Initiation of a wheat pre-breeding effort aimed at yield improvement using male-sterility marker assisted recurrent selection

by

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Declaration

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Abstract

Continous decline in South Africa's wheat production over the past few decades has lead to a corresponding rise in reliance on wheat imports to satisfy the local consumption needs. Wheat breeders need to work faster to contribute towards increasing profitability and the attractiveness of the commodity. In response to this, a study was undertaken with the aim of identifying and assessing traits that confer yield in wheat whilst investigating the possibility of a technologically-advanced high throughput phenotyping platform for assessing traits.

The first objective was to assess traits by conducting a multilocation field yield trial and a detailed study of yield attributing traits in a controlled environment. The second objective was to initiate a male sterility marker assisted recurrent selection (MS-MARS) population based on the use of wheat quality markers as well as resistance gene markers to wheat rusts and Fusarium head blight while adding high-yield traits *via* single seed descent breeding. Thirdly, a pilot study was conducted to test if a high-throughput phenotyping platform, based on remote sensing imagery and Remotely Piloted Aircraft Systems (RPAS) technology could be established for automated data collection of agronomic traits.

Results of the yield trial showed highly significant ($p=0.0000$) differences between the three localities where the trial was planted as a result of drought conditions in the season. The top ten yielding entries across the three localities did not yield significantly different from each other (LSD (5%) 1.17 t/ha) and from this set, five entries were selected for the trait study. The top two entries overall were also the best two entries at the locality that was badly affected by drought, indicating yield stability. Grain yield was positively correlated ($r>0.75$) to biomass, grain number/spike, as well as tiller number and these correlations were highly significant ($p<0.0001$).

The male sterility gene was found to be stable in the population by segregating in the expected 1:1 (sterile: fertile) ratio in the multiple cycles that were conducted. Rust resistance gene frequencies were also successfully maintained during crosses, with no significant differences between cycles (chi-square test, $\alpha=5\%$). Out of the ninety genotypes used in the field trial, forty-four were identified as crossing parents to contribute high-yield alleles in the population, with additional *Fusarium* resistance gene donor lines. In the resulting progeny of the cross, the *Fhb*1 gene was found to have been successfully transferred to 10% of the plants.

Correlations from the RPAS phenotyping study were not satisfactory, but they were promising enough to warrant further investigation towards establishing a high throughput phenotyping platform.

Opsomming

Die voortdurende afname in Suid-Afrikaanse koringproduksie oor die afgelope paar dekades het gelei tot ‘n korresponderende toename in die afhanklikheid van koringinvoere ten einde plaaslike verbruikers behoftes te kan bevredig. Koringtelers het dus nodig om vinnger te werk ten einde by te dra tot winsgewendheid en aanloklikheid van die kommiditeit. In respons is ‘n studie onderneem wat ten doel het om eienskappe te indentifiseer en assesseer wat opbrengs in koring tot gevolg het terwyl daar ook gekyk word na gevorderde tegnologie vir ‘n hoë deurset fenotiperingsplatvorm.

Die eerste doelwit van die studie was om eienskappe te assesseer deur multilokaliteitsproewe te doen asook ‘n gedetaileerde studie in ‘n beheerde-omgewing. Die tweede doelwit was om ‘n manlike steriliteits gefassiliteerde merker bemiddelde herhalende seleksie populasie gebaseer op die gebruik van koring kwaliteitsmerkers sowel as weerstandsgene vir koringroes en Fusarium te inisieer. Terwyl hoë-opbrengs eienskappe deur enkelpitnageslagteling aangespreek is. Derdens is ‘n ondersoek aangepak om te bepaal of ‘n hoë deurset fenotiperingsplatvorm gebaseer op afstandswaarneming en hommeltuig tegnologie gevestig kon word vir outomatiese data kolleksie van agronomiese kenmerke.

Die resultate van die opbrengsproef het statisties betekenisvolle verskille (p=0.0000) uitgewys tusen die drie lokaliteit waar die proef geplant was as gevolg van droogte kondisies tydens die seisoen. Die tien beste inskrywings vir opbrengs oor die drie lokaliteite het nie statisties betekenisvol verskil nie (KBV (5%) 1.17 t/ha) en vanuit die is vyf inskrywings geselekteer vir die verdere bestudering van kenmerke gekoppel aan opbrengs. Die twee beste inskrywings oor al die lokaliteite was ook die beste in die droogste lokaliteit wat opbrengs stabiliteit aandui. Graanopbrengs was positief gekorreleer (r>0.75) met biomassa, korrels/aar, getal are en die korrelasie was boonop almal statisties betekenisvol (p<0.0001).

Die manlike-steriliteitsgeen was bevind as stabiel in die segregerende populasie en het voldoen aan die 1:1 (sterile:vrugbaar) ratio oor ‘n aantal siklusse heen. Die roesweerstandsgeen frekwensies was ook suksesvol behou met geen statisties betekenisvol verskille oor siklusse Chi-kwadraattoets, α=5%). Uit die negentig genotipes gebruik in die veldproef is vier-en-veertig geidentifiseer as kruisingsouers wat hoë opbrengs allele sowel as addisionele Fusarium weerstandsgene kan bydra. In die nageslag van die kruising is die *Fhb*1-geen suksesvol oorgedra in 10% van die plante.

Korrelasies van die hommeltuig fenotiperingstudie was nie bevredigend nie, maar was des nie teenstaande nie goed genoeg om verdere oorweging te regverdig.

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List of Abbreviations

%	Percent
%w/v	Percent of weight of solution in the total volume of solution
°C	Celsius Degrees
µg/kg	Microgram per kilogram
µl	Microlitre
µM	Micro-Molar
μMs^{-1}	Micro-Molar per kcat
A	Adenine
ABA	Abscisic acid
ADON	Acetyl-Deoxynivalenol
AFLP	Amplified fragment length polymorphism
ANOVA	Analyses of variance
APR	Adult plant resistance
APS	Ammonium persulfate
APX	Ascorbate peroxidase
ARC	Agricultural Research Council
ARC-SGI	Agricultural Research Council-Small Grain Institute
B.C./BC	Before Christ
BAC	Bacterial artificial chromosome
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
bp	Base pair
C	Cytosine
CAPS	Cleaved amplified polymorphic sequences
CAT	Catalase
CEC	Crop Estimates Committee of South Africa
CHAs	Chemical hybridizing agents

cm	Centimetres
cM	Centi-Morgan
CMS	Cytoplasmic male sterility
CTAB	Cetyl trimethylammonium bromide
CV	Coeffcient of variation
DArT	Diversity array technology marker
DF	Degrees of freedom
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DTH	Days to heading
E.C.	Electrical conductivity
e.g.	*exempli gratia* (for example)
EDTA	Ethylenediamine tetracetic acid
EMS	Ethyl methan sulfonate
EPP	Extractable Polymeric Proteins
et al.	*et alibi* (and elsewhere)
Et Br	Ethidium Bromide
FHB	*Fusarium* head blight
Fig.	Figure
G	Guanine
g/l	grams per litre
GA	Gibberellic acid
Gb	Giga bases
gDNA	Genomic DNA
GLM	Generalized linear model
GMO	Genetically modified organism
GMS	Genetic male sterility

GNDVI	Green Normalized Difference Vegetation Index
GPS	Global Positioning System
GRVI	Green-Red Ratio Vegetation Index
GS	Growth stage
GSH	Glutathione
Ha/ha	Hectare
HALE	High altitude-long endurance RPAS
HI	Harvest index
HKT	High-affinity potassium transporters
HLM	Hectolitre mass
HMW	High-molecular-weight glutenins
hr/s	Hour/s
HSP	Heat shock protein
HTP	High-throughput phenotyping
Hz	Hertz
i.e.	*id est* (that is)
IPM	Intergrated pest management
kg	kilogram
kg/hl	Kilogram per hectolitre
km	kilometre
L	Litres
LAI	Leaf Area Index
LAUG	Leaf area under greenness
LEA	Late embryogenesis abundant protein
LMW	Low-molecular-weight group proteins
Locs	Locations
LSD	Least significant differences
Ltd.	Limited

LTN	Leaf tip necrosis
m	metres
M	Molar
mA	Milliamperes
MALE	Medium altitude-long endurance RPAS
MAS	Marker assisted selection
Mb	Mega bases
MDA	Malondialdehyde
mg/kg	Milligram per kilogram
MIT	Massachusetts Institute of Technology
ml	Mililitre
MLFT	Multi-location field trial
mM	mili molar
MMW	Medium-molecular-weight gladiadins
MON	Moniliformin
MS-MARS	Male sterility marker assisted recurrent selection
MT	Metric Ton/s
Mw	Molecular weight
N	North cardinal point
NDVI	Normalized Difference Vegetation Index
ng/µl	nanogram/microlitre
NGRDI	Normalized Green-Red Ratio Difference Index
NIL	Near isogenic lines
NIR	Near Infra-red
nm	Nanometres
NNA	Nearest-neighbour analyses
NUE	Nitrogen use efficiency
PBC	Pseudo-black chaff

PCR	Polymerase chain reaction
PFT	Pore-forming toxin-like
pH	Power of hydrogen
ppm	Parts per million
PTY	Proprietary company
PUE	Phosphorus use efficiency
PVC	Polyvinyl chloride
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic (DNA marker)
RCBD	Randomized complete block design
RGB	Red, Green Blue (true colour)
RIL	Recombinant inbred line
RLFP	Restriction fragment length polymorphism (DNA marker)
ROS	Reactive oxygen species
RPAS	Remotely Piloted Aircraft Systems (Drone)
RPL	Remote pilots license
rpm	Revolutions per minute
RUE	Radiation use efficiency
RWA	Russian Wheat Aphid
S	South cardinal point
SA/RSA	Republic of South Africa
SACAA	South African Civil Aviation Authority
SADC	Southern African Development Community
SAGIS	South African Grain Information Service
SAGL	The Southern African Grain Laboratory
SCAR	Sequence characterized amplified region marker
SDS-PAGE/PAGE	Sodiumdodecyl sulphate-polyacrylamide gel electrophoresis/ Polyacrylamide gel electrophoresis

SE-HPLC	Size exclusion-high performance liquid chromatography
SG	Stay green (trait)
SNP	Single nucleotide polymorphism (DNA marker)
SOD	Superoxide dismutase
SPL	Spike length
SPL	Spike length
SSD	Single seed descent
SSR	Single sequence repeat (DNA marker) (Satellites)
STS	Sequence-tagged site (DNA marker)
SU-PBL	Stellenbosch University-Plant Breeding Laboratory
T	Thymine
t/ha	Tons per hectare
TBE	Tris/Borate/EDTA buffer
TEMED	Tetramethylyethylenediamine
TF	Transcription factors
TKW	Thousand-kernel weight
Tris-Cl	Tris base and HCl buffer
UAV/S	Unmanned Aerial Vehicle/System
UK	United Kingdom
UPP	Unextractable polymeric proteins
US/USA	United States of America
UV	Ultra violet
UVHP	Unextractable very high mw polymeric proteins
VLOS	Visual line of sight
V-PPase	Vacuolar H^+-translocating pyrophosphate
VTOL	Vertical take-off and landing
VVI	Visible Vegetation Index
w/v	Percent weight/volume

WSC	Water soluble carbohydrates
WUE	Water use efficiency
Yp	Yield potential
YRTs	Yield related traits
ZEA	Zearalenones

List of species names and abbreviations

A. cristatum	*Agropyron cristatum* (L.) Gaertn.
A. elongatum	*Agropyron elongatum* (Host) P.Beauv.
Ae. kotschyi	*Aegilops kotschyi* Boiss.
Ae. neglecta	*Aegilops neglecta* Req. ex Bertol.
Ae. peregrine	*Aegilops peregrina* (Hack.) Maire & Weiller.
Ae. sharonensis	*Aegilops sharonensis* Eig.
Ae. speltoides	*Aegilops speltoides* Tausch.
Ae. squarrosa	*Aegilops squarrosa* L.
Ae. tauschii	*Aegilops tauschii* Coss.
Ae. variabilis	*Aegilops variabilis* Eig.
Ae. ventricosa	*Aegilops ventricosa* Tausch.
F. avenaceum	*Fusarium avenaceum* (Corda: Fr.) Sacc.
F. culmorum	*Fusarium culmorum* (W.G. Smith) Sacc.
F. graminearum	*Fusarium graminearum* Schwabe.
F. langsethiae	*Fusarium langsethiae* Torp and Nirenberg.
F. poae	*Fusarium poae* (Peck) Wollenw.
F. sporotrichioides	*Fusarium sporotrichioides* Sherb.
G. avenacea	*Gibberella avenacea* R.J. Cook.
G. zeae	*Gibberella zeae (Schwein.) Petch.*
Pgt/P. graminis	*Puccinia graminis* f. sp*. tritici* Eriks. & E. Henn.
Pst/P. striiformis	*Puccinia striiformis* West. f. sp. *tritici* Eriks. & E. Henn.
Pt/P. triticina	*Puccinia triticina* Eriks.
T. aestivum L.	*Triticum aestivum* L.
T. dicoccoides	*Triticum dicoccoides* (Körn. ex Asch. & Graebn.) Schweinf.
T. dicoccum	*Triticum dicoccum* Schrank.
T. durum	*Triticum durum* Desf.

T. monococcum	*Triticum monococcum* L.
T. spelta	*Triticum spelta* L.
T. timopheevii	*Triticum timopheevii* Zhuk.
T. turgidum	*Triticum turgidum* L.
T. urartu	*Triticum urartu* Thumanjan ex Gandilyan.
T. ventricosum	*Triticum ventricosum* (Tausch) Ces., Pass. & Gibelli
T. zhukovskyi	*Triticum zhukovskyi* Menabde & Ericzjan.

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Chapter 1: Introduction

Mathematical projections predict that the human population will increase by a third to approximately 9.3 billion people by the year 2050. With an increase of this magnitude, less land will be available for agricultural production (Mahon *et al*., 2017). This increase together with the continuing trends in prosperity will also drive a bigger portion of the population away from agricultural production (Singh *et al*., 2016a). There is also an increased pressure on demand with crops such as maize being used for biofuel production (UNDESA, 2016). For agricultural production to be able to meet the food demand exerted by such a population size as forecasted, food production needs to be increased by between 60-110% of the current values (Pingali, 2012; Ray *et al.*, 2013).

Ramankutty *et al.* (2018) reported that in order for production to reach the required values, there needs to be a continuous and steady annual increase of crop production at a rate of 2.4% per globally. For a crop such as wheat which is part of the top four crops (with maize, rice and soybean) that collectively supply 43% of dietary energy and 40% daily protein supply globally, demand is projected to rise at a rate of 1.6% annually (Singh *et al.,* 2016a). This, therefore, means that wheat yield per hectare need to increase from the global average of 3 to 5 t/ha (Kummu *et al*., 2017). As the situation stands currently, grain yields are no longer improving in 24-39% of the key agro-ecological regions in the world resulting in a very low rate of yield increase at 0.9% for wheat (1.6% for maize, 1.0% for rice and 1.3% for soybean) (Ramankutty *et al.*, 2018).

In conjunction with the growing population, food security is threatened even further by the rising reality of climate change and the many negative effects accompanying it (Reynolds and Ortiz, 2010). Environmental changes associated with climate change such as rising temperatures, floods, droughts, desertification and weather extremes are expected to devastate agriculture, especially in the developing world (IPCC, 2009). The effects of climate change will be experienced more intensely in the developing regions of the world due to three main factors: (a) most of the projected population increases will take place in these areas, (b) major climate change events will take place in tropical and subtropical regions where developing regions are found, and (c) most of employment opportunities in these regions are connected to agriculture (Reynolds and Ortiz, 2010; UNDESA, 2016)

Climate change effects on the environment are likely to also result in increased pests and diseases which may lead to various epidemics across the globe (Singh *et al*., 2016a). Approximately 200 plant pests and diseases have been reported and a quarter of those are economically significant in agricultural production with losses due to biotic stresses generally

ranging between 13-18% globally (Nelson *et al*., 2017). The dangers of biotic stresses are that diseases can infect all plant organs, at different stages of growth and development as well as the possibility of multiple pests and/or diseases affecting the same plants simultaneously (Singh *et al.*, 2016a). Pests and diseases of economic importance in wheat include wheat rusts, powdery mildew, fusarium head blight, spot blotch, Hessian flies, suni bugs, and the recent geographical escape of wheat blast (Singh *et al.*, 2016a; Sadat and Choi, 2017).

Based on yield data collected globally between 1989 and 2008, predictions state that wheat yield will increase by anything between 4% at worst and 76% at best (at 90% confidence) by 2050. This increase range scenario only meets part of the demand and if the lower prediction is true many people will starve (Ramankutty *et al.*, 2018). Previous yield improvement successes came as a result of the incorporation of dwarfing genes which reduced the occurrence of lodging in wheat and resulted in high input responsive germplasm as part of the green revolution (Pingali, 2012). With yield gains having levelled off, plant breeders are faced with the new challenges of improving crop productivity at a fast enough pace to meet the rising demands. This requires multiple collaborations between the various disciplines of plant sciences. Additionally, available technology should also be effectively harnessed and incorporated into these programs which promises to simplify some of the tasks that need to be undertaken. Future genetic improvement successes in wheat genetic yield potential will be better achieved *via* selection based on physiological attributes rather than empirical selection methods which have been successful in the past (Ramankutty *et al.*, 2018).

Wheat production area in South Africa has been steadily declining for almost three decades from just over 1.5 million ha to the current area of just below 500 000 ha estimated to be planted in the 2018 planting season (Grain SA, 2018). Steady yield increases due to crop improvement and an increase in production under irrigation over the same time period have not been enough to off-set the effects of this decline in area planted (Jankielsohn and Miles, 2017). This has lead to a continuous rise in the country's reliance on imports to satisfy wheat demand for local consumption (SAGL, 2017). The continuous decline in area production planted is due to producers losing interest in the crop due to low profitability. Fields where wheat had been previously grown are now planted with other economically important crops such as maize (*Zea mays* L.) and soybean (*Glycine max* L.) as the country has limited land and water resources for expansion of the crop production area (Sosibo *et al*., 2017). Therefore, it is necessary for local plant breeding efforts to work towards understanding and improving grain yield to reduce this reliance on imports.

In response to this, a study was conducted to add onto the knowledge basket towards improving wheat productivitity in South Africa. The aim of this study was to identify and assess traits that have been reported to confer yield in wheat whilst investigating the possibility of a

technologically-advanced high throughput phenotyping platform for assessment of traits. In order to achieve the aim, the following objectives were identified:

a) Assessment of yield in a high-yielding wheat population *via* a yield trial as well as a detailed study of the yield related traits (YRTs) of the population to identify crossing parents.

b) Initiation of a male sterility marker assisted recurrent selection (MS-MARS) population based on the use of wheat quality markers as well as resistance gene markers to wheat rusts and *Fusarium* head blight while adding high-yield traits *via* single seed descent breeding.

c) Investigating the possibility of a technologically-advanced high throughput phenotyping platform that is based on remote sensing imagery and remotely piloted aircraft systems (RPAS) technology.

Chapter 2: Literature review

2.1 Wheat

2.1.1 History and botany

The origin of wheat (*Triticum aestivum* L.) is not accurately known, but it is believed to have evolved from wild grasses in the Near East at a historical place known as the Fertile Crescent (Hirst, 2017). Wheat belongs to the *Triticum* genus of the Poaceae grass family, and the genus comprises of species that are diploid, tetraploid and hexaploid (Tiwari and Shoran, 2007). There are six species within the *Triticum* genus: the diploid *T. monococcum* or *T. urartu* with AA genome; the tetraploid species *T. turgidum* and *T. timopheevii* which have the AABB and AAGG genomes respectively; and the hexaploid *T. aestivum* and *T. zhukovskyi* with AABBDD and AAAAGG genomes respectively (Allen *et al.*, 2017). From the six species, the two hexaploid species are both cultivated forms, *T. urartu* is a wild species exclusively and the other three (*T. monococcum*, *T. turgidum* and *T. timopheevii*) have both wild and cultivated varieties (Allen *et al.*, 2017).

Diploid wheat together with barley are believed to have been the original species that gave rise to what would later be known as agriculture with archaeological evidence of wheat grains dating back to 15 000 BC (Ferrante *et al.*, 2017). Diploid wheat has 14 chromosomes in each of its subgenomes (i.e. A, B and D) with a complete chromosome number of 42 in its genome. The wheat genome is quite big at a size of 17 000 mega bases (Mb) which is huge when compared to maize and rice which have genome sizes of 2 500 Mb and 430 Mb respectively (Smit, 2013; Seda, 2017). Zimin *et* al. (2017) recently published the most complete wheat genome sequence which is is just above 15 Gb and covers approximately 96% of the overall wheat genome.

This large and intricate genome of wheat has resulted to it being adapted to a wide array of agro-ecological regions between 60°N and 44°S of the equator (Singh *et al*., 2011; Slafer, 2012). These regions have contrasting environments including dry, humid, high altitude to low altitudes (from sea level up to 3 000 m above sea level) as well as areas of high rainfall to irrigated areas or areas under dry-land cropping (Brenchley *et al.* 2012; Lucas, 2013).

A number of hybridization events took place that lead to the modern hexaploid species cultivated today (Figure 2.1). This began when the wild and diploid wheat *T. urartu* crossed with diploid goat grass, *Aegilops speltoides* giving it's A-genome while the latter contributed the B-genome resulting in tetraploid wild emmer wheat (*T. dicoccoides)* with the AABB genome. This was domesticated to cultivated tetraploid *T. dicoccum* (New Hall Mill, 2018).

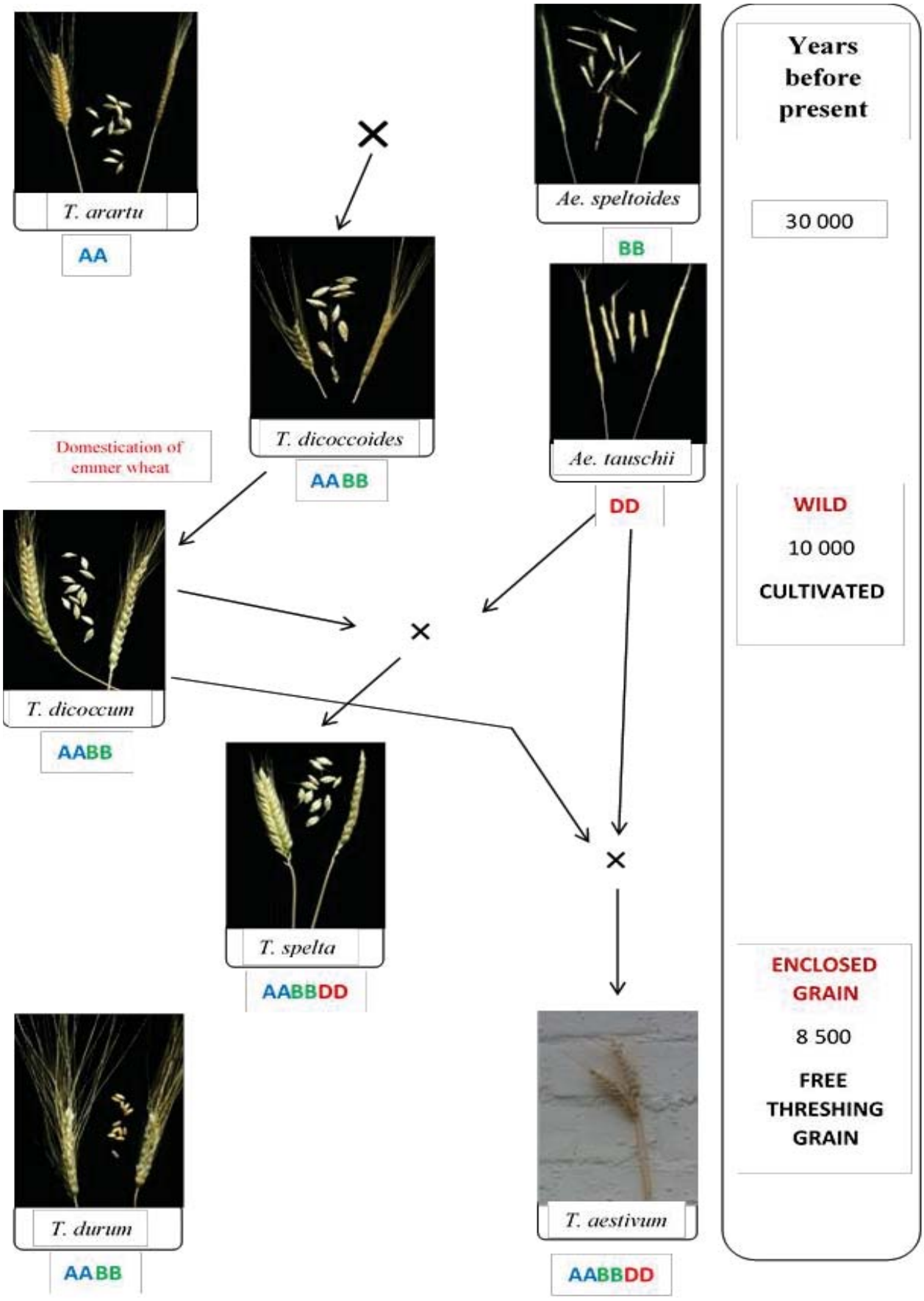

Figure 2.1 Bread wheat domestication process [Schematic presentation New Hall Mill (2018); Images contributed by author and Eversole *et al*. (2014)].

The domesticated emmer wheat (*T. dicoccum*) crossed with another diploid species of goat grass (*Ae. tauschii*) which contributed the D-genome and resulted in bread wheat (*T. aestivum*) (Peng *et al.,* 2011). The D-genome is said to be responsible for the improved tolerance to saline soil conditions in bread wheat when compared to durum wheat (*T. durum*) which is more sensitive (and lacks the D-genome). This is facilitated by lower concentrations of sodium as well as a lower sodium to potassium ratio in the leaves of bread wheat (Adu-Gyamfi, 2017). This whole domestication process took place over an extended period, beginning around 30 000 years ago from today until about 6 500 BC (Harlan, 1981; New Hall Mill, 2018).

Wheat is an annual rhizomatous grass with the above ground shoots producing culms (tillers), whose number per plant is determined by the plant genetics as well as the environmental aspects such as seeding depth, density of stand and other factors. These culms are cylindrical and can either be pithy or hollow with five to seven elongated solid nodes while the basal nodes are closely packed together and leaves develop at each of these nodes (Tiwari and Shoran, 2007). Depending on genotype and the environment, wheat can grow up to a maximum height of 1.2 m with tapering narrow leaves that are flat, extending to just under 40 cm. The cereal produces grains in spikelets with between two to five flowers in them (Du Plessis, 2010).

