCACGTCAGGGCG
WRC2051	GCGCCAGTGGGAGATGCC	GCGCCAGTGGGAGATGCT	CGGAGCAGCAGACACTGAAT
WRC2059	CGTCATCTGCCTCCTCATCA	CGTCATCTGCCTCCTCATCG	ACTTTGACCAGCTCAGCCTC
WRC2061	GGGAAAGGGGACTGGGGA	GGGAAAGGGGACTGGGGG	CACGCTCCCCGGCCTATC
WRC2068	CGGATCCTAAATCCCGTCACT	CGGATCCTAAATCCCGTCACC	GTGTAGTCATCGGGCCTTGG
WRC2071	GGTTCGGATTTTGGTTGCTAGT	GGTTCGGATTTTGGTTGCTAGC	AGCACTCAGTTCGTCTCGTT
WRC2094	CAACCACGCACACCTCCA	CAACCACGCACACCTCCG	GACTGCGCTGAAGCTGTTAC
WRC2095	GATGCTATGATCCCCGTCCC	GATGCTATGATCCCCGTCCG	AAATAAACGGCTGGCCCAAC
WRC2096	ATCACCTGGAGGCAGGGT	ATCACCTGGAGGCAGGGC	GAGGGATGAACGCGGACTAC
WRC2100	GATTGCTTATATTTCCCTATCACCT	GATTGCTTATATTTCCCTATCACCC	GATCGTCATTGTTGCCTCGC
WRC2101	GTGCTGGCGAGTTGAGGG	GTGCTGGCGAGTTGAGGC	CTGTCCACATACATGATGGCT

10.3 Appendix 3: GISH pictures of the recurrent parents, selected Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* F_1, BC_1 and BC_1F_1 metaphase spreads

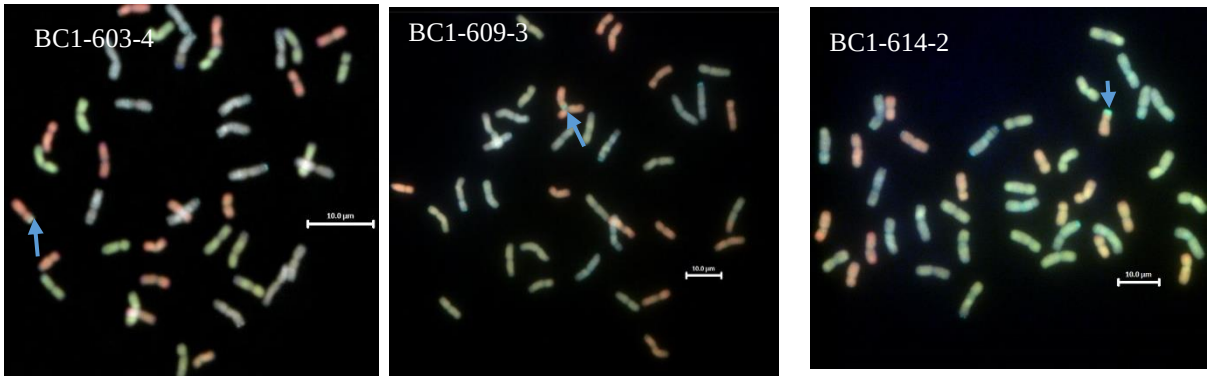
Recurrent parents



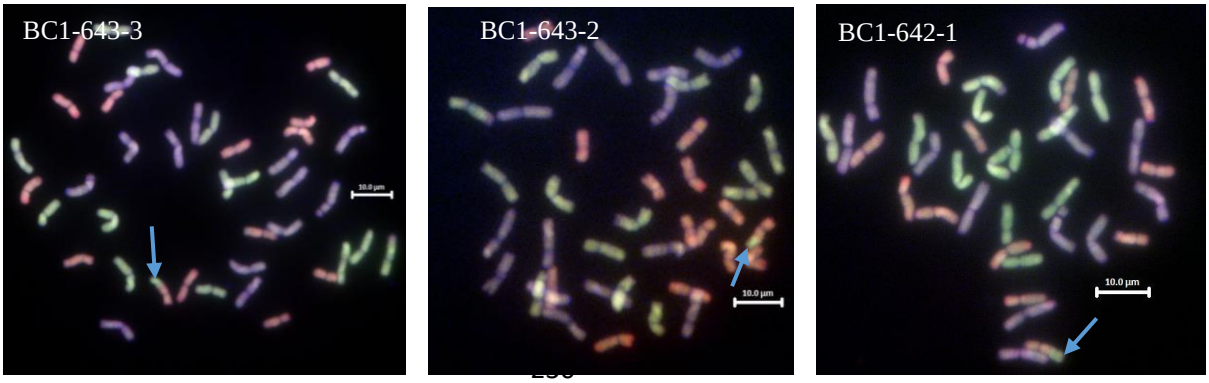
Malawian wheat/*Am. muticum* and Malawian wheat/*T. urartu* F_1