2.1.2 Wheat adaptation and development

A major factor that contributes to the wide adaptation of wheat to diverse agro-ecological regions is their response to photoperiodism and vernalization. Photoperiodism is plant response to the daylength in order for the plant to initiate flower development or reproductive growth (Slafer, 2012). Adu-Gyamfi (2017) reported that wheat's ability to reproduce at various latitudes came as a result of its very low sensitivity to photoperiod changes and as such it is able to flower even when the day-length is shorter than the ideal length. Although photoperiod sensitivity is genotype dependant, flowering will be hastened by increases in day-length yet the plants themselves will not need a specific day-length in order for flower induction to occur (Latha *et al*., 2017).

Vernalization is the requirement of a specific length of a cold period by certain plant species before any flowering may occur. Wheat adaptation to diverse environments is based on its response to cold temperature and day length to meet these vernalization and photoperiod needs, as well general temperature response of the plants to control the rate of flowering (Ochagavía *et al*., 2018). Genotype sensitivity to these two major factors is highly variable from those that are almost insensitive all the way to genotypes with quantitative (more common) or qualitative responses (Slafer, 2012).

The main driver for wheat adaptation to each environment is that development must be suited to the local environmental conditions, especially allowing anthesis or heading to occur under the best weather conditions without any risks of frost presence (Slafer, 2012). There is no vernalization requirement for seedling emergence in wheat and since photoperiod is received by plant leaves, these factors play no role in crop establishment (Evans, 1987; Slafer, 2012). Besides vernalization, temperature affects development rate before flower initiation as well as the rate at which leaves develop (Slafer, 2012).

In wheat, genotypes are separated into two groups due to their response to vernalization which are spring-type and winter-type wheat (Flood and Halloran, 1986). Spring-type wheat has a facultative need for vernilization (i.e. a shorter period of approximately five to 15 days under cool temperatures of 7°-18°C will induce flowering) and are more sensitive to photoperiod (Evans *et al*., 1975). These are sown in spring in areas where the winters are too cold for the wheat crop to survive or in areas with mild winters that aren't cold enough for winter-type wheat (Slafer, 2012). Winter-type wheats have an obligate need for cold weather in order for them to flower and will flower once they have been exposed to cold weather conditions ranging from 0°-7°C for a month or two (Evans *et al*., 1975). These are sown in areas with cold winters, but not extremely cold as to prevent crop growth and development and they evolved the need for vernilization more especially to avoid inflorescence initiation until winter has ended (Slafer, 2012). Winter-types, therefore, have a longer pre-anthesis period than spring-types. Sensitivity to photoperiod and/or vernalization is also reported to be the explanation for cultivars having diverse differences in their days to heading (Slafer, 2012).

Genes controlling plant response to these two factors are distributed in a number of chromosomes in the wheat genome. Photoperiod genes are found on the short arms of the homologous group 2 chromosomes, with the dominant alleles conferring insensitivity (Slafer, 2012). These genes are designated *Ppd-D1* (located on chromosome 2D), *Ppd-B1* (chromosome 2B), and *Ppd-A1* (chromosome 2A). The strongest effects are said to be from chromosome 2D (Worland and Law, 1985; Worland, 1999). Vernalization response genes on the other hand are found on the long arms of homologous group 5 and likewise with them, sensitivity is conferred by recessive alleles. These are designated *Vrn-A1* (chromosome 5A), *Vrn-B1* (chromosome 5B) and *Vrn-D1* (chromosome 5D) (Law *et al.,* 1975; Snape *et al.,* 2001). Winter wheat is recessive for all genes and spring-type wheat has combinations of dominant and recessive alleles resulting in some spring wheat cultivars responding to vernalization (Ochagavía *et al*., 2018).

Crop development is the continuous succession of morphological changes during which initiation and growth of various organs occurs at intervals to complete the crop's seed to seed cycle (Gonzalez-Navarro *et al.*, 2016). Grain crop development is separated into three main

component phases which are vegetative, reproductive and grain-filling. Wheat growth follows 10 developmental stages starting with germination, followed by above ground emergence, then tillering which is the last vegetative stage and this is when vernalization requirements need to be met. Reproductive stage commences once vernalization has been completed and begins with floral initiation or the double ridge stage, followed by the terminal spikelet, then stem elongation, booting, spike emergence, anthesis and finally physiological maturity (Adu-Gyamfi, 2017).

The time period between individual stages is dependent on the interaction between plant genotype, the environment it is grown in as well as the sowing date, but in general spring wheats will take about 140 days to reach maturity while winter wheat will take about 30 days longer for this (Stapper and Fischer, 1990). To keep track of growth and development there are a number of growth scales used, such as Zadoks scale, the Haun scale, The Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) scale (which is based on the Zadoks scale) and the Feekes scale. The Feekes scale is mostly used in the United States whilst the Zadoks and BBCH are common in Europe and the Zadoks scale is also commonly used in African countries such as South Africa (Wise *et al*., 2011).

2.1.3 Economic importance

Wheat domestication is postulated to have occurred in the Mesopotamian Fertile Crescent, and from there wheat moved and spread to the Middle East, North Africa, Asia and then Europe (Harlan, 1981). Today wheat is the most produced cereal in the world, and grown in over 200 million hectares of land across 120 countries with an estimated volume of around 759.6 million tonnes produced in the 2016/2017 production season (FAOSTAT, 2018) (Figure 2.2). The top wheat producing countries in the world are: The European Union (151.6 million MT), China (130 million MT), India (98.4 million MT), Russia (85 million MT), United States (47.4 million MT), and Canada (30 million million MT) (Index Mundi, 2018).

Wheat is a staple food crop for more than 35% of the world population, and is closely associated with food security at the global level as the primary source of protein and the second important source of energy (after rice) for human consumption (Tester and Langridge, 2010; Lucas, 2013). The human population is expected to reach 9.3 billion by the year 2050 and this increase will be accompanied by an increased demand of wheat supply of more than 45% to 2014's production values (Lucas, 2013). This increasing demand is worsened by the increasing temperature due to global warming which is projected to reduce wheat production in developing countries by as much as 29% (Rosegrant *et al*., 1995).

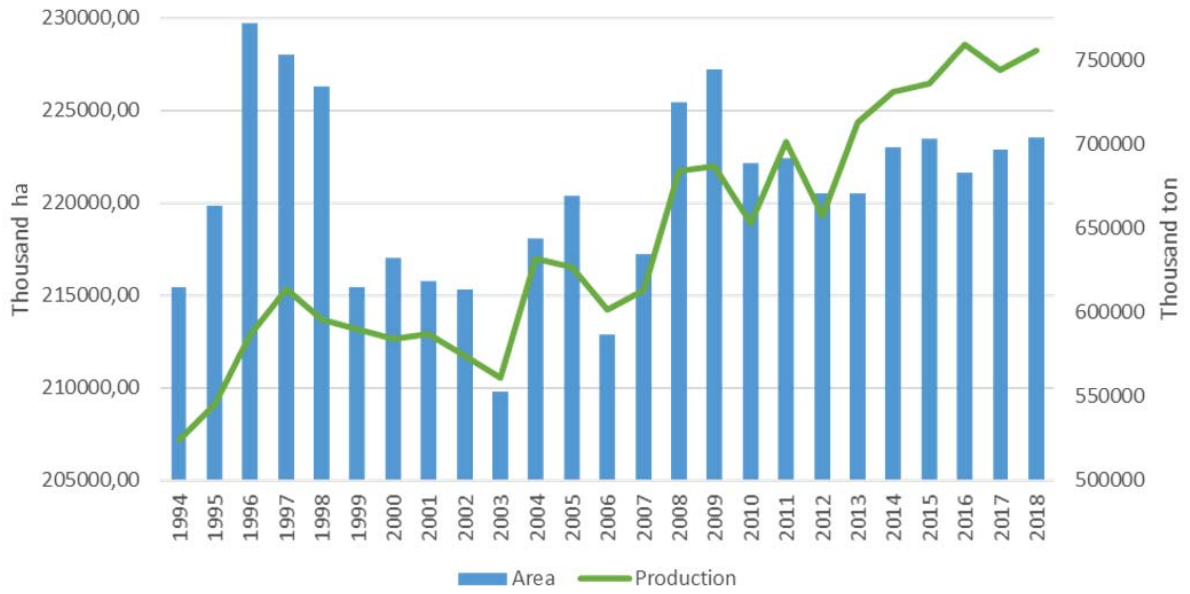

Figure 2.2 Global wheat production and area planted since 1994 (OECD.org, 2018).

There are about 21 African countries that produce a significant amount of wheat (i.e. greater than 1 000 MT), with over half of them in the sub-Saharan region (Index Mundi, 2018). The top five wheat producing countries in the continent are: Egypt (8.1 million MT), Morocco (6.25 million MT), Ethiopia (4.2 million MT), Algeria (2.4 million MT) and South Africa (1.48 million MT) contributing over 85% of the production in the continent (Index Mundi, 2018) (Figure 2.3). Productivity in sub-Saharan Africa is said to be only 10-25% of its biological potential in the region and much of the shortfall is due to lack of resources such as fertilizer in the region (Mohammed *et al.*, 2017).

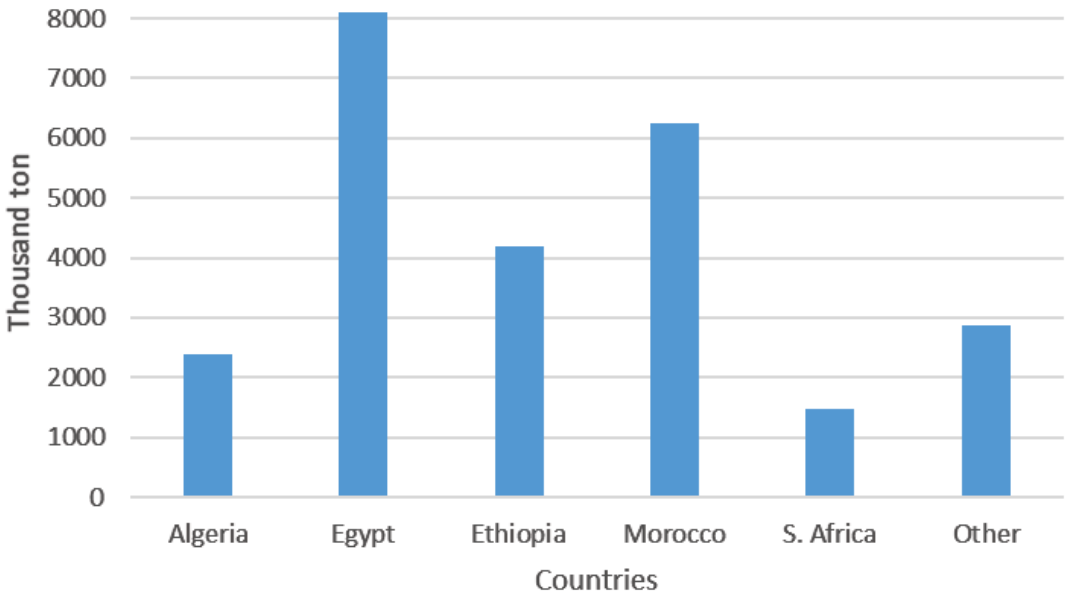

Figure 2.3 Wheat production share values in African countries (Created from information sourced from Index Mundi, 2018).

There are three main wheat-producing provinces in South Africa. Highest production is in the Western Cape (approximately 57% of total production) under winter rainfall, followed by the Free State (16%) grown under summer rainfall and the Northern Cape (14%) which uses irrigation to produce the crop. These three areas combined contribute more than 80% of the local wheat production, with the Western Cape producing approximately half of the overall country yield (CEC, 2016). Local wheat production area has been steadily declining (Figure 2.4) and currently only covers about 50% of the 3.5 million tons needed for domestic human consumption and as such, South Africa is a net importer of wheat (SAGL, 2017).

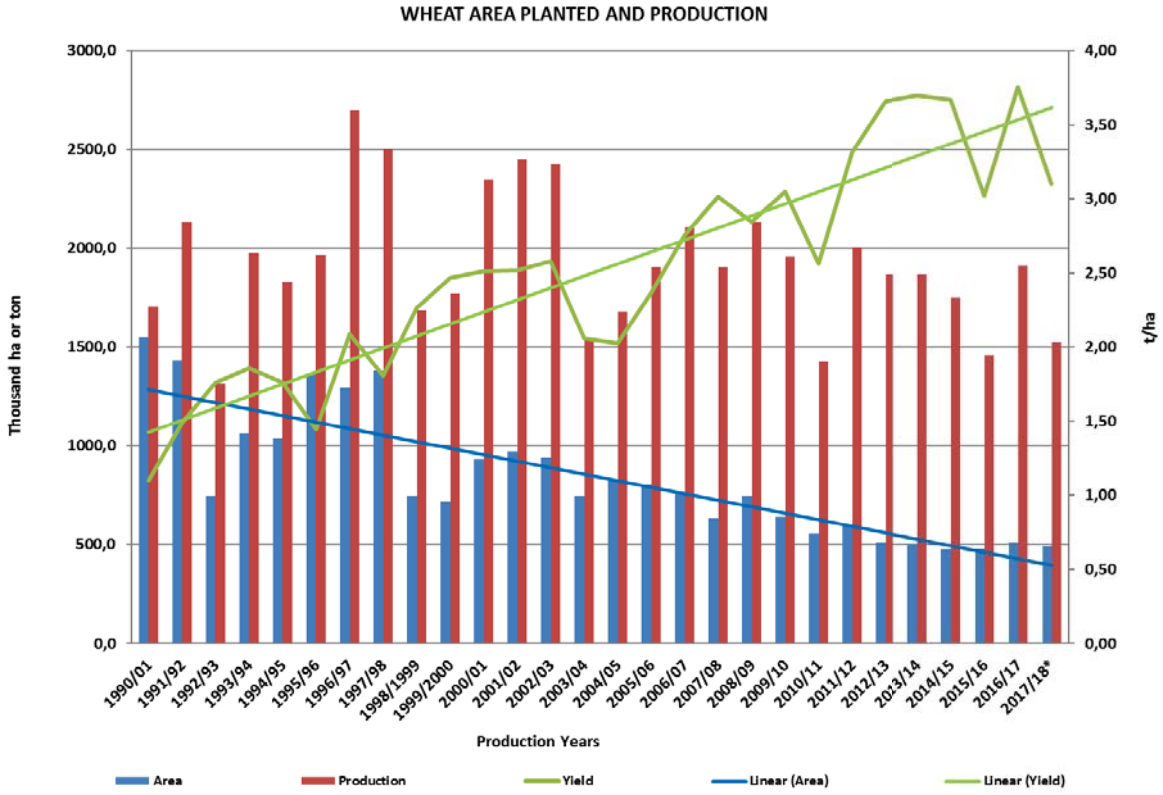

Figure 2.4 South African wheat industry outlook since 1990 (Grain SA, 2018).

2.2. Grain Yield

2.2.1. Introduction

Wheat is closely associated with food security at the global level and as such, its yield and the improvement thereof, as well as factors affecting it are of utmost importance. The current rate at which wheat is improving genetically is not adequate to meet the level of demand for the crop in the immediate to near future (Ray *et al*., 2013). Yield is the amount of crop harvested per unit of land area and it is of primary importance in breeding objective because it affects the grower's economic return as well as provide sustenance for populations (Sleper and Poehlman, 2006). Currently, there is a big gap between the actual realized yield attained by producers and the potential yield (Pradhan *et al.,* 2015).

As a quantitative trait, yield is controlled by a number of genes and its potential is expressed phenotypically through intricate morphological features and physiological functions (Sleper and Poehlman, 2006; Amelong *et al*., 2017). Genetic yield potential (Yp), therefore, is the maximum yield of a genotype grown under optimal conditions and in a conducive environment without any yield reducing stress (Gilliham *et al*., 2017). Yield gap on the other hand is the difference between the genetic yield potential of a genotype and the realized yield that the producer eventually obtains (van Ittersum *et al*., 2013). The phenotypic expression of yield is affected by the environment (e.g. photoperiod, light quality, etc.) rather than the genotype and therefore generally has low heritability (Prieto *et al*., 2018).

Yield is polygenically inherited which gives rise to complexity in the number of genotypes within a population and from allelic, non-allelic and genotype by environment interactions (Da Costa-Andrade and Miranda Filho, 2008). It is subdivided into different components which, when combined, interact to give an estimate of the final production value (Sleper and Poehlman, 2006). Yield is partitioned into components because they are reasonably easy to measure and their interpretation is intuitive (Sadras and Slafer, 2012). In wheat, these components or yield-related traits are: number of tillers, number of spikes per plant, number of spikelets per spike, number of grains per spike and grain yield (Slafer, 2012; Wu *et al.*, 2012). These yield components affect the overall yield at different levels which gives rise to some form of “hierarchy” which stands as: tiller number > inflorescence number ≥ grains per inflorescence > kernel mass (Sadras and Slafer, 2012). On top of these yield components, plant yield is also affected by a number of other related traits such as plant architecture (Wu *et al.*, 2012).

It is not possible to increase the overall yield by improving all its components at the same time because as one of the components is increased, the others decline due to competition for available growth assimilates (Sleper and Poehlman, 2006). However, breeding has been successful in keeping a continuous increase in grain yield through the accumulation of favourable alleles (i.e., additive effects characteristic of quantitative traits) which confer enhanced performance, especially under stress conditions (Hutsch and Schubert, 2017). Breeders also select for genetic yield potential, which is measured by mass of the produce per unit area of land, as well as yield stability which is the ability of the plant genotype to produce up to its genetic potential in spite of an adverse environment (Sleper and Poehlman, 2006).

2.2.2. Historical trends in wheat yield

Since the inception of the 20th century until around the early-1990s, wheat production increased more than six-fold (Lo Valvo *et al*., 2018). These increases are reported to have initially been due to extensification of agriculture with increases of production areas from 90 million hectares in the early 1900s to about 230 million hectares ninety years later; with the area doubling in the first half of the century (Lo Valvo *et al*., 2018). When land started becoming scarce and expensive to acquire for agricultural purposes, science and technology took over in contributing to further increases in wheat production and yield.

Between the years 1966 and 1985, agriculture went through the “Green Revolution” which increased wheat (and rice) yields primarily *via* intensification with production area increasing by only 30% during this period (Pingali, 2012; Reynolds, 2012a). Yield increases in wheat were initially around 1.0% per annum across all regions, and it is reported that yields increased from 0.9-2.6 t/ha (Ramankutty *et al.*, 2018). During this period, especially in the 1970s, annual increases in food production were as high as 3%, production was at its highest and has since been steadily declining to about 1% increments (Agarwal and Narayan, 2015). Lo Valvo *et al.* (2018) further reported that yield increases during the green revolution were much higher and yield increased by 250% over a period of a mere 40 years.

The total gain in yield can be sub-divided into smaller factors that contributed to the overall increase whose influences is difficult to separate into individual contributions. These factors include improvement to the grain yield potential of germplasm, other genetic gains (such as disease resistance and reduced lodging due to dwarfing genes) and gains due to improvement of management practices (improved equipment, and increased use of fertilizers) (Reynolds, 2012a). Regarding genetic improvement of germplasm, Aisawi *et al*. (2015) reported that initial increases were due to increases other than grain yield potential such as increase of protein content, and tolerance to biotic and abiotic stresses.

Lo Valvo *et al.* (2018) reported that improvements in varieties in the past century were due to increases in biological yield by improving above ground biomass (dry matter) and harvest index with regard to dry matter partitioning. This increase in harvest index also resulted in greater grain production efficiency. Depending on the crop, harvest index is reported to have improved from around 20-30% going up to 40-50% during the green revolution (Reynolds, 2012a). Most of this accumulation is reported to take place in the brief and critical period of the weeks before anthesis, with an excess of 25% of assimilates allocated to the spikes of plants (Aisawi *et al.*, 2015).

Studies between older and modern cultivars found that in the improvement of wheat germplasm, there were no changes made on the net uptake of nitrogen from the soil by plants. However, the difference is that older cultivars assimilated their nitrogen in their straw while in modern cultivars it is stored in seed (Ferrante *et al.*, 2017). This was as a result of breeding selections based on the concept of “use efficiency” developed by crop physiologists, which impacted how plants utilize resources for growth and development. This concept not only focused on nitrogen use (nitrogen use efficiency or NUE), but also on water (WUE), radiation (RUE) and phosphorus (PUE) usage (Reynolds, 2012a).

It has also been reported that modern cultivars are selected for spikes with higher sink strength than the stems, as well as higher grain density per unit area when compared to the very early cultivars (Lo Valvo *et al*., 2018). A high positive correlation was also found between time and grain number per square metre as well grain number per spike (Lo Valvo *et al*., 2018). While these traits increased with increasing yield over time, individual grain weight was reduced in the process as a result of competition for available growth assimilates (Sleper and Poehlman, 2006). Another possible reason for the reduced grain weight is that with increased number of grain per spike, more seeds moved into positions of lower grain weight potential such as basal or apical spikelets or later-order-tiller spikes (Ferrante *et al.*, 2017).

In order to understand how grain number is determined by genotypes, one needs to separate grain yield into its physical components (such as number of spikes, and spikelet number), and also following the development of organs contributing to grain yield (e.g. number of fertile florets) (Wu *et al.*, 2012). Generally, grain number per spike is determined in the early stages of reproductive stages, the spikelet number is determined early in crop phenology and the number of grains per spikelet is set by the number of fertile florets (Ferrante *et al.*, 2017).

Gonzalez-Navarro *et al*. (2016) confirmed the same information and reported that tiller number was the first component to develop, followed by spikelet number per spike, then number of grains and finally the grain mass. Floret initiation is said to take place until they reach maximum around the booting stage, then a lot of them degenerate until a few remain to be fertile at anthesis due to the active growth of stems and spikes during this period (Slafer, 2012) (Figure 2.5). Therefore, in order to improve yield, breeders need to improve the number of fertile florets at anthesis in wheat spikelets (Aisawi *et al.*, 2015).

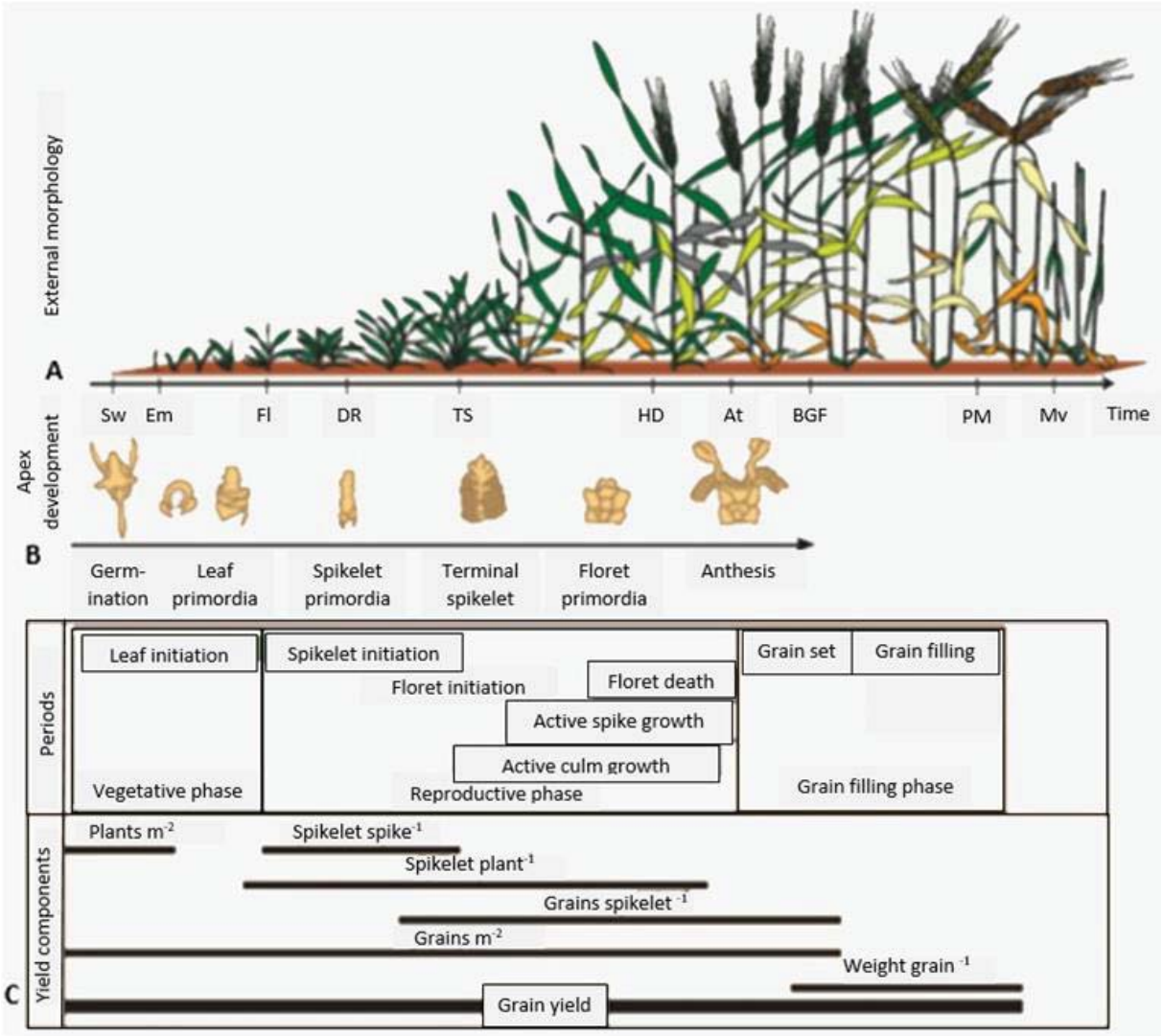

Figure 2.5 Wheat growth and development illustrating A: the various stages of sowing (Sw), emergence (Em), floral initiation (FI), first double ridge appearance (DR), terminal spikelet initiation (TS), heading (HD), anthesis (At), beginning of the grain-filling period (BGF), physiological maturity (PM) and harvest (Hv). B: Apex development in relation to the phenological stages. C: The periods of initiation and development of specific organs and those of when different grain yield components are produced in relation to the overall phonological cycle [Adapted by Reynolds *et al*. (2012a)]

2.2.3. Biotic challenges to wheat yield

The actual yield is affected by a number of factors which reduce it from what it could be as determined by the genotype's genetic potential (Chapagain and Good, 2015). These factors can be biotic and abiotic such as poor management practices. Other issues are due to socio-economic as well as political factors such as food distribution resulting in large scale food wastage predominantly in first world countries, and increasing costs of food (Gilliham *et al*., 2017). These challenges make it difficult for production to increase to meet the demands that a human population exceeding nine billion predicted to be present by mid-century. These challenges are more pronounced in developing countries because 1) most of their cereals are imported (Dixon *et al.,* 2009), 2) many of their local wheat breeding programs lack resources

to address shortages (Kosina *et al.,* 2007) and 3) they have the misfortune of being in climate vulnerable agro-ecological regions (e.g. in South Africa) (Lobell *et al.,* 2008).

One of the challenges with adapting crops to climate change is maintaining their resistance to biotic stresses since climate change will likely lead to new and unpredictable epidemiologies (Singh *et al*., 2016a). Pathogens reduce yield in a number of ways including reducing the amount of light intercepted by the plant as well as the crop stand, consuming plant tissues and assimilates (Newton, 2016). For diseases and their epidemics to occur, there need to be an interaction between the pathogen and excess amount of a susceptible host in a favourable environment (Gilbert and Tekauz, 2011). The physiological stage of the host at which infection occurs will have an effect on how much damage occurs to the host. Understanding the ecology, distribution and virulence of pathogens is essential in reducing their detrimental effects. For instance, obligate pathogens with high evolutionary rates do best in dense stand and high tiller density environments while airborne pathogens can migrate over long distances (Nelson *et al*., 2017).

2.2.3.1. Wheat rusts

Wheat is affected by a number of pest pathogens, from fungal and bacterial pathogens to viruses and insects (Duveiller *et al*., 2012). The fungus phylum *Basidiomycota* contains a genus *Puccinia,* which is comprised of about 4 000 species. Three species cause wheat rusts which are among the top ranking biotic stresses to the production of the crop (Smit, 2013; Figueroa *et al*., 2017). In South Africa, wheat rusts have made historical marks in the local production of the crop with the earliest recorded rust epidemic dating back to 1726 (Pretorius *et al.*, 2007). There are three species of wheat rust, namely, stem or black rust which is caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) (Eriks. and E. Henn), leaf or brown rust caused by *P. triticina* and stripe or yellow rust's causal organism is *P. striiformis* f. sp. *tritici* (*Pst*) (Figure 2.6) (Figueroa *et al*., 2017).

Puccinia species are obligate biotrophic parasites that evolve quickly and, therefore, have a number of races, arising from sexual recombination, mutation, or somatic hybridization (Figlan *et al.,* 2014). They also migrate easily globally which makes overcoming host resistance easy (Smit, 2013). In South Africa alone, at least 25 different races of stem rust and leaf rust have been identified over the past 30 years, with another further four races of stripe rust (Terefe, 2016). They are also heteroecious, meaning that they complete their lifecycle on two unrelated species of hosts (Lorrain *et al.*, 2017). The asexual stages of the pathogen generally occur on wheat and other species of the Poaceae family, while the sexual stage of the different rust pathogens occurs in different hosts (Bettgenhaeuser *et al*., 2014).

Pgt and *Pst* share a host for their sexual stage in barberry (*Berberis vulgaris*) and the host for *Pt*'s sexual stage is meadow rue (*Thalictrum speciosissium*) (Miedaner *et al*., 2016). Locally, the pathogens continually persist under asexual reproduction since none of their reported alternate hosts are found in South Africa (Figlan *et al.*, 2014).

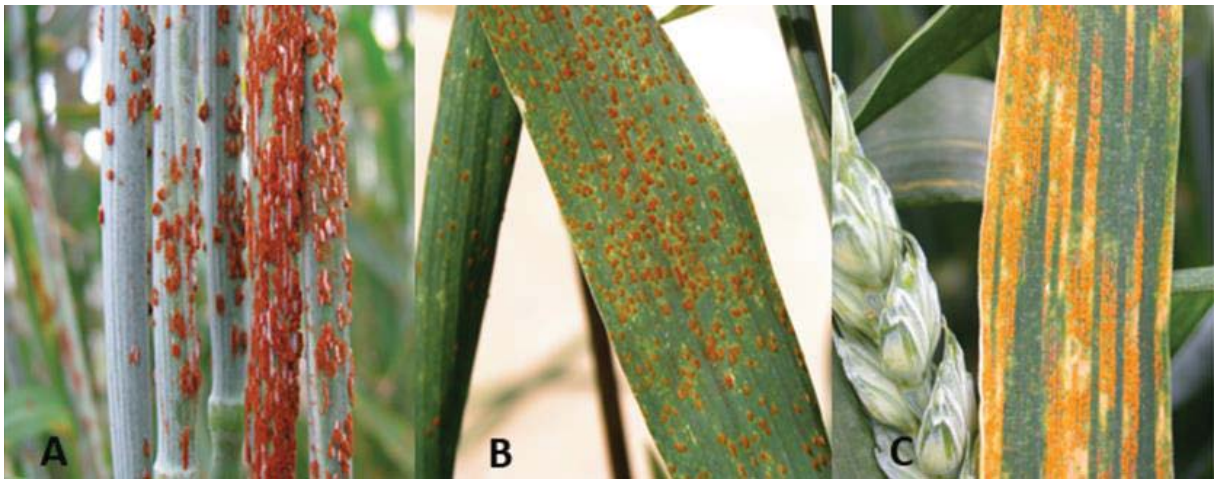

Figure 2.6 Distinct features of the three wheat rust diseases: A) Stem rust. B) Leaf rust. C) Stripe rust/ Yellow rust (CSIRO, 2013; GWA, 2016; LGSeeds, 2016).

Stem rust reduces yield by weakening the stems, thereby causing lodging and also disrupting the flow of nutrients within the infected stem, resulting in undernourished and shrivelled seeds with poor flour quality (Berlin *et al*., 2017). Stem rust can be a devastating disease depending on when the pathogen infects the host. It can result in crop damage from 35-100% of field stands, leading to it being the most feared wheat disease globally (Singh *et al*., 2011). This disease has historically been reported to be problematic all over Africa, in the Middle East, Australia, New Zealand, North and South America, and on most of the Asian continent (Liu *et al*., 2017).

The earliest local rust epidemic was due to stem rust in the Western Cape, and it is in this province as well as the Eastern Cape and Free State that the disease has caused numerous epidemics over the years with the most recent epidemic of note happening in 1985 in Albertinia, Western Cape (Pretorius *et al*. 2007). In South Africa, *Pgt* is a major disease of bread wheat (*Triticum aestivum* L.) but it has other primary hosts including durum wheat (*Triticum turgidum* var. *durum*), barley (*Hordeum vulgare*), triticale (X *Triticosecale Wittmark*), and wheat progenitors (Terefe *et al*., 2016).

A major historical event related to wheat rusts was the discovery of the stem rust pathotype *Ug99* in Uganda in 1999 which was the first race with virulence to the then widely used rye-derived resistance gene *Sr31* (Figlan *et al.*, 2014). The disease was subsequently reported to be found in Yemen, Iran in the Middle East as well as Kenya, Ethopia and Sudan in Africa (Nelson *et al.,* 2017). The pathogen is difficult to control in that it changes rapidly with at least

seven variants of *Ug99* reported, carrying different virulence genes to resistance genes such as *Sr24, Sr36* and others (Singh *et al.*, 2011).

This race group is virulent to over 90% of the commercial wheat varieties used globally (Chen *et al*., 2018) and Pretorius *et al.* (2012) reported 88% of local entries were susceptible to at least one of the *Ug99* races at seedling stage. Terefe and Pretorius (2014) reported that only about 5-10% of the bread wheat varieties grown in the 22 main wheat producing countries in Africa and Asia had acceptable resistance to *Ug*99 and that durum wheat has better resistance. *Ug*99 is difficult to contain due to its high migratory ability and was first detected in South Africa the following year after its initial detection in Uganda even though there is a distance of over 3 000 km between the two countries (Hodson, 2011).

Leaf rust caused by *P. triticina (Pt)* is also a significant pathogen of bread wheat around the world and also occurs frequently in most of South Africa's wheat growing regions (Terefe *et al.*, 2014). The disease results in yield losses through reduced quality in shrivelled seed like stem rust, and it also reduces floret setting (Smit, 2013). In South Africa, the pathogen is most commonly found in the winter rainfall production areas of Western Cape as well as areas under irrigation where it has caused a number of localised epidemics (Terefe *et al*., 2009). Terefe *et al.* (2014) reported that high incidences of the pathogen in the Western Cape are due to ideal weather conditions of the area during production. However, lower incidences of the disease were reported in 2008, which was a drier season than in 2009 and 2010. Leaf rust is adapted to a wide range of temperature conditions, from freezing temperatures of 2-35^{o}C with 15-25^{o}C being the optimum range (Smit, 2013).

Puccinia striiformis f. sp. *tritici* Eriks (or *Pst*), the causal organism for stripe rust, is more prevalent in wet high-altitude areas with cooler conditions than the two other rust pathogens with a temperature range of 0-23^{o}C and an optimal range of 9-15^{o}C (Smit, 2013; USDA, 2015). In South Africa, the disease is reported almost yearly in the production areas that have these conditions which are found in Western Cape, KwaZulu-Natal and the eastern parts of the Free State (Pretorius *et al*., 2007). *Pst* was first reported in South Africa in the Western Cape in 1996 (Pretorius *et al*., 2015). Early infestations on young plants results in underdeveloped plants, damaged tillers resulting in poor quality seed with low vigour and can result in 100% field damage in susceptible varieties (Pang *et al*., 2016). This pathogen is said to be as damaging as stem rust, but is even more lethal as it is the only rust pathogen that spreads through plant tissue beyond the infection site (Bux *et al*., 2012; Springfield, 2014). Stripe rust leads to losses in wheat yield by damaging plant respiratory systems and leaves, resulting in stunted plants with shriveled seeds (Bux *et al*., 2012).

2.2.3.2. Rust management and resistance

Reduction of primary inoculum by destroying the previous harvest's stubble, crop rotation with unrelated non-host crops such as legumes are reported to be the first steps towards control of rust diseases (Du Plessis, 2010). Farmers should also avoid growing uniform cultivars over extensive areas as this leads to epidemics if a new rust race rises which causes infection to the cultivar (Figlan *et al.,* 2014). Fungicides are also used but the financial cost associated with using them can get very high and they may have the potential detrimental effects to the environment with continued use (Figlan *et al.,* 2014).

The most effective tool against rust pathogens is the use of resistance genes which confer tolerance or resistance to commercial wheat varieties. In areas of extensive wheat production especially, only resistant varieties should be used (Dakouri *et al*., 2013). The use of major genes which confer race-specific resistance, where there is a gene-for-gene relationship between the resistance gene of the plant and a matching avirulent gene on the fungus, has been shown to be highly unreliable (Singh *et al.*, 2011). As previously stated, rust pathogens change quickly which results in new pathotypes that overcome that particular resistant gene on the host; leading to short-lived protection and a phenomenon known as the boom and bust cycle (Terefe *et al.*, 2016).

Several genes need to be combined to achieve non-race specific or durable resistance which results in medium to low infection levels (Figlan *et al.,* 2014). Durable resistance gives long-lasting protection to a cultivar which is extensively produced in an area that favours disease development (Brown, 2015). Breeders should not focus on using and relying on individual major genes but should instead select for numerous minor and slow-rusting genes which, when combined, result in best resistance against wheat rusts (Mundt, 2014). A number of resistance genes have been used successfully and are available for breeders' to use with over 58, 80 and 53 reported genes for stem-, leaf- and yellow rust, respectively (Zaman *et al.*, 2017).

The resistance genes and gene complexes are sourced from wheat relatives such as rye-derived *Sr31* (Liu *et al*., 2014a). There are some disadvantages to using wild species in that the genes may be linked to negative agronomic traits because of linkage drag (e.g. the *Sr39* gene from *Aegilops speltoides* linked to reduced flour quality) and this then requires extra work to break this drag and reduce the fragment size of the gene (Niu *et al*., 2011).

To assess the effectiveness of resistance in local cultivars as well as monitor the pathogen pathotypes found in the country, the Agricultural Research Council-Small Grain Institute (ARC-SGI) conducts annual surveys which have been mandatory since 1980 (Pretorius *et al*., 2007). At the time that these surveys were started, rust resistance wasn't the focus of breeding efforts

and as such a lot of varieties were susceptible resulting in frequent epidemics in the Western Cape and areas under irrigation around the country (Pretorius *et al*., 2007). These surveys also help breeders to know which resistance genes have been broken down by the pathogens, and which are still fit to be included in their programs (Figlan *et al.*, 2014). Mukoyi *et al.* (2011) suggested that breeding efforts should include a lot of collaboration between the neighbouring countries of South Africa, Zimbabwe and Mozambique because any new rust pathotype introductions in one of these countries results in adverse effects to the other two.

2.2.3.3. Fusarium head blight

Wheat is also affected by a few necrotrophic pathogens which are facultative parasites that live off dead plant matter (Singh *et al.,* 2016a). One of the most destructive of these is *Fusarium* head blight (FHB) (also called head scab or ear blight) and is caused by *Fusarium spp.* (Zhang *et al.*, 2011). There are almost 20 species capable of causing FHB on wheat but the most important of these globally is *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schwein.) Petch] which is also very common in nature alongside *F. culmorum* and *F. avenaceum* [teleomorph *G. avenacea*] (Zhang *et al.*, 2011; Singh *et al.,* 2016a). *F. graminearum* is more commonly found in areas with warm and humid climates; *F. culmorum*, *F. avenaceum*, *F. sporotrichioides* and *F. langsethiae* are more common in areas with cool and wet or humid climates and *F. poae* is problematic in warm and dry regions (Nicholson, 2013). *Fusarium spp.* can also cause other diseases such crown rot and root rot which are minor in comparison to FHB (Singh *et al.,* 2016a).

As a necrotroph, the pathogen survives on a wide range of plant species including those in agriculturally significant families including *Poaceae*, *Fabaceae*, *Solanaceae* and *Cucurbitaceae* and they are capable of infecting all parts of their host plant (Singh *et al.,* 2016a). FHB re-emerged as a major pathogen with severe epidemics in the last decade of the 20th century and has remained important with yield losses of between 10-70% (Buerstmayr *et al.*, 2012). Wheat is most susceptible to FHB between the stages of anthesis and soft dough especially under favourable environmental conditions for the pathogen (Zhang *et al.*, 2011). Early infection causes floret sterility, premature bleaching (Figure 2.6), reduced grain filling (shrivelled grain) resulting in yield and grain quality reduction, and under late infections, mycotoxin accumulation occurs (Buerstmayr *et al*., 2012; Singh *et al.,* 2016a).

Figure 2.7 *Fusarium* head blight of wheat on immature spikes (Science Image, 2015).

The *Fusarium* genus was described in 1809 and subsequent to that the association of FHB with mycotoxicosis (poisoning due to a fungal or bacterial toxin) in humans and animals was first reported in Russia in 1923 (Pitt and Miller, 2017). Mycotoxins are secondary metabolites produced by the fungal pathogen and they are classified into two major groups, namely; trichothecenes and zearalenones (ZEA) with a third minor group known as moniliformin (MON) (Gruber-Dorninger *et al*., 2017). *Fusarium* species produce over 20 trichothecene mycotoxins on various plant species including wheat and maize and the most common of which is deoxynivalenol (DON) which may be present on wheat without any visible symptoms (Stepień and Chelkowski, 2010). Mycotoxin content varies in wheat and this is dependent on the host genotype, weather conditions in the late grain fillings stages as well as the toxin production ability of the species because not all disease-causing *Fusarium* species are able to produce mycotoxins (Nicholson, 2013).

Small amounts (mg/kg) of trichothecenes such as DON can cause mycotoxicosis in animals when ingested as food or feed, especially monogastric animals. Mycotoxicosis leads to symptoms such as nausea, vomiting, fever, diarrhoea, and they have been shown to have associations with anaemia, immunosuppression and cancer (Zhang *et al.,* 2011). Mycotoxins are major threat because they can withstand high temperatures during baking without breaking down (Janssen *et al*., 2018). Two chemotypes of DON have been reported, 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), with the former producing nearly twice the mycotoxin content in infected grains than the latter (Ruan *et al*., 2012). DON has been shown to also play an important role in the spreading and development of the pathogen as it is also translocated through both the xylem and the phloem in wheat spikes thereby accumulating in the cells (Martin *et al.,* 2018).

Due to the danger to human and animal life, mycotoxin contamination reduces wheat marketability and as such many countries have regulations in place regarding DON content thresholds in wheat and its products (McMullen *et al*., 2012). Prat *et al.* (2014) reported that a maximum of 1.75 ppm for DON mycotoxins was allowed on unprocessed wheat (0.75 ppm for pasta) in the European Union while the US imposes a 1 ppm on finished wheat products to be fit for human consumption. In South Africa, the maximum allowable DON contamination is 2 ppm (2 000 µg/kg) and 1 ppm (1 000 µg/kg) for grain and processed wheat products respectively (Government Gazette, 2016).

Since *Fusarium* is a necrotrophic pathogen, the first line of defence in controlling the pathogen is the removal of debris in the field before planting and more importantly, rotations following rice or maize should be avoided (Zhang *et al.,* 2011). Seeds may be treated with dry heat (70^{o}C) for five days or with seed-treatment fungicide but the efficiency of the latter method depends on the germination temperature and the cultivar used (Gilbert and Tekauz, 1995; Gilbert *et al*., 2005).

Foliar fungicides applied at anthesis are not only effective against FHB, but DON accumulation as well, especially in areas known to be affected by the disease. Foliar fungicides should also be applied if weather forecasting around anthesis predicts favourable conditions for the pathogen (Gilbert and Tekauz, 2011). Biological control with *Trichoderma harzianum* Rifai (on wheat straw residues), *Clonostachys rosea* (Link : Fries.) Schroers, Samuels, Serfert and Gams (syn. *Gliocladium roseum* Bainier) and *Microdochium* have been reported (Gilbert and Tekauz, 2011; Nicholson, 2013).

Similar to wheat rusts and other diseases, the most effective and economic method of FHB and mycotoxin management is through breeding for resistance (Zhang *et al*., 2011). Tetraploid wheat such as durum is generally more susceptible to FHB than hexaploid wheat under field conditions (Ruan *et al*., 2012). This is due to the compact nature of the spikes which tends to retain anthers within and is related to faster disease development (Prat *et al*., 2014).

There are five types of resistance to *Fusarium* that have been reported in wheat. Type I resistance is resistance to initial or primary infection by the pathogen whilst Type II prevents the spread of the pathogen within the spike (Nicholson, 2013; Martin *et al.*, 2018). Type III resistance is resistance against mycotoxin accumulation, Type IV resistance is kernel infection resistance and Type V resistance reduces or limits yield losses (Zhang *et al.*, 2011; Nicholson, 2013). Types IV and V are hardly ever used in breeding programs because the mechanisms controlling them are not yet clear (Zhang *et al.*, 2011).

FHB resistance source germplasm are available that are used in breeding. These are: “Nobeokabouzu” (Japan); “Frontana” (Brazil); “Praa 8” and “Novokrumka” (Europe), and

"Wangshuibai", "Ning 7840" and the widely used "Sumai 3" (China) (Zhang *et al.*, 2011). FHB resistance is a polygenic trait and QTLs controlling it are found across all chromosomes except for chromosome 7D. There are seven QTLs designated as FHB resistance genes: *Fhb1*, *Fhb2*, *Fhb4* and *Fhb5* (sourced from wheat) and *Fhb3*, *Fhb6*, and *Fhb7* (sourced from wheat wild relatives) (Guo *et al.,* 2015). Zhang *et al.* (2011) reported that the gene pool of FHB resistant cultivars needs to be diversified outside the use of "Sumai 3" and since there is a shortage of wheat germplasm with resistance, wild relatives need to be looked into for resistance genes.

Screening of wheat wild relatives has revealed FHB resistance as high as "Sumai 3" or even higher in the genera of *Agropyron*, *Kengyilia, Roegneria, Elymus,* and *Hystrix* (Cainong, 2014). The use of wild relatives has the main limitation of linkage drag which brings other unwanted genes of poor agronomic traits with the resistance genes. This is especially prevalent in amphiploid, addition and substitution lines which also exhibit chromosome instability where an individual chromosome has been transferred (Zhang *et al.*, 2011). Wheat-alien translocation is the best method of introducing FHB resistance from wild relatives without linkage drag by translocating small segments of chromosome from the alien genotype (Cainong, 2014). Singh *et al.* (2016a) however, reported that progress in breeding for high yield-yielding FHB resistant cultivars is very slow.

2.2.4. Abiotic challenges to wheat yield

A lot of factors that lead to low yield in wheat have to do with poor management of the crop. Factors such as the use of incorrect row spacing or improper seeding rate limit agronomic traits resulting in overcrowding of plants or underutilization of the field, both of which lead to less than optimum yield (Babu *et al*., 2017). These two management practices have an effect on a number of yield related traits. Iqbal *et al*. (2010) reported that increases in seed rate reduced plant height, grain number per spike as well as 1000 kernel weight due to the competition for resources between plants. Overcrowding tends to favour tillering and the production of straw in wheat at the expense of the harvestable yield, and in the process, the harvest index (HI) is lowered (Ali *et al*., 2010). Kirkegaard *et al*. (2014) reported that improvements in crop management increased yield by 11-47% with a resultant increase on profits of 18%.

However, there are a lot of abiotic stresses the farmer or producer will not have control over, and these are factors that are brought about by changes in the weather patterns experienced globally. Cassia *et al*. (2018) reported that abiotic stresses are possibly more limiting to yield than biotic stresses due to effects of climate change. Major yield losses to wheat as well as other crops in the world come as a result of extreme temperatures, low water availability, high light intensity, high salt and mineral deficiencies or toxicities (Lucas, 2013).

Average yield decreases exceeding 50% due to abiotic stresses have been reported across all the major crops (Tuteja *et al*., 2011).

Salinity stress is reported to cost the global farming economy more than $11 billion per year (Shabala, 2013). Abiotic stresses are also more devastating in that they sometimes afflict crops simultaneously, for instance, high temperatures, high irradiance, scarcity of water, and nutrient deficiencies occur regularly in environments where wheat is grown (Lucas, 2013). Cramer *et al*. (2011) reported that 96.5% of global land area under crop production was affected by abiotic stresses, the biggest one being water shortage stress followed by temperature stress and acid soils.

The worst effects of these stresses are experienced in developing countries where farmers lack the financial resources to overcome the negative effects (Reynolds and Ortiz, 2010). Geographically, the largest portions of land at risk are found in Africa, Asia, Oceania and South America (Gilliham *et al*., 2017). This is due to some of these farmers using landraces that lack genes conferring vigour.

Worldwide, yields of the top five crops are expected to continually decrease due to the combined effect of reduced water resources, reduced arable land (as a result of increasing population) and the many negative effects of climate change (Lobell *et al.*, 2011). Wheat is generally most sensitive to stresses (abiotic and lack of management resources) during late reproductive or grain-filling stage (Slafer, 2012).

2.2.4.1. Water and salinity stress

In most regions of the world, drought is the most common and most devastating abiotic stress on wheat production and quality, and results in as much destruction as the other natural disasters combined (Nezhadahmadi *et al.*, 2013). Drought is a prolonged period of abnormally low rainfall, leading to a shortage of water (UN Water, 2018). Water variation across the globe is subject to variations in temperature and rainfall distribution in geographical regions as well as differences in soil characteristics that in turn affect water potential in these different soils and plants' ability to extract water from the soil (Des Marais and Juenger, 2010). This will be a persisting issue with reports of approximately 1.8 billion people projected to face absolute water shortage by the year 2025 (UN Water, 2018). As it stands, about two thirds of the global population currently live in areas that experience water scarcity for at least one month per year (Mekonnen and Hoekstra, 2016).

Plant response to drought stress is affected by a number of factors including the plant genetic makeup, its growth stage, the severity and duration of the stress (Tardieu *et al.*, 2018). Other factors include physiological processes of growth, different patterns of genes

expression, different respiration patterns, activity of photosynthesis machinery as well as environmental factors (Rampino *et al*., 2017).

Salinity is frequently associated with water stress in that these tend to simultaneously affect both agricultural and natural ecosystems and they are the most devastating abiotic stresses on agricultural production (Ahmed *et al*., 2013). Salinity is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death (Zhu, 2007). This close tie is due to soil water potential since salt concentration and soil water are indirectly proportional and high salt (an consecutively low soil water) has high water potential that plants need to overcome to obtain water through roots (Des Marais and Juenger, 2010). At least 22% of agricultural land i.e. approximately 6% of the total area of the world, is affected by salinity (Zhu *et al*., 2016).

Generally, the tetraploid durum wheat is less tolerant to salt stress than bread wheat (Wang and Xia, 2018). This is mainly due to durum wheat missing the D-genome which contains the functional *HKT1;5* gene, a member of the *HKT* (High-affinity potassium transporters) genes associated with salt tolerance. These genes aid in bread wheat tolerance to salinity through Na^+ exclusion at root level resulting in reduced Na^+ on the shoots (Gilliham *et al.*, 2017). This is especially important because upon entering plant roots, Na^+ accumulates on the shoots and causes yield reductions (Munns and Gilliham, 2015). In an experiment, Zhu *et al*. (2016) reported relative yields of 2.6-19.8% on bread wheat and 1.26-8.1% for durum wheat to demonstrate this phenomenon. They also noted stress symptoms on susceptible genotypes starting with wilted lower leaves, followed by leaf chlorosis and eventually necrosis and overall plant death.

At the biochemical and/or physiological level, these two stresses (amongst others) are the same in that they limit crop productivity through oxidative and/or osmotic stress. Osmotic stress reduces yields to less than half the potential yield (Szymańska *et al*., 2017). Oxidative stress occurs when there is an imbalance of reactive oxygen species (ROS; e.g. H_2O_2, $O_2^{\bullet-}$,$^{\bullet}OH$ and $^1O_2$) and antioxidants within biomolecules such as lipids, proteins and DNA (Anjum *et al*., 2015). This imbalance is due to an increase of the destructive ROS which are produced in the reduction reactions of molecular oxygen (O_2) under stress conditions resulting in reduced plant metabolic activity and development (Gill *et al.*, 2015). An example of a process that leads to the increase load of ROS is reduced photosynthetic capacity under stress, while the plant continually intercepts excess light energy (Gilliham *et al*., 2017). ROS are generated as by-products of various metabolic activities and under normal conditions they can be found at concentrations of 240 μMs^{-1} and 0.5 µM for superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) respectively. These values shoot up to 720 μMs^{-1} for $O_2^{\bullet-}$ and 5–15 µM H_2O_2 under stress conditions (Hasanuzzaman *et al*., 2012).

2.2.4.2. Plant response to abiotic stress

With plants being sessile and unable to migrate, they need to cope with the abiotic stresses that affect their ecosystems (Des Marais and Juenger, 2010). Plants respond to abiotic stresses through three main mechanisms: adaptive plastic responses, specialist strategies and generalist strategies. Specialists adapt to a narrow range of environments leading to landraces while generalists are productive in a wider range of environments and plastic responders have traits adapted to differing environments as needed (Szymańska *et al*., 2017).