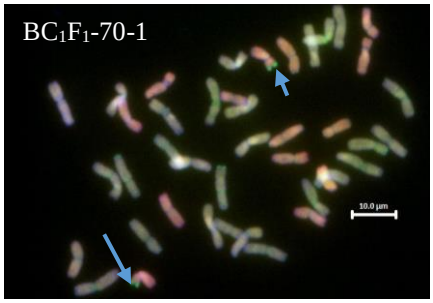
Malawian wheat/*Am. muticum* BC_1

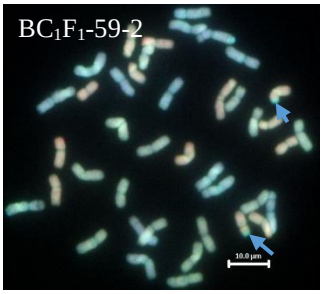


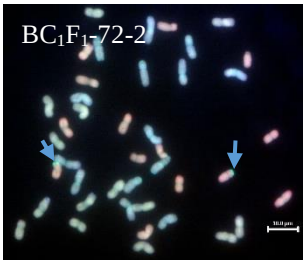
Malawian wheat/*T. urartu* BC_1

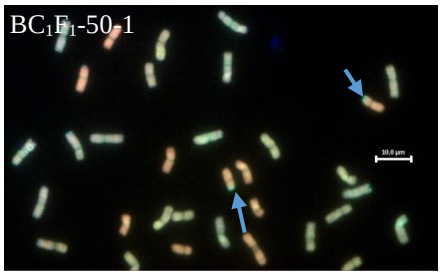


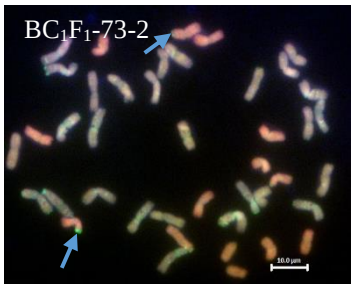
Malawian wheat/*Am. muticum* BC_1F_1

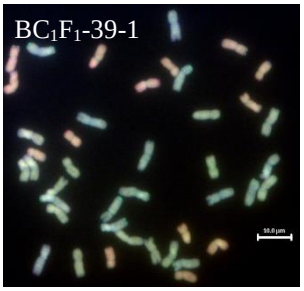


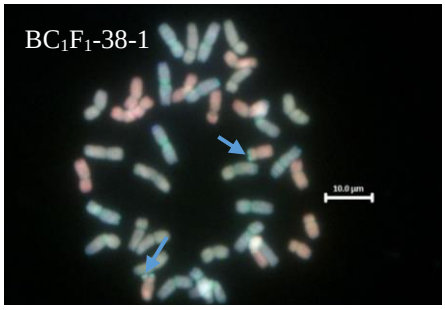


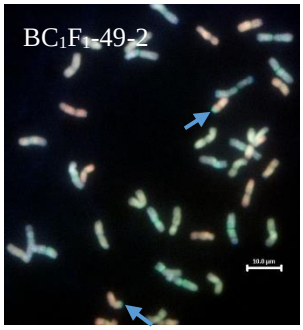


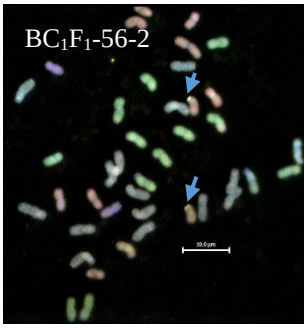




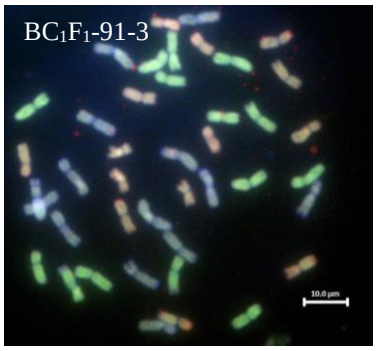


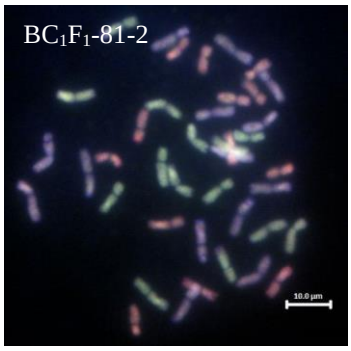


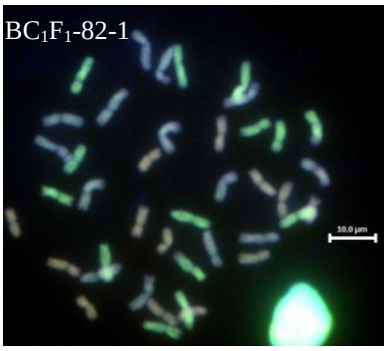




Malawian wheat/*T. urartu* BC_1F_1







10.4 Appendix 4 (for published paper): Farmer, bakeries, biscuit manufacturers and *mandazi* sellers' questionnaires

Farmers questionnaire

My name is _____ I am engaged in wheat research with a goal of developing varieties that can help promote the country's wheat production. To justify our research efforts there is also a need to assess and understand the current challenges the industry that the industry is facing. We would like to know the wheat varieties you are growing, estimated cost of production and the challenges you face in wheat production. With your consent, this chat will take a maximum of 30 minutes.