In response, plants exposed to an abiotic stress undergo a variety of changes from physiological adaptation to gene expression (Gupta and Huang, 2014). Abiotic stresses activate responses within the plant and these responses are very dynamic and complex and may be either elastic (reversible) or plastic (irreversible) (Skirycz and Inze, 2010). The level of the stress and whether it is an acute or prolonged stress will also add to the complexity of the response (Pinheiro and Chaves, 2011). For example, limited water stress at the early stages of grain-filling results in reduced sink strength and a decrease in the number of endosperm cells (Nezhadahmadi *et al.*, 2013). Early plant responses to stress are reported to be the readjustment of energy homeostasis and thereafter more stress-specific profiles develop (Cramer *et al*., 2011). Inhibition of protein synthesis is one of the initial effects of abiotic stresses as well as an increase in protein folding (Liu and Howell, 2010).

There are three physiological coping mechanisms that plants have evolved to deal with osmotic stress (Osakabe *et al*., 2014). The first is dehydration avoidance where plants change their life cycle to utilize rainfall or available water sparingly as well as morphological changes in root depth. Water uptake is adjusted alongside water loss *via* transpiration, resulting in acceptable internal water status at the cost of compromised photosynthesis and plant growth (Des Marais and Juenger, 2010). This is facilitated by stomatal regulation which reduces water loss and CO_2 absorption (Daszkowska-Golec and Szarejko, 2013). Water-use efficiency (WUE) as the efficiency of plants to fix CO_2 whilst maintaining good water loss is the ideal trait to ensure plants are still growing and developing under stress conditions.

Secondly, plants may exhibit dehydration tolerance where plants arrest growth when they have low internal water, but are able to resume growth when water becomes available on the soil (Feng *et al*., 2016). This is achieved by osmolyte accumulation on plants and reduced leaf area under water loss (Szymańska *et al*., 2017). Osmolyte accumulation also helps maintain root growth enabling plant to reach water reserves deeper into the soil profile (Qin *et al*., 2015). Lastly, plants have evolved a mechanism to escape a stress completely by accelerating growth and development, reaching reproduction before or at the onset of a stress (Des Marais and Juenger, 2010).

Reactive oxygen species (ROS) as mentioned previously are produced more in response to stress conditions and they modify enzyme activity and gene regulation (Mittler *et al.*, 2011). Plant protection from the harmful effects of ROS is through accumulation of beneficial anti-oxidants. The plant-oxidant defence is made up of antioxidant enzymes [i.e. superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)] as well as non-enzymatic antioxidants [i.e. glutathione (GSH), phenolic compounds, and alkaloids] which can all metabolize ROS and the products to prevent oxidative stress (Hasanuzzaman *et al*., 2012).

SOD is the initial defence compound which increases in ROS as a response to abiotic stress and acts as a catalyst for the dismutation of $O_2^{\bullet-}$ back to H_2O_2 and O_2 (Gill and Tuteja, 2010). SOD also protects photosystem II from damage caused by superoxides (Gill *et al.*, 2015). There are various isoforms of SOD and each one is distinguished by a different metal at their site with some specific to one of the stresses causing oxidative stress. The Cu-Zn-SOD isoform is mostly found in chloroplasts and cytosol; Mn-SOD is peroxisomes and mitochondria and FeSODs are predominantly found in chloroplast (Zhou *et al*., 2017).

Osmotic stress will either directly or indirectly affect the production of sugars as well as their concentration, metabolism, transportation and storage (Slama *et al*., 2015). Sugars such as sucrose, raffinose and trehalose as well as sugar alcohols (e.g. mannitol) and amino acids (e.g. proline) are accumulated under such conditions (Slama *et al*., 2015). Proline is an important protein that has a vital function in tolerance to a number of abiotic stresses and is formed from pyrroline-5-carboxylate synthetase (P5CR) (Kavi Kishor *et al*., 2015). Higher growth rates of crops (under drought stress) are induced by higher levels of polyamines (Pas), which affect the completeness of membranes and nucleic acid under limited water stress environments (Malabika and Wu, 2001). Osmotic regulators such as soluble sugars, aid water absorption under stress conditions and have been found to be high in tolerant wheat genotypes (Slama *et al*., 2015). Dhanda *et al.* (2004) also reported that wheat genotypes with low malondialdehyde (MDA) tend to be more tolerant to stress.

Hormones are also important in plant responses to abiotic stresses, especially abscisic acid (ABA) and ethylene (Verma *et al*., 2016). Ethylene is involved in the response to stresses such as drought, heat, chilling, and wounding. (Kurepin *et al*., 2015). ABA is involved in germination inhibition, and is also the principal regulator of many plant environmental stress responses, especially osmotic stress (Szymańska *et al*., 2017). ABA is partially responsible for stomatal closure, as result of limited water stress on plants, which therefore leads to a decrease in stomata conductance to CO_2, reduced internal CO_2 concentration and finally, reduced growth rate (Nezhadahmadi *et al.*, 2013). Plant response systems to abiotic stresses may be grouped into two pathways based on their ABA-dependency, i.e. ABA-dependent or ABA-independent pathways (Qin *et al.*, 2015). Expression of DREB1 and DREB2 proteins, the

key factors in response to drought stress, is said to be through ABA-independent pathways (Huang *et al*., 2016). Calcium ($Ca2^{+}$) is also an important signalling molecule under plant abiotic stress, a key to unlocking mechanisms to tolerance (Gilliham *et al*., 2017).

Stress-associated transcription factors (TFs) are important molecular mechanisms for adaptation and/or tolerance to stresses by plants being involved in multiple signalling pathways (Qin *et al*., 2015). These transcription factors, e.g. WRKY TFs, also have an overarching effect over osmotic stress and this specific example is more common in plant response to pathogen infection (Rushton *et al*., 2010). An excess of 15 WRKYs have been isolated from wheat, for example TaWRKY which improves tolerance to osmotic stress *via* enhanced osmotic adjustment, increased transcription of genes and sustained membrane stability (Qin *et al*., 2015). The DREB-family of transcription factors consists of 56 members, most of which have yet to be characterized (Des Marias and Juenger, 2010).

Plants contain more than 30 000 genes per cell which code for an unknown number of proteins making plant response to the environment a very complex activity (Zinta *et* al., 2018). At molecular level, limited water stress activates certain genes which in turn result in the production of different drought stress related proteins and enzymes such as dehydrins, vacuolar acid invertase, heat shock proteins (HSP), late embryo abundant (LEA) proteins and various proteinase inhibitors (Sun *et al*., 2017). Wheat growth under drought stress has been shown to be increased by the *HVA1* gene which produces a protein in group 3 LEA and has 11 amino acid motifs in nine repeats (Nezhadahmadi *et al*., 2013). *AREB/ABF* genes have been reported to be involved in osmotic stress tolerance, with reported hypersensitivity where genes are knocked out (Yoshida *et al*., 2010).

The genes involved in each of the stress responses are part of an interconnected network of genes (Szymańska *et al*., 2017). The use of high throughput omics technologies makes it possible to identify new genes and their functions for example in resistance against stresses (Cramer *et al*., 2011). These genes regulate the production of compounds that attempt to protect the plant against environmental stresses such as osmoprotectants, chaperones, and detoxification enzymes (Huseynova and Rustamova, 2010). Nezhadahmadi *et al.* (2013) reported that the expression of genes such as ABA-related genes and the production of helicase, rubisco, proline, and carbohydrates are molecular basis of drought tolerance in plants. Shi *et al.* (2010) reported that in wheat, of the 265 genes and 146 genes detected at the junction and seedling stages respectively, more than half of them are involved in responses to abiotic or biotic stresses. Another vital enzyme to plant development and resistance to abiotic stresses is vacuolar H^{+}-translocating pyrophosphatase (V-PPase) and it is controlled by the three genes; *TaVP1, TaVP2 and TaVP3* (Sun *et al*., 2017).

2.2.4.3. Breeding for stress tolerance

Abiotic stresses are generally multi-faceted in their nature, having multiple effects on plants. This means that there is no one trait or gene that could be identified or used to combat that particular stress in plants (Gilliham *et al.,* 2017). Studies that give insight into plant's response to abiotic stresses are the basis of pre-breeding and breeding exercises aimed at delivering tolerant varieties (Tuteja and Gill, 2013). Pre-breeding exercises, aimed towards this goal, have three objectives: 1) separating tolerance to a number of sub-traits working together for the overall effects, 2) identifying genes that control these sub-traits and then 3) stacking these genes to improve tolerance (Gilliham *et al.,* 2017).

Previous breeding objectives have not focused on stress tolerance improvement, but the main objective has been specific yield improvement with very low selection pressure for abiotic stress tolerance instead of yield stability which is associated with yield under stress conditions (Gilliham *et al.,* 2017). Fleury *et al*. (2010) reported that limited water stress tolerance improvement efforts are focused on plant survival at the expense of yield and suggested that objectives should aim towards ensuring continued growth and sustained yield under water-limited conditions. Reynolds (2012a) reported that breeding for genetic yield potential will assist in breeding aimed at abiotic stress, but there is a limit to its advantage.

Previous selection models have slowed down progress in abiotic stress tolerance breeding, combined with the minute economic need for such breeding objectives (Gilliham *et al.,* 2017). Reasons for delayed progress in this regard are: incomplete understanding of tolerance sub-traits and the genes controlling them which limits the use of marker-assisted selection (MAS); the perception of a lack of return in investment for such objectives and failure to commercialize genetically modified organisms (GMOs) in certain areas; and the genetic drag resulting in reduced yield where genes have been introduced (Zhu *et al*., 2016; Gilliham *et al.,* 2017). Improvements in breeding can be achieved through stress avoidance, matching crop development processes with climatic conditions to ensure crops have completed important stages when conditions are severe. Renewed interest in strategic research funding, especially pre-breeding, will lead to sustainable increases of yield under stress (Gill *et al.,* 2015).

Mutation breeding, through carcinogens and radiation, has been successfully used to develop commercial varieties with improved tolerance to various abiotic stresses in crops such wheat, rice and barley (Suprasanna *et al*., 2014). However, there are some drawbacks of non-target effects on plants that need to be removed through backcross breeding but it still remains the preferred method of creating variation for stress tolerance breeding than GM crops (Chen

et al., 2014). Where markers are available, molecular breeding can be looked into as it has added cost effective benefits.

Physiological traits have also been considered as possible ways to improve tolerance, with some general traits covering multiple stresses and some more specific. Plant WUE, also known as transpiration efficiency, measures the ratio between photosynthetic and transpiration rates and has already been mentioned as an ideal trait for ensuring plant growth and yield in stressed plants. The trait is measured by using carbon isotope discrimination, where Munjonji *et al*. (2016) noted that accumulation of ^{12}C over ^{13}C decreases with increases in photosynthesis or decreased rate of stomatal conductance.

Generally, plants with a higher WUE have poor discrimination of the two isotopes in comparison to plants that don't use water efficiently but it has been shown that under non-limiting environments, isotope discrimination does not necessarily result in yield increases (Richards *et al*., 2014). Genes underpinning the trait have yet to be discovered and need to be identified, cloned and sequenced into genetic markers so they may be used for MAS breeding exercises (Gilliham *et al.,* 2017).

The stay green trait is another set of important physiological traits of importance in stress breeding, as evidenced in its successful use on sorghum (Borrell *et al*., 2014). The trait ensures that plants maintain their chlorophyll in leaves and stems as the plant undergoes grain filling, and ensuring delayed senescence and a longer grain-filling period. Plants with the trait also have a more vertical root, reduced tiller number as well as increased leaf size on the lower leaves with a decrease in leaf size as one goes up the plant canopy (Gilliham *et al.,* 2017). Zhu *et al.* (2016) reported that physiological traits used in breeding for salinity stress include: relative water content, the lengths of the coleoptile, stem and radicle lengths as well as the dry and wet weights of roots and shoots. The trait with the biggest impact on salinity tolerance however is Na^+ exclusion from shoots which is controlled by the *Nax2* locus (Munns *et al*., 2012).

2.2.5. Wheat yield related traits

Grain yield, being controlled by a number of gentic and environmental factors, is the result of plant morphological, physiological, biochemical processes and growth parameters as well as successful conversion of solar energy *via* photosynthesis (Gutam, 2011). Initially, wheat grain yield was reported to be the result of three components: spikes per unit area, number of kernels per spike and the individual weight of kernels (Evans, 1987). Dissecting of yield into relatively simpler traits (numerical and physiological components) allows for easier assessment of yield and makes for easier selection by breeders (Xie *et al*., 2016). To use these yield related traits in breeding, they should not only have high correlations (genotypic and phenotypic) with yield,

but should also be relatively easy to assess with higher narrow sense heritability than yield per se (Savii and Nedelea, 2012).

Universally accepted yield related traits in wheat include: plant height, spike length, spike number, grain number per spike, floret number per spike, and thousand-kernel weight (TKW) (Liu *et al.*, 2014b). Quantitative Trait Loci (QTLs) controlling yield and its components are scattered on many chromosomes throughout the wheat genome and, once cloned, they can be used to improve wheat yield through MAS (Liu *et al.*, 2014b; Tahmasebi *et al*., 2017). Liu *et al.* (2014b) noted that while QTLs affecting yield-related traits may be found dispersed throughout the wheat genome, chromosomes 4A, 4B and 4D were especially rich in these. Gutam (2011) noted that while grain yield was positively associated with the rate and duration of grain-filling, the former had a bigger impact on the final grain yield.

2.2.5.1. Protein content

One of the most important yield-associated traits in wheat is the protein content as this relates to human nutrition (Löffler *et al.,* 1985). These storage proteins also give wheat its unique rheological properties that allow for the broad diversity of food that can be made with wheat (Shewry *et al*., 1995). Gluten, the main protein found in wheat, affects the bread-making and dough mixing strength properties of wheat flour (Koga *et al.*, 2017). Gluten has a lower density (1.29) when compared to starch (1.51) (Engelbrecht*,* 2008).

There are two major groups of these proteins which are the polymeric glutenins and the monomeric gladiadins (An *et al*., 2006). These are also grouped into three groups based on their mobility during sodiumdodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Payne and Corfield, 1979). The first are high-molecular-weight (HMW) glutenins with x and y-type HMW subunits, then the low-molecular-weight group (LMW) (made up of α-type gladiadins, γ-type gladiadins) and gladiadins which are also called medium-molecular-weight gladiadins (MMW) (e.g. ω-type gliadins) (Payne *et al*., 1984).

Glutenin subunits are mostly made up of HMW and LMW subunits but there is a small portion of ω-gliadins bound to glutenins by a disulphide bond and are called glutenin-bound (ωb) gliadins (Wieser *et al.,* 1994). Among the glutenin subunits, LMW are most abundant, followed by HMW and ωb are the least (Wieser *et al.*, 2000). The MMW gladiadins include ω5-type and ω1,2-type (ω-gladiadins, ω-secalins) the former of which have high glutamine contents (Shewry *et al*., 1986). In the presence of water, the glutenin polymer interacts non-covalently to form gluten, the unique protein of wheat (Shewry *et al.*, 2001).

HMW glutenin genes are found on the long arms of 1A, 1B and 1D chromosomes (locus *Glu*-1), MMW glutenin genes are found on the short arm of chromosome 1B (*Gli*-B1) and LMW

group is controlled by the *Glu*-3 loci found on the short arms of group 1 chromosomes (*Glu-A3, Glu-B3* and *Glu-D3*). Genes controlling α-type gladiadins are in the *Gli*-2 locus on the short arms of chromosomes 6A, 6B and 6D (Wieser *et al*., 2000).

As an important trait, grain protein content has been subjected to a lot of breeding efforts in previous breeding programs but simultaneous increases of yield and protein content have not been possible (Slafer *et al*., 2015). The main reason for this is that there is a negative correlation between yield and protein content (Slafer and Andrade, 1993; Wang *et al.*, 2012b; Tsilo *et al*. 2013), although some authors have suggested that since this relationship is normally, weak simultaneous improvements may be possible (Johnson *et al*., 1973; Levy and Feldman, 1987). Another impending factor is the low heritability estimates associated with the trait which is controlled mostly by the environment and is generally found to be higher in low-yielding environments (Slafer, 2012).

Tsilo *et al*. (2013) reported that protein content could be improved simultaneously with yield by separating the overall proteins into its different constituents and selecting for the proteins that are positively correlated to grain yield. This is done by analysing the gluten/endosperm proteins using size-exclusion high performance liquid chromatography (SE-HPLC) which split them up according to their molecular weight (Mw), from the biggest to the smallest. This process splits the gluten proteins into three fractions: extractable polymeric proteins (EPP), unextractable polymeric proteins (UPP) and unextractable very high Mw polymeric proteins (UVHP) (Bietz, 1984). EPP was found to be negatively correlated to yield and accounted for the well-known negative correlation between yield and protein content. As such, Tsilo *et al*. (2013) concluded that selecting for unextractable proteins, especially UVHP will result in improved protein content and bread-making quality without the reduction in grain yield which was in agreement with earlier work by Charmet *et al*. (2005). Other authors have suggested and facilitated improving protein content *via* the use of wild relatives such as *T. turgidum, T. dicoccoides* and *Ae. squarrosa* (Ogbonnaya *et al*., 2013).

2.2.5.2. Hectolitre mass

Hectolitre mass (HLM) or test weight (bushel weight), as it is referred to in other countries, is a measure of the volume of grain per unit volume and it is the function of the density of wheat (Donelson *et al*., 2002). HLM is an important wheat quality parameter and is one of the earliest used for quality assessment (Posner and Hibbs, 2005). HLM is also a good indicator of grain-soundness or the wheat flour yield to be expected (Posner and Hibbs, 2005). For wheat millers, flour extraction is an important factor and as such HLM is part of the grading regulations in wheat and the grade assigned also determines the profit a farmer makes (Makgoba, 2013). South African millers assign wheat quality grade based on HLM first, which in turn determines

how much they will pay the farmer for his grain, before other quality parameters like protein content are factored in (Engelbecht, 2008).

HLM testing is non-destructive, conducted by using 1 kg of clean seed using various specialized equipment in different countries and the units reported are kilograms per hectolitre (kg hl^{-1}) (Engelbecht, 2008). Newer technological advancements have been made such as Perten near infra-red (NIR) equipment that uses less mass to make measurements than the older methods, as well as obtaining multiple parameters at the same time which saves time (GME, 2017). HLM evaluation is said to have begun in the 17th or 18th centuries in Britain, and normally ranges between 70-80 kg/hl which can be exceed in either extreme based on the wheat growing environment (Triccoli and Di Fonzo, 1999).

The South African wheat industry considers wheat to be suitable for bread making if it has a test weight of at least 74 kg/hl (Miles *et al*., 2013). HLM is mostly affected by the environment during the grain-filling stages of wheat maturation than genetic control (Evans *et al.*, 1975; Makgoba, 2013). Evans *et al.* (1975) reported that HLM is affected by moisture availability, temperature, nitrogen and biotic stresses. Gaines *et al.* (1996) further reported that drier areas generally have higher HLM values than humid areas.

Engelbecht (2008) reported that correlations between grain yield and HLM ranged from very low (r=0.32) to high (r=0.82). There is no correlation between HLM and thousand kernel weight (TKW) and there are no reported genes that link the two (Ghaderi and Everson, 1971). Although some negative correlations have been reported, HLM and protein content usually have low to moderate correlations (0.11-0.64) with each other (Engelbecht, 2008).

2.2.5.3. Tiller number

Tillers are defined as shoots arising from buds in the axils of plant leaves and tillering is an essential aspect of yield determination with about 30-50% of wheat yield obtained from the main stem and the remainder from tillers (Thiry *et al*., 2002). This trait contributes to yield by affecting the canopy size, the area undergoing photosynthesis and the number of spikes that produce grain yield as well determining genetic yield potential differences among genotypes (Xie *et al.*, 2015). Tillering contributes to wheat's ability to adapt to changing environments, resulting in increased tillering when there is plenty resources and reduced tillering for survival under stresses such as drought conditions (Elhani *et al*., 2007).

In wheat, tillering begins after the two or three leaf stage, and the formation of new tillers thereafter is synced to further leaf formation with early or primary tillers producing secondary tillers on top of the main stem contribution (Evers and Vos, 2013). The duration of tiller formation ends just before stem elongation with the tillers at their maximum possible number,

and the remaining axillary buds enter into dormancy (Sylvester-Bradley *et al*., 2008; Xie *et al.*, 2015). Environmental conditions such as plant density and light availability or quality also give cues for plants to stop tillering (Toyota *et al*., 2014). As an example, R:FR [red (R) light to far-red (FR) light] ratio which is a measure of light quality, will cause tillering cessation when its value is between 0.20 and 0.40, but if the ratio is higher, tillering is prolonged which results in increased tiller number (Toyota *et al*., 2014; Xie *et al.*, 2015).

At the end of tillering some tillers fail to develop a spike and they start dying off before the stem matures leaving only a few to produce grain and contribute to the final yield (Thiry *et al*., 2002). Between 10 and 80% of the produced tillers survive to produce grain and the surviving proportion is determined by the genotype, and the environment's effects (favourable or unfavourable) on available resources (Thiry *et al*., 2002; Elhani *et al.,* 2007). Tiller abortion occurs in reverse order to tiller development with the youngest tillers dying off first and this takes place between stem elongation and anthesis (Sylvester-Bradley *et al*., 2008). There are contrasting ideas on whether this abortion is an advantage or disadvatage with some stating it reduces grain yield potential by decreasing the harvest index (Foulkes *et al*., 2011) and some suggesting that nutrients are absorbed by the surviving tillers from the senescing tillers that are aborted (Thiry *et al*., 2002). Tiller survival or reduced tiller abortion is considered a very important aspect under favourable environments and the ability to reduce tillering when the environment is harsh (Thiry *et al*., 2002).

Xie *et al*. (2015) reported positive contributions of tillers to yield by increasing the grain number harvested per plant with a slight reduction on thousand-kernel weight (TKW). The relationship between grain yield, and the maximum number of tillers has been reported to be very weak and the significant one being with surviving tillers (Elhani *et al.,* 2007). Tillering has also been reported to be related to photoperiod response and a QTL controlling initial shoots per plant found on chromosome 2D that corresponds to the *Ppd*-D1 gene (Xie *et al.*, 2015). Thirty-four QTLs were identified to be related to tillering traits and these are scattered across 10 chromosomes of which most (76%) are on the A-genome of wheat (Xie *et al.*, 2015). Three genes have also been reported that have a major effect on final tiller number in wheat, as well as many more minor QTLs (Zhang *et al*., 2013). The three major genes are *tin*1 located on chromosome 1AS (Richards, 1988), *tin*2 on chromosome 2A (Peng *et al*., 1998), and gene *tin*3 located on 3AL (Kuraparthy *et al*., 2007).

2.2.5.4. Days to heading

Days to heading (DTH) is defined as the number of days required for the inflorescence (head/cob/panicle) to emerge from the flag leaf of a plant or a group of plants in a study (Gramene, 2017). The stage occurs during the rapid stem elongation phase of wheat growth

and development (Zadoks 50). Flexibility in heading or flowering has enabled wheat to be adaptable in a wide range of environments in diverse geographical positions throughout the globe. Vernalization (*Vrn-A1*, *Vrn-B1* and *Vrn-D1*) and photoperiod (*Ppd-D1*, *Ppd-B1*, and *Ppd-A1*) genes are the main drivers of this trait, ensuring that the plant transition from vegetative stage to reproductive takes place when conditions (temperature and moisture) are best suited for this (Slafer, 2012; Guedira *et al*., 2016). This adaptation of wheat to growing conditions contributes largely to increases in wheat yields (Kamran *et al.*, 2014).

Adu-Gyamfi (2017) reported that days to heading ranged from 90 days (for spring wheat) to 120 days (for winter wheat) but this is a highly variable trait based on vernalization and photoperiodism. In South Africa this range is slightly shortened to between 90 and 110 days. Days to heading QTLs are located in similar regions as QTLs controlling protein content in wheat grain, post-anthesis leaf senescence and even grain yield (Liang *et al*., 2018).

2.2.5.5. Flag leaf area

In wheat, and related cereals, the last leaf to emerge on the plant tillers is referred to as the flag leaf (Barnard, 2012). In general, increases in the overall leaf area in wheat results in increases of photosynthetically active surface area and contributes to canopy development (Al-Tahir, 2014). Leaf area, especially flag leaf area, is a great indicator of genetic yield potential in wheat with higher potential reported in broader and longer leaves which have larger areas (Barnard, 2012). Genetic yield potential and grain protein content are determined during the flag leaf stage (until anthesis) (Barnard, 2012). Most of grain yield in wheat is contributed by the top three leaves, with flag leaf area contributing up to 75% of the leaf area supporting grain filling (Al-Tahir, 2014).

In order to increase grain productivity in wheat breeding programs, it is essential that the genetic mechanisms controlling flag leaf morphology be well-understood (Fan *et al.*, 2015). Flag leaf morphology traits include flag leaf angle, flag leaf area as well as its determinant traits which are flag leaf length and flag leaf width (Wang *et al*. 2011; Chen *et al*. 2012). Regarding the leaf angle, Innes and Blackwell (1983) reported that erect upper leaves in wheat resulted in higher grain yields when compared to genotypes with horizontal leaves. The ideal leaf structure to be selected for is dynamic leaves where the upper leaves are erect before anthesis to ensure maximum permeability of the sun and increased photosynthetically active area, which then become droopy or horizontal during grain-filling when lower leaves are rapidly senescing (Slafer and Andrade, 1993). Flag leaf traits are complex quantitative traits and strongly influenced by environmental factors (Kobayashi *et al*., 2003).

The size of the flag area is influenced more by the flag leaf width than its length as reported by Fan *et al.* (2015) who found higher correlations and shared QTLs between flag leaf area

and its width than with the length. At least 38 putative QTLs have been detected that are associated with flag leaf morphology, and these are distributed across more than 12 of the 21 wheat chromosomes (Fan *et al.*, 2015; Wu *et al*., 2016). Most of these QTLs are spread through the B-genome and the traits reported moderate to high broad sense heritability estimates. There are 13 QTLs specifically linked to the flag leaf area trait, the most commonly expressed QTLs are *QFla.cau*-2D and *QFla.cau*-5A.1 with the former having the strongest effect on the trait (Fan *et al.*, 2015; Wu *et al*., 2016).

Flag leaf morphology traits are generally related to yield attributing traits but have also been shown to be linked to a major QTL for type I resistance to *Fusarium* head blight (Xue *et al*., 2013). Fan *et al.* (2015) reported 12 QTL clusters that simultaneously affected flag morphology traits and yield related traits like spike length, spikelet number, kernel number and kernel weight, and these were found on chromosomes 1B, 2D, 4A, 4B, 5B, 6B and 7D. Positive correlations have been reported between flag leaf area and yield attributing traits in various studies (Wang *et al*., 2011, Xue *et al*. 2013; Fan *et al.*, 2015; Wu *et al*., 2016).

2.2.5.6. Plant height

Wheat plant height is an important agronomic trait, not only for morphogenesis and plant architecture, but also for its significant and positive influence on grain yield (Cui *et al.,* 2011; Wang *et al*., 2012a). Plant height in wheat is comprised of the spike length and lengths of all the internodes above ground (Cui *et al.,* 2011). Bruening (2005) reported that farmers tend to prefer taller genotypes if they are interested in straw production, but shorter varieties ensure a smaller amount of post-harvest field debris. Tall plants have the advantage of having improved light distribution within the canopy but they are highly correlated with lodging (Wang *et al*., 2012a). Lodging causes significant losses in wheat grain yield (of up to 80%) (Berry *et al.,* 2004). Lodging sensitivity is associated with the length of the basal internodes, where plants with longer internodes are more sensitive to lodging and shorter plants are insensitive (Tian *et al*., 2015). Very short genotypes can lead to increased spread of fungal pathogens which leads to decreased yields and/or increased use of chemical fungicides (Zapata *et al*., 2004).

Any breeding strategy aimed at genetic yield potential improvement in wheat should take into account selection for the appropriate plant height as well as lodging resistance since higher plant yields will require stocky stems to carry the load (Cui *et al.,* 2011; Reynolds *et al*., 2012a). The optimum plant height for wheat is between 70 and 100 cm, which ensures maximum yield without lodging risks and easier mechanical harvesting (Rebetzke *et al*., 2012). Most modern cultivars are within this yield-optimising height range (Ferrante *et al.*, 2017). The “Green Revolution” was built on the introduction of dwarfing genes into cereal crops, resulting in high yielding semi-dwarf genotypes with much reduced lodging tendencies (Jamali and Ali, 2008).

At least 25 dwarfing genes have been named; most of which have been characterized with linked markers (Ellis *et al*., 2005; Zhang *et al*., 2008). Dwarfing genes and their alleles have an effect on grain yield in wheat by lowering internal competition for assimilates between the developing spikes and stems (Zhang *et al.*, 2010). Of these, *Rht-B1b* and *Rht-D1b* are the most widely used in breeding programs globally and carried by about 90% of the semi-dwarf varieties (Borlaug, 1968). *Rht*-B1 and *Rht*-D1 are located on the short arms of chromosomes 4B and 4D, respectively (Huang *et al*., 2003). Both genes were resulted from spontaneous mutations and control gibberellic acid (GA) insensitivity (Ellis *et al*., 2005).

Plant height is a complex and quantitatively controlled trait (believed to also have some Mendelian gene control) with genes controlling the trait distributed across almost all the 42 chromosomes in wheat (Wang *et al*., 2010; Cui *et al.,* 2011). In wheat, plant height is more associated with the length of internodes and has a very weak genetic association with the spike length (Cui *et al.,* 2011; Wang *et al*., 2012a). Wang *et al.* (2012a) also reported that QTL activity is low in the early stages of wheat development, increasing with development before decreasing again towards the end. GA-insensitive dwarfing genes such as *Rht-B1b* and *Rht-D1b* are associated with increased grain number per spike which has contributed to yield increases during the green revolution (Miralles and Slafer, 1995). Jamali and Ali (2008) also reported significant and positive correlations between plant height and spikelets per spike, grain number and weight per spike, respectively.

2.2.5.7. Spike length

Spike length (SPL) forms part of the overall plant height in cereal plants and as the grain producing area, they have an influence on two of the three main yield components (Wu *et al*., 2014, Zhang *et al*., 2015). At the heading stage, the green spikes contribute to grain-filling by their photosynthetic capacity, this is especially important under drought stress conditions (Maydup *et al*., 2010). The trait has been shown to have positive correlations with traits that include harvest index (HI), shoot biomass, spike density, and most importantly grain yield (Moghaddam *et al*., 1997; Donmez *et al*., 2001). Longer spikes are also associated with reduced spike compactness which improves resistance to FHB and in turn increases yields that would have otherwise been lost to the pathogen (Buerstmayr *et al*., 2009).

As with most traits of agronomic importance, spike length is a polygenic trait that is affected by both genetic and environmental factors with the latter having a more pronounced effect (Wu *et al.*, 2014; Zhang *et al*., 2015). At least 15 spike length QTLs have been reported in wheat and they are located on chromosomes 1A, 1B, 2A, 2B, 2D, 3D, 4A, 5A, 5B, 5D, 6A, 6B, 6D, 7A and 7D (Ma *et al*., 2007; Cui *et et al.*, 2012; Wu *et al*., 2012). Wu *et al*. (2006) reported that the 6P chromosome of *Agropyron cristatum* L. Gaertn contained an agronomic

trait associated with increased floret number and number of kernels, and the species was later crossed with wheat to form a number of wheat- *A. cristatum* 6P translocation lines (Luan *et al*., 2010). Huang *et al.* (2013) then found that the translocation lines with the 6P translocation chromosome segment had higher thousand-grain weight and spike length in two populations. Zhang *et al*. (2015) further studied this and summarized that the chromosomal segments of *A. cristatum* 6P positively regulated TKW and spike length. QTLs, for the translocation controlling the traits, were located on chromosome 1AS and were localised around single sequence repeats (SSR) loci *Agc*7155 to *SSR*263 (Zhang *et al*., 2015).

Another QTL of importance controlling spike length is *QSpl.nau*-2D which was identified in a "Nanda2419" × "Wangshuibai" recombinant inbred line (RIL) population, and this explained up to 20% of the phenotypic variation of the trait (Ma *et al*., 2007). Wu *et al*. (2014) confirmed the location of this QTL to be on the 0.9 cM long *Xcfd*53-DG371 interval of chromosome 2DS, with its position closer to DG371. The QTL functioned like a major gene with partial dominance and was designated *HL1* for head length. The authors also found higher TKW associated with longer spikes, with the trait having QTLs controlling it near the *HL1* gene. Another gene located near *HL1* is *Rht-8*, but previous independent studies demonstrated that this specific dwarfing gene has no effect on spike length (Gasperini *et al*., 2012). The *Ppd-D1* gene for photoperiod sensitivity is found on the same chromosome with pleiotropic effects on plant height, DTH, tillering, spikelet number and number of grains per spike (Worland, 1996; Worland *et al*., 1998).

The *HL1* gene was reported to be the first SPL QTL to be precisely mapped and has the great advantage of not having an adverse effect on spikelet number per spike. The gene affects spike morphology by regulating the distance between spikelets which in turn changes the spike length and compactness as well as spikelet density. The gene is located in a conserved region with reduced number of markers, and it is yet to be cloned so it can be used in plant breeding exercises (Wu *et al*., 2014).

2.2.5.8. Spikelet number

Peel's (2000) method of assessing spikelet number excludes the top and the bottom spikelets as these do not make any significant contribution to the grain yield and are also the first to die if plants experience stressful conditions. Spikelet number is one of the yield related traits (YRTs) that are developed very early in crop phenology, after leaf initiation (Reynolds *et al*., 2012a). QTLs related to spikelet number are mostly found in the A- and B-genome of wheat, especially on chromosome 5A (Zhang *et al*., 2010; Tahmasebi *et al*., 2017). Savii and Nadelea (2012) found moderate correlation (0.62) between spikelet number and plant height. They also reported that the correlation of spikelet number with grain number per spike was higher than with grain weight per spike but both correlations were positive.

2.2.5.9. Plant biomass

Plant biomass is another trait that is very important in yield determination as it encompasses a lot of the other traits as the assimilate source. While harvest index measures the effectiveness of plants during assimilate partitioning, biomass is important because it produces and houses these assimilates. The “Green Revolution” gave rise to high yielding semi-dwarf cultivars which had an improved yield due to increased harvest index (assimilate partitioning) where grain yield increased at the expense of straw yield (Townsend *et al.,* 2017). While yield increase was previously due to significant jumps in harvest index, this trait has now been maximised and further increases in yield will need to look into improved biomass production (Reynolds *et al*., 2012b).

Plant biomass has other important uses in agriculture even after harvest where plant residues will provide cover for the soil, thereby retaining water and reducing soil erosion. The decomposing residues return nutrients back into the soil; improve soil organic matter, as well as other soil characteristics (Huggins *et al*., 2011). Fossil fuel effects on climate change have sparked an interest in biofuels which are produced from crop biomass. The principal crop is maize, with other crops including wheat are being investigated as additional candidates (Townsend *et al*., 2017).

Biomass and its accumulation in plants is affected by a number of biotic and abiotic factors such as pests and diseases (especially foliar pests) as well as environmental cues such as water availability. Management factors that affect biomass include sowing date, seeding density, and nutrient availability. Crop biomass has a positive curvilinear relationship with nitrogen (much like grain yield), but there are limits to this with regards to lodging effects and the cost benefit (White and Wilson, 2006). Sandaña and Pinochet (2011) investigated the effect of phosphorus deficiency on wheat yield and its contributing traits. Their results showed that harvest index and water use efficiency were less affected by the deficiency and yield losses were mostly attributed to reduced biomass. This reduction in biomass decreased the size of leaves, thereby reducing intercepted radiation and consequently yield.

Townsend *et al*. (2017) reviewed a number of studies and found a very strong and positive relationship between plant biomass and plant height, which explains why increased biomass is limited by lodging in tall plants. The introduction of semi-dwarfism also reduced both plant height and biomass. Reduction in grain yield is more affected by reduced biomass yield in P-deficient material and therefore grain number is closely associated with plant biomass (Sandaña and Pinochet, 2011). Further gains in wheat grain yield are driven by increasing biomass in the crop (Quintero *et al*., 2018).

2.2.5.10. Grain number per spike

Before anthesis, spikelets will have a maximum of 10 florets, with fewer than half of these setting grains and this floret survival within the spikelet determines the final number of grains found in a plant. Some spikelets survive to set seeds while another portion gets aborted (Xie *et al*., 2016). Floret survival itself is affected by the dry weight of the spikes at anthesis (Rebetzke *et al*., 2012). The process of grain-filling in wheat is determined by photosynthesis in leaves (with a slight contribution from spikes) taking place at the time of filling and the mobilization of stem-stored water-soluble carbohydrates (WSC) (Xie *et al*., 2016). These are the two main carbon sources in plants (Ehdaie *et al*., 2008). This then explains why water availability from late vegetative stage to grain-filling has a big impact on yield, affecting the number of spikelets, TKW and the grain number per spike (Elhani *et al*., 2007). The authors also reported a much stronger association between yield and grain number under conditions where water is not limited. The grain number of wheat is determined from tillering to just before anthesis commences. This makes the trait especially vulnerable to environmental conditions between these stages (Reynolds *et al.*, 2012a).

Tahmasebi *et al*. (2017) reported that because of the close relationship between yield and grain number, as possibly its main determinant, there is also a negative association between grain number and grain weight. Grain number is more plastic than grain weight, responding to photosynthate availability which makes it attractive for trait selection in non-limiting production areas (Xie *et al*., 2016). The dwarfing gene used in the “Green Revolution” also led to yield increases due to pleiotropic gene effects that increased assimilate partitioning to developing spikes and increased the number of grains per spike (Nadolska-Orczyk *et al*., 2017). Another important gene in wheat adaptation, the *Ppd-1* gene, has major effects on inflorescence architecture and spikelet development. Boden *et al*. (2015) proposed breeding for improved inflorescence and grain number should involve the use of the *Ppd-1* gene and the key flowering regulator *FLOWERING LOCUS* (*FT*).

Genes affecting plant hormones have an effect on the YRTs with cytokinin metabolism genes affecting grain number, and gibberellin-related genes affecting spike length (Nadolska-Orczyk *et al*., 2017). Nadolska‑Orczyk *et al*. (2017) also listed the transcript elongation factors *TaTEF-7A*, *TaGW2,* and two TKW related transcription factors: *TatGW6-b* (Indel mutant) and *TaTGW6-c* (null mutant) to have significant effects on grain number. Liu *et al*. (2014b) mentioned that using *TaANT* on chromosome 4A, and developing a marker from this gene (related to ovule primordial cells) would give breeders a marker to be used for grain number per spike. Tahmasebi *et al*. (2017) confirmed previously reported QTLs (related to grain number per spike) on chromosomes 1A, 5A and 6B as well as reporting additional ones on

chromosomes 2B and 7D. Other QTLs have been reported on chromosomes 2D, 4A, 4B, 5D, 7A and 7B (Zhang *et al*., 2010; Liu *et al*., 2014b).

2.2.5.11. Grain weight per spike

Grain weight has much more implications in wheat than just yield as it will also affect the vigour of progeny seedlings, and their early growth before they can produce their own food through photosynthesis (Tahmasebi *et al*., 2017). Seed size was also an important factor or selection criterion during wheat domestication and in modern day breeding programs (Peng *et al*., 2011). While protein quality is an important trait for nutritional purposes, starch makes up approximately 70% of grain endosperm and, therefore, has a great influence on the resulting seed size and weight (Nadolska-Orczyk *et al*., 2017). Under non-limiting conditions, stem carbohydrates will contribute between 10 and 62% to the final grain weight, while this value increases from 40 to 100% under stressful conditions (Ehdaie *et al*., 2008).

The maximum grain weight is limited by the carpel size before anthesis occurs, and the final weight is determined during grain-filling i.e. approximately from just before anthesis to around two weeks after. Additional weight will be accumulated in plants with delayed senescence (Xie *et al*., 2016). In a study, Xie *et al*. (2016) reported that TKW and grains per spike were determined by dry weight and these two traits in turn determined the final grain weight per spike. Genes controlling TKW, *TaSus1* (chromosome 7 loci) and *TaSus2* (chromosome 2 loci), which are integral in the sucrose conversion pathway where sucrose is converted to starch, are very important in grain weight selection (Hou *et al*., 2014). QTLs related to grain weight per spike have been reported on chromosomes 3A, 3B, 4A, 4B, 4D, 5A, 5B, 5D, 6B and 6D (Marza *et al*., 2006; Liu *et al*., 2014b).

2.2.5.12. Harvest index

Harvest index (HI) is the ratio of harvested yield, such as grain in the case of cereals like wheat, to the total aboveground biomass, and it is used as measure of how effective a crop is in translocating its assimilates to harvestable yield (Reynolds *et al*., 2012b). This is a very important trait in crop improvement and substantial yield increases in wheat and other cereals has been through its alteration (Peltonen-Sainio *et al.*, 2008).

The introduction of dwarfing genes which spurred the “Green Revolution” resulted in an improvement in HI (Sadras and Slafer, 2012). This led to significant increases in HI from around 0.3 to the ranges of 0.45-0.50, and 0.50-0.55 for spring and winter wheat cultivars, respectively (Xie *et al*., 2016). While these values may seem high, they have been stagnant

since the early 1990s, and it is believed that wheat is still quite below its potential ceiling of approximately 0.64 (Foulkes *et al*., 2011; Reynolds *et al*., 2012b).

HI is complex because it depends on a balance between individual components (biomass and grain yield), and its selection is further complicated by shortened straw (not always being profitable) as well as high environmental effects on HI (Peltonen-Sainio *et al.*, 2008). Source-and-sink demands are constantly changing during crop development. The trade-offs in partitioning must be understood to ensure maximum assimilate partitioning towards grains without underinvesting in the roots, stems and leaves required for physiological and structural integrity (Reynolds *et al*., 2012b). Peltonen-Sainio *et al.* (2008) also found that oat cultivars, with a high HI fail to have constant ranking in yield.

HI is determined over long periods, which is why it is affected by changes in the environment (positive or negative) depending on their timing and duration (Peltonen-Sainio *et al.*, 2008). An example of good timing and duration would be mild unfavourable conditions before anthesis, followed by favourable conditions during grain-filling, which will favour grain weight and result in high HI (Peltonen-Sainio *et al.*, 2008).

HI generally has a positive relationship with yield. The trait is also associated with a number of YRTs, having a weaker (but positive) relationship with grain number rather than with grain weight (Peltonen-Sainio *et al.*, 2008). Tiller number has a positive relationship with HI stability (Peltonen-Sainio *et al.*, 2008). Lodging is also closely related since reducing vegetative biomass weakens plant structure making it unable to withstand increased grain mass. The best option for avoiding lodging is improving the root plate spread while stem width is being reduced to ensure anchorage (Reynolds *et al.*, 2012b). Plant height and HI however, are negatively associated in not only wheat but also in barley and oats as well (Peltonen-Sainio *et al.*, 2008; Xie *et al*., 2016).

2.3. Wheat breeding

As a result of the “Green Revolution”, staple foods were more abundant which lead to cheaper prices and in the process the number of undernourished people was largely reduced globally (Webb, 2009). The advancements during this period reduced world poverty and avoided the conversion of massive areas of lands to agricultural cultivation (Pingali, 2012). This was done through a combination of breeding and crop husbandry practices (Pingali, 2012). The impact of the “Green Revolution”, however, was more pronounced in non-limiting agro-ecological regions with adequate resources and less so in marginal environments (e.g. Africa) where yield gains to the high-yielding germplasm were less than 10% compared to the 40% of the former (Pingali and Kelley, 2007).

With yield increases having reached a plateau (when yield is the main selection criteria), and the ever-increasing population putting increasing pressure on food production, there needs to be a second "revolution" to boost production. Pingali (2012) reported that the "Green Revolution 2.0" has already started, especially in developing countries. Furthermore, this new revolution should be aimed at improving stress tolerance of crops, especially with the current scope of global climate change. Ferrante *et al.* (2017) suggested that any further improvements to wheat yield would be by selecting for physiological traits that can be used in both selecting of parental lines and progeny selections. Suggestions into breeding for stress tolerance included selecting for vigorous early growth to ensure adequate root development and establishment (Turner and Nicolas, 1987). This trait also has the added advantage of limiting water loss due to transpiration.

Now plant breeding is defined as the "art and science of improving the genetic pattern of plants in relation to their economic use" (Sleper and Poehlman, 2006). Plant breeding began in the early days of human civilizations as humans ceased their nomadic lifestyle, selected food suited for their needs in a purely artistic manner and in so doing they improved, and domesticated a lot of crops (Ram, 2014). It is said this period was around 12,000 years ago with the domestication of primitive wheat in fertile Mesopotamia (Hirst, 2017). This meant that farmers themselves were also breeders and selection was done almost exclusively *via* mass selection. This resulted in certain individual plants making greater contributions to the progeny and generations thereafter than others (Smýkal *et al.*, 2016). Over multiple generations through centuries, the plant breeding tools were constantly improving but it still remained an "artform" and they resulted in improved varieties and improved seed quality (Hirst, 2017).

The most important of these discoveries was that of Gregor Mendel who is attributed as the father of genetics due to his insights from his work with peas and other plants, thus beginning the science aspect of plant breeding. Published in 1865, the importance of Mendel's work was only understood in 1900 and soon after, many discoveries were made in the field of plant breeding (Smýkal *et al.*, 2016). Milestone examples include Wilhelm Johannsen developing the Pure-Line theory as well as coining the terms "genotype" and "phenotype", Shull exploiting heterosis in making hybrid crosses from inbred lines, and Jones developing the first commercial hybrid maize (Table 2.1) (e-Krishi Shiksha, 2012). A number of new plant breeding techniques were established following the discovery of Mendelian principles and they were developed on the basis of these principles, including pedigree and backcrossing approaches (Kingsbury, 2009).

Over many generations of “artistic” breeding, yield was the main goal for the farmer breeders when it came to food crops. Singh *et al*. (2016b) reported that in wheat (and related crops) the hierarchy of farmer needs on a cultivar start with grain yield as the highest ranked trait, followed by quality parameters and then disease resistance. The main focus of breeding is the development of higher yielding varieties with acceptable end use quality (Tsilo *et al*., 2013). In the process of domesticating wheat, the accessions of donor species with their fixed genetic backgrounds as well as selected traits resulted in reduced genetic variation in the crop, a factor that is hindering the current rate of genetic gain (Yang *et al*., 2009; Singh *et al*., 2015; Murchie, 2016).

Due to this reduced variation which is a result of intense breeding, further improvements in yield will not come from direct yield selections, but from a good understanding of morphological and physiological basis of yield and then come up with new selection criteria (Hutsch and Schubert, 2017). This requires a multi-disciplinary effort involving crop physiologists whose role is to study physiological traits related to yield. These traits should then be grouped into four different classes, namely: dry matter economy of the wheat crop, grain yield components, water and nitrogen economy of the crop (Ferrante *et al.,* 2017). Reynolds *et al*. (2012a) suggested that output-driven breeding programs, although needing to be multi-disciplinary, should be led by plant breeders and crop physiologists who can navigate the project towards the desired goals.

Table 2.1 Significant plant breeding milestones (adapted from e-Krishi Shiksha, 2012)

Year	Milestone
9 000 BC	First evidence of plant domestication in the hills above the Tigris river.
3 000 BC	Domestication of all important food crops in the Old World.
1 000 BC	Domestication of all important food crops in the New World.
700 BC	Assyrians and Babylonians hand pollinated date palms.
1694	Camerarius demonstrated sex in (monoecious) plants and suggested crossing as a method to obtain new plant types.
1716	Mather of USA observed natural crossing in maize.
1719	Fairchild created first artificial hybrid of dianthus sp. (Carnation × Sweet williams).
1727	Vilmorin introduced the concept of progeny testing.
1753	Linnaeus published Species plantarium. Binomial nomenclature was born.
1866	Mendel published his discoveries in Experiments in plant hybridization, culminating in inheritance and discovery of unit factors (genes).
1899	Hopkins described the ear-to-row selection method of breeding in maize.
1908–1909	Hardy of England and Weinberg of Germany developed the law of equilibrium of populations.
1908	Nilsson Ehle proposed the concept of multiple factor hypotheses.
1909	Shull conducted extensive research to develop inbreds to produce maize hybrids.
1917	Jones developed first commercial hybrid maize.
1926	Pioneer Hi-bred corn company established as first seed company.
1934	Dustin discovered colchicines.
1935	Vavilov published The scientific basis of plant breeding.
1940	Harlan used the bulk breeding selection method in breeding.
1943	Establishment of CIMMYT.
1944	Avery, MacLeod, and McCarty discovered DNA is hereditary material.
1945	Hull proposed recurrent selection method of breeding.
1950	McClintock discovered the Ac-Ds system of transposable elements.
1952	Introduction of *Rht* genes in the US *via* the “Norin 10” variety.
1953	Watson, Crick and Wilkins proposed a model for DNA structure.
1970	Borlaug received Nobel Prize for the “Green Revolution”.
1970	Berg, Cohen and Boyer introduced the recombinant DNA technology.
1995	*Bt* corn developed.
1996	Roundup Ready® soybean introduced.
2004	Roundup Ready® wheat developed.
2009	Schnable *et al*. - Sequence of the first crop genome (*Zea mays*) published.
2012	Jinek *et al*. - CRISPR programmed for targeted *in vitro* DNA cleavage.

Wheat breeding efforts in South Africa started at the beginning of the 20th century with the introgression of rust resistance of “Reiti” in locally adapted landraces. This was followed with attempts at adding rust resistance sourced from *A. elongatum*, *T. timopheevii*, “H44–24”, and other sources but because this followed vertical resistance selection, there was always a bust

after a boom in wheat resistance (Pretorius *et al.*, 2007). In the 1960s, breeding for rust resistance resulted in the successful release of cultivars with improved resistance worldwide. This together with reduced inoculum and the introduction of fungicides, moved the focus away from rust resistance breeding and more towards increasing yield as part of the Green Revolution (Pretorius *et al.*, 2007, 2012; Singh *et al.*, 2016a). The discovery of the *Ug*99 (TTKSK) pathotype in 2000, revived the interest in breeding for increased disease resistance in wheat globally (Pretorius *et al.*, 2012).

The renewed interest in breeding for disease resistance as well as the current trend of breeding for abiotic stresses in international public wheat breeding, has resulted in reduced attention towards increasing genetic yield potential (Reynolds and Borlaug, 2006; Braun *et al.,* 2010). Future yield gains are also negatively-affected by global climate change, diminishing natural resources, increasing costs inputs, and competition for arable land (Reynolds *et al*., 2012a).

2.3.1. Breeding selection methods used in the study

2.3.1.1. Recurrent selection

Recurrent selection was initially used as an important procedure for breeding cross-pollinated crops and the name was initially given in 1945 by F.H. Hull. As one of the oldest selection methods, the procedure now has increasing use in the improvement of self-pollinating crops such as wheat and the aim is to gradually concentrate desirable alleles in a population. This is done through cyclic repetition of selecting desirable individuals (with trait/s of interest) from the population which are crossed to form a new population (Acquaah, 2007). Recurrent selection is especially important in the improvement of quantitative traits (Marais and Botes, 2009). Singh *et al.* (2015) reported that the procedure was ideal for enhancing frequencies of desired trait alleles in populations, breaking the current limitations of a narrow genetic base on commercialized cultivars (Yang *et al*., 2009). This selection method also has the advantage of improving populations without compromising genetic variability (Acquaah, 2007).

The method requires extensive hybridization, which may be a limitation in selfing crops but the use of male sterility genes has removed this shortcoming. Another disadvantage of the procedure is the possibility of breaking genetic links between desirable traits due to the constant recombinantion as a result of crossing. There are four main recurrent selection schemes used: simple recurrent selection, recurrent selection for general combining ability as well as specific combing ability and the last being reciprocal recurrent selection which exploits both specific and general combining ability (Acquaah, 2007).

2.3.1.2. Single-seed descent

The single-seed descent selection method (also modified pedigree) may be used with other bulk population breeding methods as a means to reduce the chance of genetic drift within the population. The method was first proposed in 1941 by C.H. Goulden to speed up breeding programs before individual plant selection starts, while maintaining genetic diversity. One seed is randomly selected per plant in early segregation stages and its main objective is to attain homozygous plants quickly and only thereafter begin making selections. The procedure is ideal for selfing small grains or pulses such as wheat and soybean, which can be grown in high density populations and still produce seed. Advantages of the procedure are that it doesn't need a lot of space in the early stages and these are usually done in greenhouses and it is best used in programs where the cultivar is not bred in the same locality it will be ultimately commercialised in. Disadvantages associated with the method are that there's no effect of natural selection on the method so any possible advantages of natural selection are lost, and also selections are based on the selected plant phenotype without progeny performance tests (Acquaah, 2007).

2.3.2. Use of male sterility in wheat

2.3.2.1. Reproduction in plants

Plant reproduction is broadly divided into two, sexual reproduction and secondly, asexual or vegetative reproduction which leads to progeny that resemble the mother plant. For sexual reproduction to occur, male and female gametes fuse to form a zygote which in turn goes through cell division to form the plant offspring (Acquaah, 2007). There are different rates of cross fertilization within sexually reproducing crops: more than 95% (outcrossing or allogamous), between 5% and 94% (mixed mating) and less than 5% (self-fertilizing or autogamous) (Gniech Kurasowa, 2015). In angiosperm (flowering) plant species, 9% reproduce asexually, 62% use outcross, 12% have mixed mating and 17% of them self (Fryxel, 1957). Acquaah (2007) reported that it is important for reproductive systems to be known, especially by plant breeders because it determines the structuring and maintenance of the species genetic diversity as well as the choice of breeding method applied to it.