Section A: Interviewee's details

Name: _____ Sex: _____

Village: _____ Age: _____

Section B: Wheat production

1) What varieties of wheat do you grow currently?
    1. Kenya nyati
    2. Kadzibonga
    3. Sepita
    4. Nduna
    5. Kanyale
    6. Others _____

2) Do you buy seeds every year or you use saved seeds

    We buy every year …….. ☐

    We use saved seeds…….. ☐

    If you buy seed, where do you buy? _____

3) What are some of the challenges to wheat production in your area (Rate the challenges according to their importance's)
    1. Lack of extension services _____
    2. Limited access to seeds _____
    3. Pest and diseases _____
    4. Lack of markets _____
    5. Others _____

4) What is the estimated annual cost of producing wheat on your farm (seed, fertiliser, ploughing, weeding, harvesting, threshing)

	Units/Quantity	Cost in MK
1. Land	_____	_____
2. Seed	_____	_____
3. Ploughing	_____	_____
4. Fertilizer	_____	_____
5. Weeding	_____	_____
6. Harvesting/ threshing	_____	_____
7. Others	_____	_____

5) Where do you sell your wheat grain

1. Local market
2. Vendors
3. Farmers
4. Admarc
5. Others _____

6) How much is the selling price per kilogram

_____

7) What are the other uses of wheat in your area

1. Bread
2. Samoosa
3. Biscuits
4. Mandasi
5. Scones
6. Others Nsima and thobwa

8) Additional Comments:

_____

Biscuit manufacturers' questionnaire

My name is _____ I am engaged in wheat research with a goal of developing wheat varieties that can help promote the country's wheat production. To justify our research efforts there is also a need to assess and understand the current challenges the industry that uses the wheat grain/processing/products face. We would like to have some current estimated costs/margins of wheat flour as we justify the need to promote local wheat production. With your consent, this chat will take a maximum of 30 minutes.

Company Name/ respondent……

1) What products do you make (List)

    1. High value biscuits
    2. Low value biscuits
    3. Others…… ……

2) What are the cost of producing a set of products (preferably high value biscuits)

    …… ……

    a. Cost of flour (50 kg bag/per tonne)
        …… ……
    b. How many products are made from a tonne/50kgs of flour
        …… ……
    c. Transportation costs of flour
        …… ……
    d. Costs of ingredients used by product

Ingredient	Cost/Price
Flour	……
Yeast/soda	……
Salt/Sugar	……
Cooking oil	……
Others	……

    e. Operation costs/administrative cost
        …… ……

3) Give a list of your distribution channels

    _____

    _____

    _____

    _____

    _____

4) Costs of transporting products

5) Selling price (wholesale and retail)

4a).Wholesale……
4b).Retail……..

Commercial bakery and local bakeries questionnaire

My name is _____ I am engaged in wheat research with a goal of developing wheat varieties that can help promote the country's wheat production. To justify our research efforts there is also a need to assess and understand the current challenges the industry that uses the wheat grain/processing/products face. We would like to have some current estimated costs/margins of wheat flour as we justify the need to promote local wheat production. With your consent, this chat will take a maximum of 30 minutes.

Company Name/ respondent……

6) What products do you make (List)

1. Bread
2. Mandasi
3. Scones
4. Biscuits
5. Chapati
6. Others…… ……

7) What are the cost of producing 1 product (preferably bread)

…… ……

f. Cost of flour (50 kg bag/per tonne)

……

g. How many products are made from a tonne/50kgs of flour …… …….

h. Transportation costs of flour …… ……

i. Costs of all ingredients by product)

Ingredient	Cost/Price
Flour	……
Yeast/soda	……
Salt/Sugar	……
Cooking oil	……
Others	……

Operation costs/administrative cost

……
……

8) A list of distribution channels

_____

_____

_____

_____

_____

9) Selling price (wholesale and retail)

4a).Wholesale……
……

4b).Retail……
……

***Mandazi*/ *madonasi*/samoosa sellers questionnaire**

My name is _____ I am engaged in wheat research with a goal of developing wheat varieties that can help promote the country's wheat production. To justify our research efforts there is also a need to assess and understand the current challenges the industry that uses the wheat grain/processing/products face. We would like to have some current estimated costs/margins of wheat flour as we justify the need to promote local wheat production. I will need 20 minutes of your time to ask you the following questions.

Section A: Interviewee's details

Name: _____ Sex: _____

Village: _____ Age: _____

Section B: Estimating profit margins

10) What products do you make (List)

7. Mandazi
8. Others (mention)……..

11) What are the cost of producing mandazi)

j. Costs of ingredients by product

Ingredient	Cost/Price
Flour	……
Yeast/baking soda	……..
Salt/Sugar	……..
Cooking oil	……
Firewood/electricity	……..

12) Where do you sell your products

1. Roadside
2. Market
3. Door to door
4. Others

(mention)…….

13) Costs of transporting products (if any)……

14) Selling price ……..