Wheat falls under autogamous plant species with between 1% and 4% naturally occurring cross pollination (Singh *et al*., 2015). A narrow genetic diversity is one of the characteristic consequences of self-pollinating species as alleles get fixed. This loss of diversity reduces response to environmental changes in plants, and in extreme cases can lead to extinction of populations (Herlihy and Erkert, 2002).

Naturally outcrossing species employ several mechanisms to ensure that outcrossing occurs and it does so between unrelated individuals. These include self-incompatibility or chemical systems, physical systems such as unisexuality (monoecy and dioecy) and temproral systems (protandry and protogyny) (Gniech Kurasowa *et al.*, 2015). The use of hybrids in plant breeding allows for the combination of desirable traits, and when genetically unrelated parental lines are hybridized, the resulting progeny is usually superior to both parents due to heterosis or hybrid vigour.

Hybrid vigour is also expressed in self-fertilizing crops such as wheat and rice but the process of hybridization in these crops is labour-intensive which in turn leads to high costs of hybrid seeds (Singh *et al*., 2015). Well executed exploitation of hybrid vigour is believed to improve wheat quality parameters as well as increase the crop yield with up to 15% (Singh *et al*., 2015). This is more-so the case with wheat where adoption of hybrid seeds is quite low, covering only about 0.2-0.3 million ha (less than 1% of total area) compared to 17-20 million ha planted with hybrid rice seed (Longin *et al*., 2012).

The main adopters of hybrid wheat are France and Germany in Europe which account for about half of the production, as well as India and China which produce above 30 000 ha of hybrid wheat each (Longin *et al*., 2012). In order to produce hybrids from selfing crops, breeders need to limit self-pollination in female parental lines using mechanisms such as emasculation, chemically and genetically induced male sterility, temperature and/or photoperiod sensitive male sterility, cytoplasmic male sterility, self-incompatibility, and biotechnological techniques that induce pollen abortion (Singh *et al.*, 2015).

2.3.2.2. Male sterility systems used in wheat

a) Emasculation

Emasculation in wheat is done *via* physical clipping of the florets just below the anther tips using scissors a few days before the anthers shed their pollen and in so doing, remove both the top half of the glumes and the anthers (Wells and Caffey, 1956). Another method of emasculating wheat flowers is the use of water treatments. This is done by immersing wheat spikes into hot water (40-45^{o}C) for about 5 minutes (Otsuka *et al*., 2010). Both procedures are labour-intensive and also time-consuming and cannot, therefore, be used in large scale breeding programs.

b) Chemically induced male sterility

Certain chemicals, referred to as gametocides, have the ability to cause physiological abnormalities in wheat pollen, resulting in disruption of pollen development, shedding and its viability (Singh *et al.*, 2015). Examples of such chemical sterilization or hybridizing agents

(CHAs) include: DPX-3778, ethrel, pyridine mono-carboxylates and Clofencet (Genesis) which is mostly used by Monsanto (Adugna *et al*., 2004; Parodi and Gaju, 2009). The most important aspect in selecting the appropriate CHA is that it should have minimum negative effects on plant growth and grain yield (Singh *et al*., 2015). Hormones such as gibberellic acid (GA3) and deficiency of the micro nutrient boron induce male sterility in wheat (Chowdhury *et al*., 2008). Limiting factors to the adoption of CHAs in developing wheat hybrids include bio-safety concerns as well as the variable effect, optimum dosage and cost-effectiveness in achieving complete sterility in female lines (Murai *et al*., 2008). There have also been reports of poor seed germination, poor vigour and general poor performance in CHA-derived wheat hybrids (Adugna *et al*., 2004). The adoption of this technology is prevalent in European hybrid production (Singh *et al.*, 2015).

c) Cytoplasmic male sterility

Cytoplasmic male sterility (CMS) arises as a result of defective signalling between genes located in the nucleus and those found in the cytoplasm of plant cells (Chase, 2007). CMS was first reported in 1951 (Kihara, 1951) and since then, over 70 cytoplasm male sterility genes have been studied for use in wheat (Singh *et al*., 2010). There are three main groups of wheat CMS: T-CMS which carries cytoplasm from *T. timopheevii* (Wilson and Ross, 1962), K-CMS with *Ae. kotschyi* cytoplasm, and V-CMS that has cytoplasm from *Ae. variabilis* (Lucken, 1987). T-type CMS is the most common and widely used form of cytoplasmic male sterility (Adugna *et al*., 2004; Singh *et al*., 2010). For this system to be used in hybrid development, fertility must be restored in the F1 generation and as such, restoration genes are incorporated into male-fertile pollinator lines such as *T. timopheevi, T. dicoccoides,* and *T. spelta* genotypes (Panayotov *et al*., 1986).

The need for restorer lines means that there is a third line that breeders need to maintain on top of the two main ones, namely: male sterile, maintainer and restorer lines, which increases the costs of hybrid development by this method. Other disadvantages of the method include: unstable male sterility, negative alloplasmic and cytoplasm effects and limited options of sources of fertility restorer genes (Ikeguchi *et al*., 1999; Liu *et al*., 2002). Regardless of such shortcomings, CMS is used for hybrid development in both China and India (Singh *et al*., 2010; Longin *et al*., 2012).

d) Photo/thermo/photo-thermo/thermo-photo sensitive male sterility

Photosensitive male-sterile plants are usually responsive to long-day photoperiods which leads to pollen abortion. Therefore, breeders induce cytoplasmically controlled male sterility by exposing them to long days of more than 14 hours and at stages where selfing and seed formation on the plants needs to occur, thus, they are exposed to short day environments (Murai, 2001). Thermosensitive wheat genotypes express male sterility when exposed to low

temperatures of below 4°C or high temperatures above 25°C during meiosis (Fotiou *et al*., 2010; Ji *et al*., 2010). Other plants combine these two temperature cues resulting in two kinds of ecological-genic male sterility. One is affected to a greater degree by the photoperiod, with some degree attributed to temperature and the other is the opposite, with temperature having a greater effect (Li *et al*., 2006; Jian-Kui *et al*., 2009).

This method of using environmental cues to control male sterility is a great improvement on the use of CMS because it removes the need for maintaining three lines (Zhou *et al*., 2011). Other advantages of this system are that it is maintained easily and is relatively cheaper than other systems (Li *et al*., 2006). Thermosensitive male sterility is said to be better suited to tropical regions that have fairly constant day length and aren't therefore suitable for photoperiod-sensitive sterility (Virmani and Donald 1996). The two main shortcomings are partial sterility issues and low resistance to diseases that are associated with environmentally controlled male sterility (Dai *et al*. 2008). Photoperiod sensitivity-based male sterility has been successfully used to develop hybrids in China (Singh *et al*., 2015).

2.3.2.3. Genetic male sterility in wheat

Genetic male sterility (GMS) has simple or Mendelian inheritance since the genes that control it are found in the nucleus of gametes and not in the cytoplasm like CMS. This sterility system is more common since it arises as a result of spontaneous mutational events and has been identified in at least 175 plant species (Chaudhury, 1993). GMS is normally controlled by single male sterility genes with two main alleles (*Ms* is the dominant and *ms* is the recessive) though some plants have exhibited nonallelic gene action (maize and tomato) and some multiple gene action (Horner and Palmer, 1995). Most of the genetic male sterility genes affect gamete development rather than the structure of the flowers or flower parts. These genes act in early pollen development where they affect the development of the tapetum which is the inner wall of microsporangium that provides enzymes and hormones for microspores (Chaudhury, 1993; Horner and Palmer, 1995).

The first documented male sterile wheat plant came about as a spontaneous mutation found in a wheat field in 1972 in China (Gao, 1987). This was noticed in one plant which, at flowering stage, formed semi-transparent and widely-open glumes which attracted the interest of an agricultural technician. The plant was named "*Taigu*" and its anthers were small and yellow-white, with a normal pistil and where spikes were covered with a bag to enforce self-pollination, no seeds were recovered. Where pollen was transferred from plants with normal anthers, the resultant F1 seeds segregated into a 1:1 ratio of normal (male fertile): mutant (male-sterile) progeny (Deng and Gao, 1982). This segregation pattern was indicative of a trait controlled by a single dominant gene which was named *Ta1* (new nomenclature *Ms*2), and

mapped onto the short arm of chromosome 4D, 31.1 cM from the centromere (Deng and Gao, 1982; Liu and Deng, 1986). To further explain, male sterile plants are heterozygote (*Ms2ms2*) and when pollinated by male-fertile plants (*ms2m2*) their progeny is 50% sterile and heterozygous (*Ms2ms2*) and 50% fertile (*ms2m2*) (Deng and Gao, 1982; Zhai and Liu, 2009).

Since GMS plants do not exhibit a different phenotype (other than non-viable pollen) from those that are fertile within the same population, removing unwanted plants was previously problematic until the *Ms2* gene was combined with dwarfing genes *Rht-D1c* (previously *Rht10*) (Cao *et al*., 2009). The two genes, *Ms*2 and *Rht-10,* are 0.18-1 cM apart (Liu *et al*., 2002). Cloning the *Ms2* gene has been difficult because the male sterile gene cannot be fixed and is always mixed with the *ms2* from the sterile paternal parent (Cao *et al*., 2009). The gene was only recently cloned, but no markers have been made available for use in breeding (Ni *et al*., 2017).

Many reports of GMS in wheat have been reported but only five GMS loci have been located. Two of these are recessive which are *ms1* and *ms5* and the other three are dominant including *Ms2* as well as *Ms3* and *Ms4* (Singh *et al*., 2016b). The *Ms3* gene was found in the nuclear-cytoplasm hybrid of wheat and *Ae. squarrosa* after a ethyl methane sulfonate (EMS) treatment (Maan *et al*., 1987). This *Ms3* gene is located on chromosome 5AS while *Ms4* is on chromosome 4DS (Maan and Kianian, 2001). The recessive *ms1* locus has has seven allelic mutants: Pugsley's (*ms1a*), Probus (*ms1b*), Cornest one (*ms1c*), *FS2* (*ms1d*), *FS3* (*ms1e*), *FS24* (*ms1f*) and *LZ* (*ms1g*) (Zhou *et al*., 2008).

2.3.3. Marker use in breeding

Genetic markers highlight a specific aspect on a genome and can be in the form of a nucleotide or a short sequence of DNA subunits. DNA is deoxyribonucleic acid. DNA's helical structure is made of many nucleotides that have a pentose sugar, a phosphate group and one of four bases as their building blocks. The four bases are adenine (A), guanine (G), thymine (T) and cytosine (C) and the DNA structure is held together by strong hydrogen bonds to form the double helix structure of DNA. Two complementary nucleotides (either A and T or G and C) bind together to form a base pair (bp) and the DNA sequence of an organism carries its genetic information required for the organism's function (Dreisigacker, 2012). Of the overall wheat DNA sequence, a very small portion contains genes and a majority of it is non-coding DNA made up of repetitive sequences or microsatellites. Genetic markers are a variation in sequences and arise as a result of mutations or alterations to genome (William *et al*., 2007).

Genetic markers have become a vital resource in plant breeding programs in a number of ways. Using markers, breeders can keep track of trait based genes over breeding generations which increases the speed and efficiency of releasing breeding material. Markers have also

be used to uncover the phylogeny and population structure of crop germplam as well as identify genes controlling traits *via* QTL or association mapping (Dreisigacker, 2012). The use of genetic markers carries a number of advantages over selections conducted on the bases of phenotypic or morphological markers. Markers are not affected by changes in environments (selective neutral behaviour), are unlimited in number and distributed all over plant genomes and they reduce costs in breeding programs by allowing for early selections of material (Herrera-Foessel *et al*., 2011; Dreisigacker, 2012).

In choosing the best marker to use, multiple factors need to be considered. The markers should be easily accessible as well as cheap to use. In diploid plants they should be able to distinguish between homozygous and heterozygous states (co-dominant inheritance) and should also be highly polymorphic (William *et al*., 2007). Ideal markers should also be frequently showing on the genome and have high reproducibility, i.e. analysis conducted with them should be easily duplicated elsewhere (Dreisigacker, 2012).

There are various types of genetic markers that have been developed and used in breeding exercises around the world. For example, simple sequence repeat (SSR) or microsatellite markers are tandem repeats of sequences made up of 1-6 bp long monomers repeated several times. They are dispersed through the genome, are highly co-dominant and they are the most polymorphic marker system with differences in alleles arising from differences in the length of repeats (Plaschke *et al.*, 1995; Dreisigacker, 2012). Plaschke *et al.* (1995) and another independent study reported higher intraspecific polymorphism in SSRs than amplified fragment length polymorphism (AFLP) (Röder *et al.,* 1998).

SSR markers also gained popularity due to their reproducibility and the information they provide (De Loose and Gheysen, 1995). The most commonly used SSR markers in breeding were developed by Röder *et al.* (1998) and are identified by the suffix ‘GWM’. Röder *et al.* (1998) discovered that the highest number of microsatellite markers was found in the B-genome, followed by the A-genome then the D-genome with 115, 93 and 71 SSR markers, respectively. Pallavi *et al*. (2015) reported that co-dominant SSR markers are extremely useful in large scale pre-breeding efforts because they identify plants with resistance in the homozygous state very early on which can be selected without mistakenly selecting heterozygotes as well.

Sequence characterized amplified regions (SCARs) markers were developed from RAPD (random amplified polymorphic DNA) markers by designing longer primers (22–24 bp long) for specific amplification of a particular locus (Michelmore *et al.*, 1991). There are also single nucleotide polymporphism (SNP) markers which are single base pair mutations at a specific locus, giving rise to typically two alleles. They are advantageous because they have numerous detection systems with various throughput and multiplexing levels already commercially available (William *et al*., 2007). Cleaved amplified polymorphic sequences (CAPS) markers are generated by restriction enzyme digestion of polymerase chain reaction (PCR) and are polyphormic in nature. These markers can easily detect mutations such as insertions, deletions as SNPs. Other examples of markers include diversity array technology (DArT) marker, sequence-tagged site (STS) markers, and many more (Dreisigacker, 2012).

Molecular markers are the basis of MAS. Markers for disease resistance are particularly important as they allow for breeding programs to breed for resistance in areas which are not disease hotspots and make it possible to stack multiple genes on the same germplasm (Herrera-Foessel *et al*., 2011). Because wheat was domesticated through multiple crossing events, it has a large gene pool of related plant species. Inter-chromosomal translocations from these species carrying useful genes (with available markers) enable breeders to transfer segments containing the genes of interest into elite wheat lines (Dreisigacker, 2012).

2.3.3.1. Wheat quality markers

Protein content is one of the most important yield-associated traits of wheat because it relates to human nutrition. High molecular weight (HMW) glutenins only make up around 10% (3-5 subunit per hexaploid variety) of the total seed storage proteins, but they are the main determinants of end-use quality of wheat grain because they affect the viscosity and elasticity of wheat dough (Payne, 1987; Shewry and Halford, 2002; Koga *et al*., 2017). They alone account for 47–60% of variation in wheat bread making quality (Payne *et al*., 1987; Lukow *et al*., 1989). Using nullisomic, tetrasomic, nulli-tetrasomic, and ditelocentric series lines, HMW glutenins were shown to be encoded by the *Glu-1* loci located in the long arms of 1A, 1B, and 1D chromosome (designated *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively) near the centromere (Payne *et al.*, 1984; Liu *et al*., 2008).

Each locus has two tightly linked HMW glutenin genes which are named x-type and y-type and they have different molecular weights (Liu *et al.*, 2008). The y-gene on chromosome 1A is silent on most wheat cultivars (hexaploid), but active in several diploid and tetraploid wheats (Payne and Lawrence, 1983). This silencing of certain genes results in only three to five HMW protein subunits from the potential six (Zamani *et al*., 2014). Due to the close link between the x-type and y-type units, it is sometimes difficult to determine which subunit is related to baking

quality (D'Ovidio and Anderson, 1994). Subunits linked to good baking quality are: Ax1 and Ax2∗ in *Glu-1A* locus; *Bx17* + *By18*, *Bx7* + *By8*, or *By9* are in the *Glu-1B* locus, and *Dx5* + *Dy10* are in the *Glu-1D* locus (Payne and Lawrence, 1983; Payne *et al.,* 1987; Radovanovic *et al*., 2002; Butow *et al*., 2004). On the other hand, AxNull (*Glu-1A*), *Bx6* + *By8*, *Bx20* + *By20* (*Glu-1B*), and *Dx2* + *Dy12* (*Glu-1D*) are related to undesirable baking quality (Payne and Lawrence, 1983; Payne *et al.,* 1987; Shewry *et al.,* 2003). *Glu-1D* has the largest effect on baking quality with the *Dx5* and *Dy10* combination (Liu *et al.,* 2008).

Previous methods that were used by plant breeders in selecting wheat varieties for baking quality are SDS tests, Zeleny sedimentation and mixograph assessment. These methods were not effective because they required large amounts of seed to run tests and they were destructive procedures (Gale, 2005). However, all of the HMW glutenin genes have been cloned and sequenced and bacterial artificial chromosome (BAC) clones are available for use in marker assisted selection (Ragupathy *et al*., 2008).

Most HMW glutenin subunits are identified by SDS-PAGE which separates the different alleles of baking quality markers based on their mobility or molecular weight (Liu *et al.*, 2008). Other subunits such as Dx2, Dx5, Dy10 and Dy12 have been cloned to markers such as SNPs that are easily identified in agarose gels (Giancaspro *et al*., 2016b). However, agarose markers are mostly dominant markers which make it difficult to tell heterozygous and homozygous species apart as well as failed PCR from negative reactions. There is also presence of non-genome specific amplification resulting in multiple PCR products and high quality DNA is often required to track certain markers (Liu *et al*., 2008).

Low-molecular-weight subunits are the most prevalent, making up about a third of the total seed proteins, and almost two thirds of the glutenins (Bietz and Wall, 1973). They significantly contribute to grain quality, not to the degree of HMW glutenins, but through additive and epistatic interactions (Koga *et al*., 2017). The *Glu-3* loci has many genes on it, with between 20 and 40 genes in hexaploid wheats (An *et al*., 2006). This large number of genes limits the use of LMW glutens in SDS-PAGE because of their complex banding patterns with overlaps in LMW genes as well as gliadins (Koga *et al*., 2017). There are at least seven, nine and five alleles respectively for *Glu-A3*, *Glu-B3* and *Glu-3*, respectively (Zhang *et al*., 2004; McIntosh *et al*., 2008). Zhang *et al*. (2004) identified one multi-allelic gene controlling LMW glutenins in the *Glu-A3* locus and developed seven PCR markers for these alleles. Wang *et al*. (2010) also isolated LMW genes in the locus and developed STS markers for a multiplex PCR reaction. The marker used in this study, however, was a microsatellite reported by Manifesto *et al.* (2001) to be linked to the LMW genes.

2.3.3.2. Rust resistance markers

The best resistance to pathogens is achieved by combining multiple non-specific genes within the same genotype. Adult plant resistance (APR) involves quantitative resistance with a number of minor genes that limit disease progression in adult plants (Gustafson and Shaner, 1982). Marker use is central in successful pyramiding of genes, and to confirm presence of specific genes with high accuracy. With the use of markers there is no need for disease inoculation trials (Tsilo *et al*., 2009; Pumphrey, 2012). Pyramiding is done *via* backcross breeding adding genes in a relatively short time, especially when used with MAS (Figlan *et al*., 2014). The major disadvantage associated with this method is that when adding new genes onto existing gene pyramids, there may be a slight disruption onto the existing pyramid (Pretorius *et al*., 2007). At times, there may be issues with expensive genotyping costs and marker reliability versus phenotyping accuracy (Terefe *et al.*, 2016).

With regards to the local South African wheat breeding history, one of the oldest breeding exercises ever conducted was to introduce rust resistance genes into local germplasm (Pakendorf, 1977). Breeding for both yield and rust resistance is done in a number of breeding programs around the globe. The efficiency in such program is reduced in that selection for both these traits reduces genetic gain, but to combat this, CIMMYT expanded its population size (Terefe *et al.*, 2016).

e) Stem rust resistance genes

Resistance against stem rust in host plants is separated into two major groups. Firstly, there is seedling resistance, which is conferred by major genes and is effective from the seedling stage. Secondly, adult plant resistance which is conferred by minor genes and starts functioning at later stages of growth (Figlan *et al*., 2014). Genotypes that have APR usually have moderate susceptibility to the pathogen, but accumulation of favourable QTLs leads to genotypes with higher resistance (Singh *et al*., 2016a). Five wheat stem rust genes have been identified to confer high levels of quantitative APR: *Sr2*, *Sr55* (*Lr67/Yr46/Pm46*), *Sr56*, *Sr57* (*Lr34/Yr18/Pm38*), and *Sr58* (*Lr46/ Yr29/Pm39*) (Lagudah *et al.,* 2006; Yu *et al.,* 2014).

The major gene, *Sr2*, was first used in the 1920s by McFadden who selected it from “Yaraslav” emmer wheat and at a later stage was mapped onto the short arm of chromosome 3B near the *csSr2* marker (Jia *et al*., 2018). *Sr2* is widely deployed in rust resistance programs as it has additional resistance to other diseases such as leaf rust, stripe rust and powdery mildew (McIntosh *et al.,* 1995). Additive gene effects have been reported when *Sr2* was used with *Sr33* on Chinese Spring seedlings (Ayliffe *et al*., 2013). *Sr2* has been successfully used in wheat breeding programs for more than 80 years and has even been effective against *Ug*99 and its variants (Jia *et al*., 2018).

Sr2 is also closely linked with pseudo-black chaff (PBC) expression which is a melanin pigmentation in wheat glumes and towards the nodes of the stems (Jia *et al*., 2018). PBC expression levels are variable and are affected by plant genotype as well as the environment (Singh *et al.,* 2008). This phenotypic expression has been used as a morphological marker for the gene, but not extensively due to the variable expression (Sharp *et al*., 2001). Molecular markers used to keep track of the gene in breeding programs include *xgwm533* and the CAPS based *csSr2* marker (Spielmyer *et al*., 2003; Jia *et al*., 2018). *Xgwm533* reveals *Sr2* presence as a 120 bp fragment but the marker gives problems with false positive results (Hayden *et al.*, 2004). The CAPS marker is much more accurate for *Sr2* detection, gives 53 bp, 112 bp and 172 bp fragments in *Sr2*-carrying genotypes, as well as 112 bp and 225 bp fragments in the absence of *Sr2*. This marker is also closer to the *Sr2* gene than the former (Jia *et al*., 2018).

The *Sr31* resistance gene was sourced from the rye 1BS.1RS translocation which was derived from a cross between “Petkus” rye and Russian wheat cultivars “Kavkaz” and “Avrora” (Purnhauser *et al*., 2011). While certain portions have been transferred from all seven rye chromosomes into wheat, this specific translocation is of great importance to breeding because it carries resistance genes to a number of pests. Besides *Sr31*, other resistance genes associated with the translocation are *Lr26* for leaf rust resistance, the *Yr9* gene for stripe rust resistance and the *Pm8* gene for resistance to powdery mildew (Zeller, 1973). These genes have been mapped to near the end of chromosome 1RS, specifically around 5 cM distal to the *Sec-1* genes for seed-storage protein and additionally, no recombination events have been reported between the “Petkus” rye genes due to a tight linkage (Singh *et al*., 1990; Mago *et al*., 2002).

The 1RS chromosome segment caused reduced grain quality resulting in sticky dough and reduced dough strength. These issues were the result of monomeric secalins from rye and the substitution of glutenins and gliadins (*Glu-3* and *Gli-1*) (Dhaliwal and MacRitchie, 1990). To remedy this, multiple cycles of homeologous recombination between chromosome arms 1RS of “Petkus” rye and 1BS of wheat variety “Pavon” resulted in recombinant lines with a number of breakpoints between the *Sec-1* locus, disease resistance genes and the *Gli-1*/*Glu-3* loci (Lukaszewski, 2000). *Sr31*, having a high degree of resistance to stem rust was widely deployed all over the world with high success until it was overcome by *Ug99* (Pretorius *et al.*, 2000). Although having been overcome by the *Ug99* race of stem rust, the gene is still used widely in breeding programmes that aim to pyramid rust resistance genes into their germplasm across the world (Das *et al*., 2006). Encouraging results have been shown where the gene has enhanced resistance, more so in germplasm with either *Sr25* or *Sr24* (Menon and Tomar, 2001).

Das *et al*. (2006) cloned and validated SCAR markers that were successfully optimized for use in identifying both *Sr*31-carrying (SCSS30.2$_{576}$) and deficient genotypes (SCSS26.2$_{1100}$) by producing PCR products of 576 bp and 1 100 bp, respectively. These markers have an added benefit in that they do not amplify for the *Sr*24 gene in the same region meaning they could be used in pyramiding these genes without false positives. Mago *et al.* (2002) also developed specific STS marker from an iag95 probe which could amplify PCR products containing the "Petkus" rye segment with a 1 030 bp fragment size.

Wheat relatives with homeologous or partly homologous genomes have provided other rust resistance genes such as *Sr26*, which was sourced from *A. elongatum* and introgressed *via* irradiation into 6AS.6AL-6Ae#1 translocation chromosome close to the centromere on the long arm (Knott, 1961). Dundas *et al*. (2007) further reduced the gene location to the extreme distal portion of the 6Ae#1 chromosome, stating a linkage to loci *Xmwg573-6Ae#1*, *Xmwg798-6Ae#1*, and *Xmwg2053-6Ae#1*. The initial segment transferred resulted in a yield penalty of about 9% in wheat, and these negative effects were attributed to linkage which led to the development of new translocation lines with shortened *A. elongatum* segments that did not reduce wheat yield (The *et al*., 1988; Dundas *et al*., 2001).

Sr26 has the advantage of having low frequencies among modern cultivars and providing effective resistance against a number of the *Ug99* derivatives (Lowe and Soria, 2010). The low frequency ensures reduced interaction between the gene and *Ug99* which in turn prolongs its durability but this can also be ensured through gene pyramiding.

f) Leaf rust resistance genes

Leaf rust has been less of a problem globally in recent years due to successful deployment of slow rusting resistance (APR) genes onto cultivars (Singh *et al*., 2016a). Around 80 leaf rust resistance genes have been described in wheat, and most of these were sourced from germplasm related to wheat (Zaman *et al.*, 2017). The *Lr24* gene is one such gene which has been used in breeding programs and is tightly linked to the *Sr*24 gene and therefore germplasm with the gene has some resistance to both rusts. *Sr24*/*Lr24* was transferred into wheat from the 3Ag chromosome of *A. elongatum*, a wheat relative, with the transferred chromosome segment located on the satellite of chromosome 1B and named T1BL.1BS-3Ae#1L (Menon and Tomar, 2001). The initial segment containing the gene was linked to red grain colour but with the use of recombinant lines, Sears (1973) broke the linkage drag and shortened the gene segment so it could be deployed to white grain cultivars.

Another *Sr24*/*Lr24* gene combination was discovered in a wheat variety called "*Amigo*", from a translocation that was first use in rye before being transferred into the wheat variety. In this variety the segment was located on the short arm of chromosome 1B and not chromosome

3DL (Lowe and Soria, 2010). The gene gives resistance to rust from the seedling stage and is still functional at the adult stage of wheat plants, making it an attractive candidate for gene pyramiding (Pallavi *et al.,* 2015). However, both genes in the gene complex have been overcome by their respective pathogens, with *Sr24* not being effective against *TTKST*, a *Ug99* variant, and virulence to *Lr24* reported in South Africa as well as North and South America (Pretorius and Kemp, 1990; Long *et al*., 1994). *Sr24/Lr24* can still be used effectively by combining the gene with other rust genes to increase resistance abilities, e.g. a combination of *Lr9* and *Lr24* is said to be effective (Pallavi *et al.*, 2015).

McIntosh *et al*. (2011) reported that the location of the gene on chromosome 3DL was closely linked to *Xgwm114*, and a number of markers for the gene have been reported as well. Mago *et al*. (2005) catalogues an SSR marker (*BARC71*) as well as two AFLP markers (*Sr24#12* and *Sr24#50*) that can be used to confirm presence of the gene. The *Sr24#50* (200 bp) was reported to be a more reliable marker for scoring *Sr24/Lr24* than the *Sr24#12* marker (500 bp) because the former is a dominant marker and the latter marker amplified a band with lower intensity for non-carriers (Pretorius *et al.*, 2012). Pallavi *et al*. (2015) also reported a RAPD marker (OJP-09), a DNA-STS marker and six restriction fragment length polymorphism (RFLP) markers, all linked to the gene. A RAPD marker ($S73_{728}$) was used to develop a new SCAR marker, $SCS73_{719}$, which can successfully detect the *Sr24/Lr24* translocation by amplifying a 719 bp band (Cherukuri *et al.,* 2003).

Lr34 is a multi-fungal resistance gene that confers adult plant resistance and is widely used in breeding programs for its slow rusting effects that is not easily broken down by new leaf rust races (Terefe *et al.*, 2014). The gene was first reported in “Frontana” in 1966 (Dyck *et al*., 1966) and has been effective for a long time and are now used in cultivars planted on at least 26 million ha of land (Krattinger *et al.*, 2009). *Lr34* resistance is achieved through a longer latent period, by reducing the development rate of haustoria and hyphae between plant cells leading to uredia spores that are smaller in size and number (Krattinger *et al.*, 2009). Keller *et al*. (2013) reported that *Lr34* confers resistance to other diseases such as stripe rust (*Yr18*), powdery mildew (*Pm38*) and increased tolerance to barley yellow dwarf virus (*Bdv1*) as well as resistance to stem rust pathogens in selected genetic backgrounds.

Singh and Huerta Espino (1997) reported a small but significant reduction in biomass and other YRTs for *Lr34*-carrying near isogenic lines (NIL) (compared to those not carrying the gene) under disease-free environments. On the other hand, *Lr34* had a positive effect on flour protein content and water absorption values of NILs carying the gene (Labuschagne *et al*., 2002). The gene has a tight linkage to the leaf tip necrosis (LTN) locus in wheat, allowing this phenotypic expression to be used as a morphological marker in some CIMMYT lines but this expression is affected by the environment and genetic backgrounds (Keller *et al*., 2013).

The *Lr34* gene has been successfully cloned and found to be located on chromosome 7DS, with a nucleotide sequence of 11 805 bp and it is close to locus *Xgwm295* (Krattinger *et al.*, 2009). The authors found that only three polymorphisms separate the alleles of the resistant and susceptible cultivars: An A/T single nucleotide polymorphism in intron 4, a 3 bp (TTC) deletion in exon 11 and a C/T single nucleotide polymorphism in exon 12. This allowed for the development of a gene-specific marker, *cssfr1*. Lagudah *et al*. (2006) had previously developed an STS marker, *csLV34*, which is 0.4 cM from *Lr34*, and when these two markers are used together in a multiplex reaction, co-dominance can be observed.

Another leaf rust resistance gene sourced from a wild relative is *Lr37*, which was sourced from the 2NS chromosome of *T. ventricosum* Ces. (syn. *Ae. ventricosa* Tausch, genome designation NvDv) which was transferred to chromosome 2AS of bread wheat (Seah *et al*., 2000). The fragment is about 25 to 38 cM long and contains other resistance genes for diseases such as powdery mildew, rusts (*Sr38, Lr37, Yr17*), eyespot as well as resistance genes to pests like cereal cyst nematode and Hessian fly (Seah *et al*., 2000). The fragment was initially transferred into a wheat cultivar named “VPM1” and since the segment does not recombine with wheat chromosomes, the linked genes are always transferred together (Bariana and McIntosh, 1993).

2.3.3.3. *Fusarium* resistance markers

Buerstmayr *et al*. (2009) reported that resistance to FHB is polygenic, being conferred by a number of genes and also quantitatively inherited. Furthermore, it was reported that QTLs controlling resistance to FHB are found throughout the bread wheat genome outside of chromosome 7D. There are seven QTLs linked to FHB resistance which are: *Fhb1, Fhb2, Fhb4*, and *Fhb5* (sourced from wheat) as well as *Fhb3, Fhb6,* and *Fhb7* (from wheat wild relatives) (Buerstmayr *et al*., 2009; Ruan *et al*., 2012). At least 100 FHB resistance QTLs (mostly related to Type II resistance) have been found in “Sumai #3” and other resistance sources through the use of RILs and double haploid technology (Buerstmayr *et al*., 2009). Although this number is very high, progress in transferring these QTLs for use in breeding programs has been slow due to some QTLs being specific to populations (Lv *et al*., 2014).

Fhb1 is the most widely used QTL in breeding programmes and it is found on the short arm of chromosome 3B and previously designated *Qfhs*.ndsu-3BS (Xue *et al*., 2010). The QTL was sourced from “Sumai #3” and it is flanked by three markers *Xgwm533* (above) and two tightly linked markers (*Barc133* and *Xgwm493*) at the bottom (Anderson *et al*., 2001). Using mutation analysis, gene silencing as well as transgenic overexpression, Rawat *et al*. (2016) were able to show that FHB resistance by *Fhb1* was due to a pore-forming toxin-like (PFT) gene. *Fhb1*, together with *Fhb2*, confer Type II resistance on wheat plants which prevents the

spread of the pathogen within the head (Martin *et al.*, 2018). Type II and Type I (resistance to initial infection conferred by *Fhb4*) contribute to the field resistance that is observable in released cultivars (Prat *et al*., 2014). To evaluate the presence of Type II resistance, single-floret inoculation is done where the rate of disease spread along the ear is measured (Prat *et al*., 2014).

Type II resistance has been favoured in many breeding programs because it is easier to assess than Type I and there is a lack of germplasm exhibiting good Type I resistance (Ruan *et al*., 2012). Lemmens *et al*. (2005) reported the involvement of *Fhb1* in detoxifying the DON mycotoxin into deoxynivalenol-3-glucoside (DON-3G), i.e. Type III FHB resistance. Some negative effects such as decreases in flour yield and the protein content have been associated with *Fhb1* (McCartney *et al*., 2007).

Fhb5 (initially named *Qfh.ifa*-5A or *Qfhi.nau*-5A) on chromosome 5A typically confers Type I resistance but has some smaller contributions to Type II resistance as well (Prat *et al*., 2014). To test for Type I resistance, spray inoculation is done followed by measuring the proportion of spikes showing FHB symptoms are measured (Prat *et al*., 2014). Xue *et al*. (2011) discovered that the 5A QTL was inherited as a single Mendelian gene, i.e. a 3:1 ratio in the F1 population, and thereafter it was named *Fhb5*. The gene is located in a 0.3 cM interval between two markers: *Xgwm304* and *Xgwm415*.

A number of studies into FHB resistance have reported QTLs with major effects to host resistance on the 7A chromosome of wheat (Jia *et al*., 2005; Li *et al*., 2016; Kumar *et al*., 2007; Jayatilake *et al*., 2011; Giancaspro *et al*., 2016a). These QTLs are mostly associated with Type II and Type III resistance (Jayatilake *et al*., 2011), however, Giancaspro *et al*. (2016a) reported involvement of a QTL on the same chromosome associated with Type I resistance.

Conflicting results have been published about the actual location of the QTL on chromosome 7A with some results mapping it in the short arm (Mardi *et al*., 2006), the long arm (Jia *et al*., 2005; Kumar *et al*., 2007) or in the proximity of the centromere (Jayatilake *et al*., 2011). This suggested multiple QTLs on the chromosome: a) the 7AS QTL was mapped into the *Xe77m47-22–Xgwm233* interval, b) 7AL is in the 15 cM distance between markers *Xgwm276* and *Xgwm282* with a tight linkage to *Xbarc121* and c) the 7AC QTL is flanked by *Xbarc174* and *Xwmc17* (Jia *et al*., 2005; Mardi *et al*., 2006; Jayatilake *et al*., 2011).

The conflicting results on the QTL location is most likely due to the QTL being close to the centromere and thus could easily be mistaken to fall on either side of the chromosome. Kumar *et al*. (2007) named this QTL *Qfhs.fcu*-7AL which spans 39.6 cM with its location very close to the centromere. They also postulated a possible presence of other minor QTLs on the chromosome but suggested they were undetected due to a small population in their project.

The QTL is associated with either the FHB resistance QTL found on the 7E chromosome of *Thinopyrum* or *T. turgidum* sp. *dicoccoides* (Shen and Ohm, 2007). Jayatilake *et al*. (2011) named the QTL *Fhb7AC* due to its inheritance and further explained that even though it did not outperform *Fhb1* in terms of infection rate, *Fhb7AC* resulted in lower DON content and the two expressed additive gene effects.

2.4. Remote sensing based phenotyping

In breeding and pre-breeding programs, plant breeders select genotypes based on their physical performance or in other terms, their phenotype. The phenotype of a plant is defined as a set of traits related to a specific genotype's structure, morphology, physiology and performance in a specific environment (Granier and Vile, 2014). Breeders observe this aspect in the field and is a result of the plants interacting with the environment in which it is grown, taking into account the biotic and abiotic factors (Haghighattalab *et al*., 2016). The study of these traits is referred to as phenotyping and entails timely measurements of plant performance, physiology as well as response to stresses and the ultimate yield produced at harvest (Haghighattalab *et al*., 2016). Genetic variation is the keystone of plant breeding and once it has been identified or generated in a selection population, precise phenotyping is integral in developing new varieties (Gilliham *et al*., 2017).

The challenges for breeders that they are working with enormous amounts of genotypes, especially in the early stages of their programs and the traits that need to be studied are time sensitive. To overcome this, high-throughput phenotyping platforms need to be developed and utilized which will not only analyse large numbers of entries simultaneously, but will also be quick in collecting precise and valid data of plant performance (Haghighattalab *et al*., 2016). Reynolds (2012b) reported that the phenotyping protocol used by breeders is dependent on three factors: the target environment (and trait), scale of operation, and the degree of precision required for the trait.

With the expansion and adoption of technology in all fields, as a result of the "Digital Revolution", there are opportunities for agriculture to take part and utilize these advancements through the use of remote sensing and spatial mapping technology to improve targeting and monitoring of agricultural resources (Pingali, 2012). Developments in non-invasive imaging, image analysis and computer processing allow for assessment of plant height, transpiration, growth and biomass in both field and controlled environmental conditions (White *et al*., 2012; Sankaran *et al*., 2015). Functional phenotyping approaches don't only help in making the job of the breeder easier, but also help in gene discovery of the underlying genetics controlling the phenotype (Pinto *et al.,* 2010).

2.4.1. Remote sensing in agriculture

Remote sensing refers to measurements of reflected radiation without any physical contact made between the measuring sensor and the source (Mulla, 2013). In remote sensing, transmitted or absorbed radiation is neglected. In agriculture, remote sensing use focuses on radiation reflected by soil and plant matter (Mulla, 2013). Remote sensing in agriculture has been used to study plant properties such as crop yield (Reynolds, 2016), biomass (Price, 2014) and plant nutrients (Möller *et al.*, 2007), biotic and abiotic stresses such as water stress (Allen, 2016), weed stress (Thorp and Tian, 2004), diseases (Kumar *et al.*, 2016; Mills, 2016) and also soil properties like organic matter, pH (Christy, 2008), and the amounts of water, clay, and salt in the soil (Hairmansis *et al*., 2014). Remote sensing in Agriculture came about due to the increasing adoption of precision agriculture in the United States in the 1900s (Mulla, 2013).

Precision agriculture involves farming management practices that are applied at the right place, at the right time and right amounts for that specific place, thereby reducing wastage of agricultural inputs (Mulla, 2013). To do this, intensive data and information are collected and processed to determine what inputs need to be applied, as well where and how much needs to be applied. By doing so, crop productivity is improved and the environment is protected from any harm (Harmon *et al*., 2005). For precision agriculture to be possible, there needs to be technological advances in computer processing, field positioning, yield monitoring, remote sensing and sensor design (Mulla and Schepers, 1997). Effective sensors for remote sensing need to be strong, use renewable energy where possible as well as continuously collect data in a short space of time and passing it onto analyses computing platforms wirelessly for reliable up-to-date information (O'Shaughnessy and Evett, 2010).

2.4.1.1. History of remote sensing in agriculture

Remote sensing is classified according to the sensor platform used. Historically it began with the use of satellites but has since expanded to aerial and ground based (proximal) platforms (Mulla, 2013). Quiroz (2016) reported that the most basic use for remote sensing in agriculture is observation of crops and estimating yield. Satellite imagery use in agriculture first started with the launch of Landsat 1 (originally known as Earth Resources Technology Satellite 1) in 1972 (Doraiswamy *et al.*, 2003). This technology has a lot of disadvantages, the first being the distance between the sensor and source resulting in cloud cover being a limiting factor on the use of the technology, especially under rain-fed plantations (Quiroz, 2016). The cost of getting quality data from satellite imagery is beyond what most farmers can afford in developing countries (Mulla, 2013). These two factors are combined in areas close to the equator and the cloud cover problem is persistent (Hall, 2016).

Satellite platforms also tend to have very low spatial resolutions in the images captured, with the Landsat 1 resolution being 80 m which gives very little detail on fields (Price, 2014; Hall, 2016). Another reported issue has been the return visit frequency of these satellites which makes it difficult to use them in time-sensitive measurements (Hall, 2016). These issues are addressed in newer satellites like GeoEye, with sub-metre resolution, as well as the Worldview satellite which improved return visit frequency from the 18 days of Landsat 1 to just one day (Mulla, 2013). The progress made on the satellite platforms is still not enough. Issues such as the correction of imagery for atmospheric interferences still need to be addressed and, therefore, better platforms need to be brought forward (Mulla, 2013).

Ground-based or proximal or close-range remote sensing makes use of sensors that are mounted on tractors, spreaders, and irrigation booms or sometimes hand-held like the Trimble® GreenSeeker® crop sensing system (Mulla, 2013; Price, 2014). Another example of proximal sensing is the Lamnatec Scanlyzer which is a gantry with a multiple-sensor imaging platform. It was developed to help identify new traits that would be sufficiently robust to be introduced into breeding programs using imagery (Dornbusch *et al.*, 2015).

Proximal sensing doesn't always involve measuring reflected radiation in that measurements can be done directly from crop canopies (Haghighattalab *et al*., 2016). The main advantages of obtaining measurements directly are that it improves resolution, allows for data to be collected from multiple angles, and the distance between the plants and the sensor is known and, therefore, can be kept constant (White *et al.*, 2012). Ground-based platforms are limited in the scale at which they can be used, they may be bulky and may take a long time to make measurements whilst resulting in soil compaction during the process (Vroegindeweij *et al*., 2014). These platforms need to be used in combination or replaced with aerial-based phenotyping platforms to address limitations (Haghighattalab *et al*., 2016).

2.4.2. Remotely piloted aerial systems-based phenotyping

2.4.2.1. History and classification

The most increasingly widely adopted form of remote sensing is aerial remote sensing, using Unmanned Aerial Vehicles (UAV) also known as Remotely Piloted Aircraft Systems (RPAS) or drones. The United States Department of Defence (2007) defines a UAV as "a powered, aerial vehicle that doesn't carry a human operator, uses aerodynamic forces to provide vehicle lift, can fly autonomously or be piloted remotely, can be expendable or recoverable and can carry a lethal or nonlethal payload". They vary in shape and size and autonomous flights are done through flight-planning and control software based on global positioning system (GPS) co-ordinates (Simelli and Tsagaris, 2015).

The first idea of developing RPAS was from the legendary inventor Nikola Tesla who postulated a remotely controlled aircraft with the abilities to change direction mid-flight, be exploded if needed and didn't miss its intended target. In 1889 he developed his aircraft named the "Telautomaton" which was operated on multiple radio frequencies that controlled specific actions of the craft (Vroegindeweij *et al*., 2014). After the invention of gyrostabilizers, for use in manned aircrafts by Lawrence Sperry to be automatically controlled, the possibility of applying this technology gave rise to the development and use of unmanned flying bombs since the early 1900s. Following this, RPAS technology was used extensively in military activities such as bombing and targets for anti-aircraft training as well as assault RPAS in World War II (Newcome, 2004).

RPAS used in remote sensing need to have at least three key components: the RPAS craft, a sensor (or sensors) and a platform allowing communication between the drone and its radio controller (Quiroz, 2016). The RPAS itself has a GPS navigation system, fully-intergrated multi-axis gyroscopes and accelerometers, pressure and airspeed sensors and most importantly, a reliable power-source like a battery or internal combustion engine. With automation tools, RPAS can take off, follow flight plan waypoints and once completed, land where they took off. Constant communication between the device and the remote allows the device to follow programmed fail-safe commands such as returning to its launch point when there are issues in-flight such as loss of GPS signal or altitude (Simelli and Tsagaris, 2015).

RPAS are separated into different classes based on different criteria, for example in the military they are classified their on size and flight duration. Using this criterion, there are four classes: a) high altitude-long endurance (HALE) UAVs, b) medium altitude-long endurance (MALE) UAVs, c) Regular use UAVs and d) small and portable UAVs. HALE UAVs reach altitudes of 20 km with a 850 kg payload and a 35 hour flight time, while MALE UAVs reach 8 km altitudes with loads of 200 kg for approximately 30 hours. Regular use UAVs reach heights of 4.5 km with a payload of 25 kg for up to six hours and portable UAVs are operated at visual line of sight (VLOS) so they reach up to 120 m (Simelli and Tsagaris, 2015).

RPAS have since been adopted for daily use in various personal and commercial uses around the world, from the media industry, in healthcare and emergency rescue missions, delivery of food and medical aid (Anderson, 2014). They are also used in security purposes at various levels and even in agriculture (Simelli and Tsagaris, 2015). RPAS use in agriculture was ranked as first in "2014 Breakthrough Technologies" by Massachusetts Institute of Technology (MIT) (Price, 2014). By late 2014, Grand View Research reported the UAV industry to be at $552 million globally and with its increasingly fast adoption to various uses, it is expected to go as high as $2 billion by the year 2022, with agriculture dominating most of the market share and an estimated 103776 new jobs by the year 2025 (Price, 2014).

The commercial RPAS industry has been active in South Africa for more than 20 years (Reitz, 2017). Industries that have or should be soon introducing the use of commercial RPAS in the country include: military, agriculture, architecture, media, as well as wildlife surveillance (Kapdi *et al*., 2018). The industry has huge potential in the country, with an estimated turnover of around R6 billion in 2016 and creating over 30 000 jobs in the process (Botha, 2017).

2.4.2.2. RPAS in agriculture

The first reported use of RPAS in agriculture has been in precision agriculture where they were used for chemical spraying purposes, where cloudy conditions affected visibility and nullified the use of manned aircrafts (Suguira *et al*., 2005). There are five RPAS categories according to their application in agriculture. Fixed and flying wing RPAS are similar to manned crafts and use wings and propellers for flights and have the advantage of being able to cover large areas of up to 400 ha (Price, 2014; Vroegindeweij *et al*., 2014).

Vertical take-off and landing (VTOL) RPAS use multi-rotors for mobility and, while they cannot cover long distances, they have the advantage of being easily controllable for diverse tasks such as hovering if needed (Vroegindeweij *et al*., 2014). They cover smaller areas of around 20 ha and can be fitted with gimbals to support heavier payloads (Price, 2014). Micro UAVs are used for monitoring of small spaces with limited flight range and can be propelled by flapping wings or rotors. Parafoils and airships glide using parachutes or balloons and therefore have longer flights and are used mostly for deliveries. Combination and concept RPAS are a blend of the above-mentioned principles and are tailor-made for their specific use (Vroegindeweij *et al*., 2014).

The use of manned aeroplanes in agriculture is a practice that began in the 1920s. The basic purpose for airborne systems is observation and provision of accurate and up-to-date data on crop status (Greenwood, 2016). With removing the pilot from inside the craft, specialized technologies have been developed that are based on sensors and microcontrollers as well as communication systems of ground control stations, to allow for control at ground level (Simelli and Tsagaris, 2015). Vroegindeweij *et al*. (2014) reported that the use of machines is usually reserved for jobs that are dull, dirty, dangerous or dependable and thus, RPAS are suited for field observation jobs that are time consuming and subjective to the observer (Greenwood, 2016). Since 2001, Yamaha has been marketing a remote-controlled helicopter-RPAS that can perform tasks such as seeding and chemical spraying (Vroegindeweij *et al*., 2014).

Reynolds (2016) reported a simple use of RPAS fitted with a red, green and blue (RGB) camera to count the number of coconuts in farmer plantations in Western Samoa. This was used to estimate the age of the palm trees and, consecutively, virgin coconut oil yield estimates could be made. In India, insurance companies use RPAS to quickly assess crop damage on their clients' fields in order to ensure speedy pay-outs for replanting, when possible (Garg, 2016). RPAS flights were conducted in Nigeria over a proposed rice paddy field with the purpose of assessing the land's geography and elevation which allowed agriculturists to plan irrigation infrastructure beforehand (Le, 2016). RPAS are not confined to plant production but have been used in animal production as well. Livestock farmers with expansive land use RPAS technology to locate their livestock in their land, to move them from one area to another (as a herding tool) and also for assessing the status of their fences. In the fisheries industry they are used to scout for illegal fishing vessels in protected water sources (Greenwood, 2016).

2.4.2.3. Indices and sensors used in RPAS-based phenotyping

Images taken with RPAS can be manipulated to give specific data by using multiple vegetation indices to compute the values, or in certain instances, other sensors may be mounted on the RPAS with the normal RGB camera. These indices are based on the ratios of the amount of plant reflected waves in the visible light (RGB, 550–700 nm) and/or near infrared (NIR, 700–1300 nm) wavelengths of the electromagnetic spectrum (Kumar *et al.*, 2016). This reflectance is a result of the optical properties (transmission, reflection and absorption) of leaf parts such as the cell wall, chloroplast and protoplasm (Figure 2.8) (Kumar and Silva, 1973; Carter and Knapp, 2001). Plants tend to strongly absorb visible light, more especially in the red and green wavelengths, but near infrared light is strongly reflected (Hall, 2016).

The Green-Red Ratio Vegetation Index (GRVI) and the Normalized Green-Red Ratio Difference Index (NGRDI) are based on reflectance of the green and red parts of the visible light spectrums. The Visible Vegetation Index (VVI) measures the amount of vegetation or greenness of an RGB image. Leaf Area Index (LAI) characterizes plant canopies and is a ratio between the area covered by a canopy and the ground the plant is on (Simelli and Tsagaris, 2015). Mulla (2013) gives a more detailed comparison of the different indices and their practical applications in agriculture studies.

The most commonly used vegetation index is by far the Normalized Difference Vegetation Index (NDVI) which is the reflectance ratio between red visible (550-700 nm) and near infrared (700–1300 nm) light waves (Simelli and Tsagaris, 2015; Hall, 2016). The basic use of this index is to give a measure of photosynthesizing leaves in a canopy or give the health status of plants built on the basis that healthier vegetation will absorb more light in the visible (green) spectrum and reflect more NIR light. NDVI values range from -1 (water) to +1 (plant with strong

vegetative growth) and the higher and the more positive the value, the healthier the plant is (Kumar *et al.*, 2016).

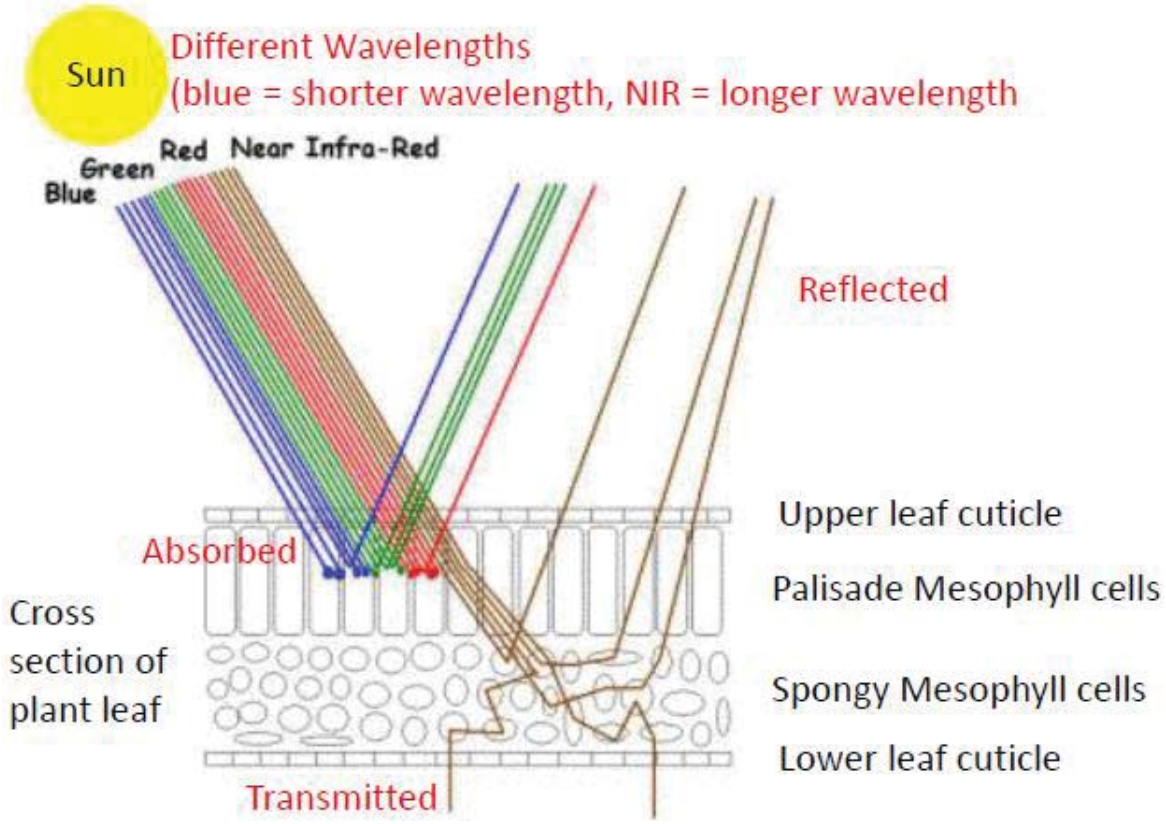

Figure 2.8 Light spectra and its interaction with plant leaves from which vegetation indices are based on (SEOS Project, 2018)

NDVI is used in a number of practical applications such as assessing fields and in the process identifying problem areas within the field; an application that is very important in precision agriculture more especially (Price, 2014; Allen, 2016). Kumar *et al.* (2016) used NDVI to assess crop response to spot blotch and reported that sensor-based tools can be used for disease scoring and report scores accurately (Pretorius *et al*., 2016). Use of NDVI for disease scoring is advantageous in that it is not affected by light changes through the day and it removes any bias on the data (as compared to human scoring) (Kumar *et al.,* 2016).

NDVI has also been used in non-destructive assessments of plant response to salinity (Hairmansis *et al*., 2014), measurement of LAI and biomass (Mulla, 2013; Jansen *et al*. 2014). Other uses include continuous assessment of crop health (Carter and Knapp, 2001; Allen, 2016) and potential yield forecasting up to two and a half months before harvesting (Price, 2014). By non-destructive assessment of plants, multiple measurements can be made with the same plants over time to get reliable time-series data (Furbank and Tester, 2011).

Although useful, NDVI has a few shortcomings like being influenced by soil reflectance in short crop populations as well as its insensitivity to changes in chlorophyll content as plants

approach senescence. This is evident especially in crops of area index beyond 2.0 (Mulla, 2013). To mitigate this limitation, the Green Normalized Difference Vegetation Index (GNDVI) is used, which substitutes red light reflectance for green light reflectance which is more sensitive to chlorophyll loss in senescing plants (Sripada *et al*., 2008).

Besides manipulation of the normal or true colour (RGB) images, specialized cameras or sensors have been developed and/or adapted for use in RPAS. Specialized NDVI cameras and infrared cameras have been used and they allow for visualization of crop status invisible to the naked eye (Hunt *et al.,* 2010; Milla, 2016). Low-cost consumer grade cameras can be converted to capture NIR images and their appeal is in their low cost and size, but they have the challenge of not being radiometrically calibrated (Haghighattalab *et al*., 2016). Thermal cameras may also be used, especially in studies that have to do with plant water status or canopy temperature (White *et al.*, 2012; Milla, 2016).

Multispectral cameras such as the MultiSpec 4C as well as hyperspectral cameras are also used (Busemeyer *et al.,* 2013; Chapman *et al.*, 2014; Haghighattalab *et al*., 2016; Milla, 2016). Multispectral imaging collects reflectance data with wider bands of more than 40 nm in the visible and NIR light spectrum whilst hyperspectral imaging collects data of narrower bands (10 nm) in a wider spectrum than the former. This allows for data to be collected from not only vegetation but soil as well (Mulla, 2013). Hyperspectral remote sensing is considered the future sensor of choice because it reveals more detailed data such as soil moisture and nutrients as well as plant chlorophyll and carotenoids (Milla, 2016).

2.4.2.4. Advantages to RPAS-based phenotyping

RPAS-related technology has been changing rapidly in recent years, and thereby elevating the field to perhaps the most rapidly advancing sensor-based platform for remote sensing uses in both agriculture as well as general environmental studies (Chapman *et al.,* 2014; Haghighattalab *et al*., 2016). RPAS have been miniaturized and materials used on them, such as carbon fibre, result in vehicles that are not only lightweight for easy transportation but also very durable (Simelli and Tsagaris, 2015). With the automation capabilities, RPAS have become much easier to use, and data processing costs are being reduced by the day as some image-processing programs are available as open-source software (Reynolds, 2016). A decade ago, RPAS would have been as expensive as a 120 kW tractor but nowadays a useful mapping RPAS can be purchased for below R10 000 and can even be custom-built by user for an even lower amount (Greenwood, 2016).

When compared to other remote sensing platforms, RPAS-based remote sensing has multiple advantages and is the best form of remote sensing. When compared to manned aircrafts, RPAS are better in that with their design they don’t need to accommodate for any

added space taken up and additional weight of the pilot (Sosa, 1997). Without any fragile life carried within the craft, RPAS are therefore able to perform dangerous missions and actions wherever needed and can cover entire plant breeding experiments in a short space of time (Vroegindeweij *et al*. 2014; Haghighattalab *et al*., 2016). RPAS can be programmed for flights at different height and speed levels, as required by the task at hand, and are usually not affected by minor changes in weather conditions (Haghighattalab *et al*., 2016).

Images taken by RPAS are much closer to the ground compared to satellite-derived images and, therefore, have a much higher resolution at a much lower cost and are also not affected by environmental conditions such as cloud cover (Vroegindeweij *et al*. 2014; Simelli and Tsagaris, 2015; Milla, 2016; Reynolds, 2016). Multi-rotor RPAS can approach their target to within a few centimetres during their flights (Xiang and Tian, 2011). Even with the improved return frequency of one day in satellites, it is still much less than what RPAS do since they can fly over whole experimental sites in a matter of minutes, and when necessary, their batteries can easily be changed (Mulla, 2013).

When compared to land-based phenotyping platforms, no wheel tracks are necessary for RPAS phenotyping platforms which releases more land for planting. Additionally, since there is no need for heavy machinery such as tractors to mount the platform, soil properties don't get affected by compaction. Difficult terrain conditions such as muddy fields are removed in aerial remote sensing. They are also very agile when compared to land-based platforms which allows them to approach their target (Vroegindeweij *et al*. 2014).

2.4.2.5. Challenges to RPAS-based phenotyping

Although useful, UAVs are not likely to replace all other traditional remote-sensing methods, especially manned aircrafts and/or satellites (Milla, 2016). Problems that may be encountered with the use of RPAS, include their payload limitation which (below 25 kg), especially on VTOL RPAS, which limits their use to mostly observational purposes (Xiang and Tian, 2011; Vroegindeweij *et al*., 2014). In developing countries that lack adequate infrastructure for uninterrupted electricity supply for battery charging purposes, RPAS adoption may be a challenge (Greenwood, 2016). Other issues in developing countries may be lacking infrastructure for quick internet access for the cloud computing tasks associated with RPAS data processing (Greenwood, 2016).

For automation, RPAS can follow GPS waypoints of planned missions but where problems arise, pilots need to intervene thus automation needs improvement (Vroegindeweij *et al*., 2014). Changes in weather conditions such as high wind speeds and precipitation will affect

the flying ability of RPAS, and at times camera settings may need to be adjusted to allow for continuity of data collection under different light conditions. Under controlled growing conditions such as greenhouses, light intensity varies substantially which has an influence on camera performance and in situations where animals are under buildings dust and greenhouse gases affect RPAS performance (Vroegindeweij *et al*., 2014).

The largest challenges around RPAS use are the laws and regulations applied by the governments and civil aviation organisations around the globe. This is due to RPAS usage being previously restricted for military use only, and only recently allowed for civilian and commercial use. Such legislation regarding their use is still not well established (Vroegindeweij *et al*. 2014). The need for regulation is due to issues related to the privacy, safety and security of society at large (Jeanneret, 2016).

Furthermore, in the United Kingdom, there was 23 near-misses between UAVs and airlines within a six-month period in 2015, which was a huge concern for commercial pilots (Jeanneret, 2016). Several countries, especially in the developing side such as India, and Kenya, have banned the use of RPAS by civilians; requiring explicit permission from authorities (Garg, 2016). A few countries have working legislation around the commercial use of RPAS (Jeanneret, 2016). In July 2015, the South African Civil Aviation Authority (SACAA) rolled out a set of regulatory laws regarding the use of RPAS which include the requirement of a remote pilot's license (RPL) for users at various levels (CAA, 2017).

2.4.2.6. RPAS Regulations in South Africa

Regulations released by the SACAA in 2015 are governed by Act No 13 of 2009 and they regulate RPAS usage for private, commercial, and research. Under private use a RPAS may only be used for one's personal and private purposes, and no commercial outcome or gain may arise from that. Private-use pilots still need to adhere to all statutory requirements relating to liability, privacy and other laws enforceable by any other authorities. For all other users, the RPAS must be registered and only operated in terms of the Part 101 of the South African Civil Aviation Regulations. RPAS should not be flown within 50 m of groups of people or property where permission has not been granted by the property owner. RPAS flights should be done on clear day conditions, with the system remaining within visual line of VLOS of the pilot and the maximum height allowable is 50 m above the ground. Permission is needed before pilots can conduct flights in proximity of manned aircrafts or controlled/restricted/prohibited airspace (airport, helipad and airfield) (CAA, 2017).

Chapter 3: Materials and methods

3.1. Introduction

Wheat production is faced with a number of challenges such as stagnant yields and reduced profitability. The collective field of plant breeding has a lot of work to do in order to remedy this. The aims of this study were to identify and assess traits that have been reported to confer yield in wheat and also to investigate the feasibility of a technologically-advanced high throughput phenotyping platform for assessing traits.

The first objective was to assess yield and its related traits in a population of 90 high-yielding wheat lines in order to identify possible crossing parents. This objective was addressed in multiple approaches. The first was conducting a multi-location field yield trial (MLFT) whilst simultaneously screening for genes linked to rust resistance and baking quality using genetic markers. The field trial was planted in three wheat producing localities of the Western Cape in the 2015 production season. A hydroponic study was also conducted in a controlled environment to study YRTs.

The second objective was to initiate a MS-MARS population aimed at increasing yield. A male sterile population (female) was crossed with a selected population of high-yielding germplasm. An initial cross was done between two nursery populations, a male-fertile (pollen donor) population and a male-sterile (female population). In the second cycle, progeny from the initial cross were the female population. The high yielding population was used as pollen donors and were selected from the initial population used in objective one. A total of 44 genotypes were selected based on results of objective one and 12 genotypes were also added from the Stellenbosch University-Plant Breeding Laboratory (SU-PBL) nursery which produced good yields. Progeny from this cross was grown under greenhouse conditions to initiate a single seed descent selection scheme.

The third objective was a pilot study into the feasibility of establishing a high throughput phenotyping platform that is based on RPAS technology. This was tested on the field trial of objective one where RPAS flights were programmed and conducted during the plant growth and development of genotypes. During these flights images were captured from which data were extrapolated on the performance of the genotypes. This data were correlated with actual field data that were collected at the same time as the flights to investigate if RPAS can be used to collect routine data.

Figure 3.1 shows a flowchart diagram with the inter-connected relationships between the aim and objectives that were undertaken in the study.

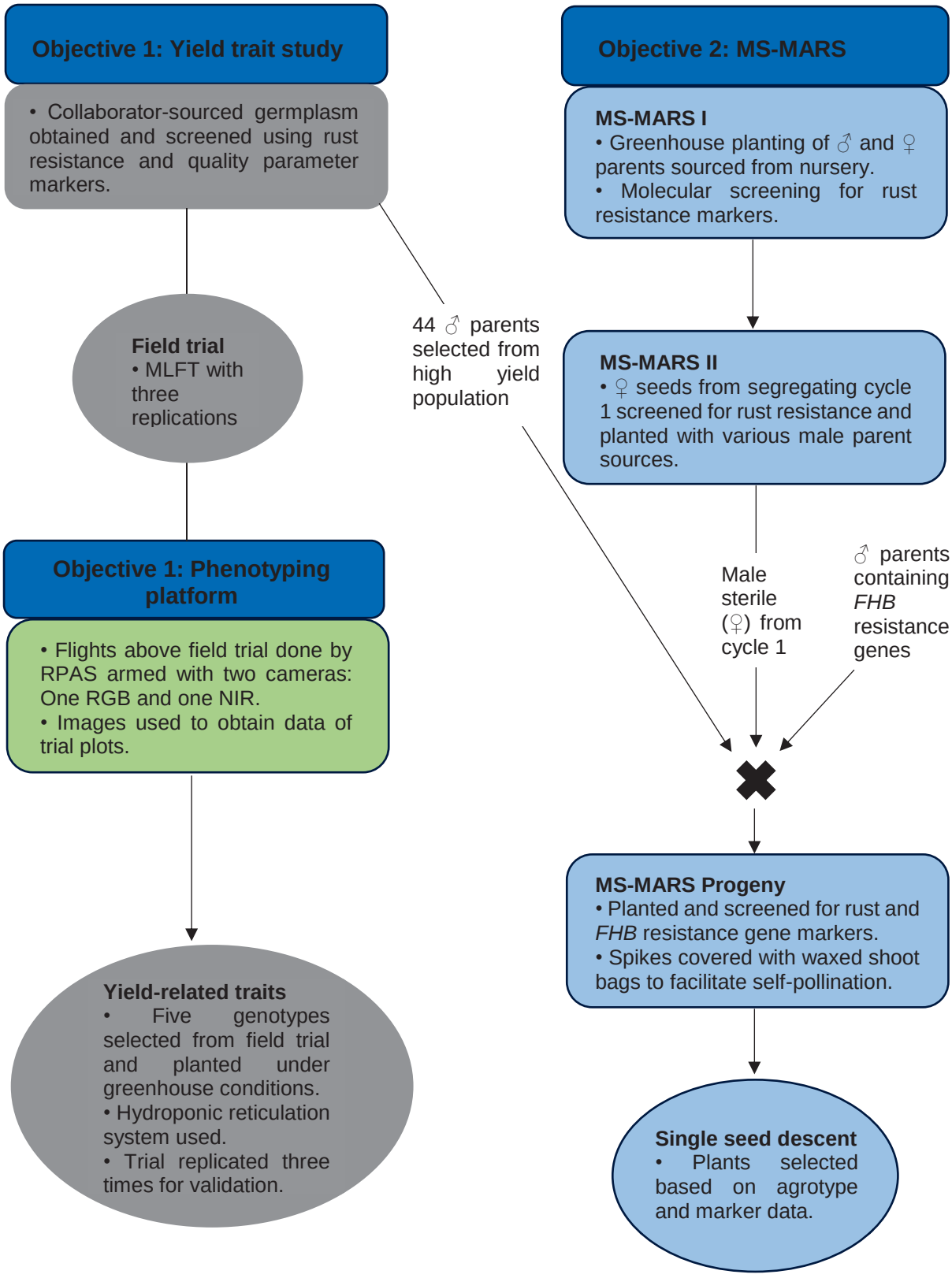

Figure 3.1 Flowchart of objectives and activities for the study.

3.2. Assessment of yield and related traits

3.2.1. Plant material

For the first objective of assessing yield and its related traits, germplasm was identified from local and international collaborators. In total, 90 genotypes (Addendum 1) were selected for the overall study. Five of the top 10 yielding genotypes were selected for a physiological trait study using hydroponics under greenhouse conditions (based on field trial data analyses).

3.2.2. Molecular marker assessment

Sixty genotypes received from collaborators with good YRTs were sown in two, 96-hole polystyrene growing trays as three replications for the collection of genomic DNA (gDNA) samples to be used for marker assessment. The extra 30 genotypes included in the field trial had already been screened for markers in a previous study.

3.2.2.1. Plant DNA extractions

DNA extractions were done using the cetyl trimethylammonium bromide (CTAB) method which was adapted and shortened from Doyle and Doyle (1990). Leaf sample cuttings of approximately 5 mm each were made and placed into into 2 ml micro centrifuge tubes using stainless steel scissors. Three stainless steel ball bearings and 500 µl 2% CTAB buffer [100mM Tris-Cl (pH 8.0), 1.4 M NaCl, 20mM EDTA (pH 8.0)] were added to each tube containing leaf sample. Tubes were closed and placed in a Qiagen® Tissue Lyser (Qiagen, Southern Cross Biotech, Claremont, South Africa), to grind the leaf material. This was done for three successive periods of 60 seconds at a shaking frequency of 30 Hz. The lysed mixture was then incubated in a 60°C water bath for 15 minutes, followed by an addition of 500 µl of chloroform: isoamylalcohol (24:1) in a fumehood. The mixture was centrifuged using a Microfuge® 18 Centrifuge (Beckman Coulter, Lonsdale, Pinelands, Cape Town, South Africa) at 14 000 rpm for 10 minutes.

Approximately 400 µl supernatant (of the centrifuged mixture) was transferred into clean 1.5 ml micro centrifuge tubes in which 400 µl chloroform: isoamylalcohol (24:1) was again added. Tubes were closed and centrifuged for another 10 minutes at 14 000 rpm, then the supernatant transferred into new 1.5 ml micro centrifuge tubes. Fifty microliters of 3M ammonium acetate, followed by 500 µl ice cold 100% ethanol were added to the tubes, which were thereafter inverted several times to precipitate the DNA.

Tubes were centrifuged with an Allegra™ X-22R Centrifuge (Beckman Coulter, Lonsdale, Pinelands, Cape Town, RSA) for two minutes (12000 rpm at 4°C) to coagulate the DNA strands into pellets and then the supernatant was discarded. DNA pellets were rinsed twice using cold

70% ethanol, and the alcohol was discarded afterwards leaving the pellets to air dry inside the tubes for an hour. Once dry, the pellet was re-suspended in 30 µl distilled water (dH_2O) and stored at -20°C as stock DNA. DNA concentrations were quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Kempton Park, RSA). Concentrated DNA was then diluted with water into 500 µl microcentrifuge tubes, to make a volume of 50 µl working DNA (100 ng/µl).

3.2.2.2. Polymerase Chain Reactions for molecular markers

Molecular markers for resistance to diseases as well as for quality parameters were screened in all the genotypic material using an updated standard panel of markers to the one previously used by Wessels and Botes (2014). Resistance markers for the rust pathogens that were screened for were the *Lr34*, *Sr2* markers as well as markers for the *Sr31*, *Lr24*/*Sr24*, *Lr37*/*Sr38*/*Yr17*, *Sr26* and *Lr19* which were multiplexed into one PCR (Table 3.1). For the *Fusarium* resistance genes, *Xgwm493* and *Xgwm533*, PCRs were performed individually then the markers *Xgwm130, Xgwm156, Xgwm233, Xgwm293, Xgwm304* and *Barc133* were combined into one multiplex PCR (Table 3.5). Quality parameter markers were *Dy10, Dy12, Dx5* which were run as a multiplex, and *GluA3* for flour quality. Genotypes were also screened for markers of the dwarfing genes *Rht-D1b* and *Rht-B1b* (Table 3.9). For all reactions, unless otherwise mentioned, the PCR primers used were at a concentration of 10 µM.

The rust resistance marker PCR work was done in three separate reactions. The first of these was the *Lr34* marker which was run as multiplex of the two different markers for the gene *cssfr1* and *csLV34*, respectively. The reaction conditions for this reaction are shown in table 3.2. The second reaction was for the *Sr2* marker which was run in two stages as shown in Table 3.4. The remaining rust resistance gene markers were run as a multiplex under the conditions shown in Table 3.3. All PCRs were performed using a 2720 Thermal Cycler (Applied Biosystems, Fairlands, RSA).

Table 3.1 Rust resistance molecular markers used in the study.

Rust resistance gene	Marker name	Primer sequences (5' → 3')	Ta (°C)	Expected fragment size (bp)	References
Lr34/Yr18/Pm38	*cssfr1* (*L34DINT9F* and *L34PLUSR*)	F: TTGATGAAACCAGTTTTTTTTCTA	58	517 +ve	Krattinger *et al*. (2009)
		R: GCCATTTAACATAATCATGATGGA			
	csLV34	F: GTTGGTTAAGACTGGTGATGG	56	150 +ve	Lagudah *et al*. (2006)
		R: TGCTTGCTATTGCTGAATAGT		229 -ve	
Sr2 (CAPS)	*csSr2*	F: CAAGGGTTGCTAGGATTGGAAAAC	60	53, 112, 172	Mago *et al*. (2011)
		R: AGATAACTCTTATGATCTTACATTTTTCTG			
Sr31	*Iag95*-STS	F: CTCTGTGGATAGTTACTTGATCGA	55	1 030	Mago *et al*. (2002)
		R: CCTAGAACATGCATGGCTGTTACA			
Lr24/Sr24	*SCS737 19*	F: TCGTCCAGATCAGAATGTG	55	719	Cherukuri *et al*. (2003), Prabhu *et al*. (2004)
		R: CTCGTCGATTAGCAGTGAG			
Lr37/Sr38/Yr17	VENTRUIP	F: AGGGGCTACTGACCAAGGCT	65	259	Helguera *et al*. (2003)
	LN2	R: TGCAGCTACAGCAGTATGTACAC AAAA			
Sr26	Sr26#43	F: AATCGTCCACATTGGCTTCT	60	207	Mago *et al*. (2005)
		R: CGCAACAAAATCATGCACTA			
Lr19-149	*STSLr19 130*	F: CATCCTTGGGGACCTC	60	119	Prins *et al*. (2001)
		R: CCAGCTCGCATACATCCA			

*Ta – annealing temperature

The PCR cycle for *Lr34* (Table 3.2) was as follows: 5 minutes at 94°C, 35 cycles of 1 minute at 94°C, 1 minutes at 57°C and 1 minute at 72°C, and a final extension step of 7 minutes at 72°C.

The *Lr34* PCR samples were ran on a 1.8% (w/v) SeaKem® LE Agarose (Lonza, Arch Chemicals Inc., Cape Town, RSA) (dissolved in 1X TBE buffer diluted from 5X TBE stock solution [0.5 M Tris (hydroxymetyl) aminomethane, 0.5 M boric acid, 0.5 M ethylenediamine tetra acetic acid disodium salt dehydrate (EDTA)]) gel with added 0.05g/l ethidium bromide

(*EtBr*) for staining. Gels were viewed under UV- light using a Uvitec gel imaging system (distributed by Whitehead Scientific Inc, Stikland, RSA).

Table 3.2 PCR volumes for *Lr34* marker.

Reagents	**Volume (µl)**
dH2O	4.6
Kapa® GreenMix	10
Din9F	0.6
Lr34+R	0.6
CSLV 34F	0.25
CSLV 34R	0.25
Total	**16.3**
DNA (100ng/µl)	1.5
TOTAL	**17.8**

The rust multiplex PCR cycle (Table 3.3) was as follows: 3 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 1 minute at 72°C, and a final extension step of 10 minutes at 72°C. After amplification, the PCR product was mixed with 5 µl Cressol loading dye thereafter ran on a 1.6% (w/v) gel with added 0.05g/l *EtBr* staining for visualization.

Table 3.3 Multiplex PCR volumes for markers *Sr31, Lr24/Sr24, Lr37/Sr38/Yr17, Sr26* and *Lr19*.

Reagents	**Volume (µl)**
2GFastMix	12.5
Iag 95F	1
Iag 95R	1
719F	0.5
719R	0.5
VENT	0.5
LN2	0.5
Sr26#43F	0.5
Sr26#43R	0.5
12CF	0.5
12CR	0.5
Total	**18.5**
DNA (100ng/µl)	1.2
TOTAL	**19.7**

The PCR cycle for *Sr2* (Table 3.4) was as follows: 5 minutes at 95ºC, 40 cycles of 30 seconds at 92ºC, 40 seconds at 60ºC and 50 seconds at 72ºC, and a final extension step of 5 minutes at 72ºC. After PCR, 7 µl of each sample was run on a 1.5% (w/v) agarose gel with 0.05g/l *EtBr* to visualize a 337 bp band. Samples which were negative or didn't show this band were discarded as negatives for the marker. To the remaining 5.9 µl PCR samples that had shown the first band, 2.5 µl of 10U PagI (BspHI) enzyme [made by 1 µl nuclease-free water, 1.25 µl 10X Buffer O and 0.25 µl restriction enzyme (PagI)] (Fermentas Life Sciences, Burlington, Ontario, CA) was added to each tube to digest the positive band. The mixture was

then incubated at 37°C for one hour and ran on a 2% (w/v) agarose gel with *EtBr* for visualization of the positive 172 bp band.

Table 3.4 PCR volumes for marker for *Sr2* (CAPS) marker.

Reagents	**Volume (µl)**
dH2O	3
Kapa® GreenMix	7.5
Cssr2F	0.45
Cssr2R	0.45
Total	**11.4**
DNA	1.5
Total	**12.9**

Table 3.5 Fusarium head blight resistance molecular markers used in the study.

QTL name	**Marker name**	**Primer sequences (5' → 3')**	**Ta (°C)**	**Expected fragment size (bp)**
Qfhs.ifa-5A-1	*Xgwm304*	F: AGGAAACAGAAATATCGCGG R: AGGACTGTGGGGAATGAATG	60	219
	Xgwm156	F: CCAACCGTGCTATTAGTCATTC R: CAATGCAGGCCCTCCTAAC	60	330
	Xgwm293	F: TACTGGTTCACATTGGTGCG R: TCGCCATCACTCGTTCAAG	60	207
7AQTL	*Xgwm130*	F: AGCTCTGCTTCACGAGGAAG R: CTCCTCTTTATATCGCGTCCC	60	126
	Xgwm233	F: TCAAAACATAAATGTTCATTGGA R: TCAACCGTGTGTAATTTTGTCC	60	248
Qfhs.ndsu-3BS	*Xgwm493*	F: TTCCCATAACTAAAACCGCG R: GGAACATCATTTCTGGACTTTG	60	211
	Xgwm533	F: AAGGCGAATCAAACGGAATA R: GTTGCTTTAGGGGAAAAGCC	60	160
	Barc133	F: AGCGCTCGAAAAGTCAG R: GGCAGGTCCAACTCCAG	60	125

* Adapted from Röder *et al*. (1998).

The PCR cycle for *Xgwm493* and *Xgwm533* (Table 3.6) was as follow: 2 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 40 seconds at 60°C and 50 seconds at 72°C, and a final extension step of 5 minutes at 72°C. The PCR samples were ran on a 1.5% (w/v) agarose gel with added 0.05 g/l *EtBr* for staining and then visualized under UV-light using a Uvitec gel imaging system.

The *Fusarium* marker (Table 3.7) cycle was as follows: 3 minutes at 94°C, 44 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, with a final extension step of 7 minutes at 72°C. The individual PCRs were optimized as a multiplex.

Table 3.6 PCR volumes for *Xgwm493* and *Xgwm533* markers when run individually.

Reagents	**Volume (µl)**
dH2O	5.75
Kapa® GreenMix	6.25
Primer F	0.5
Primer R	0.5
Total	**13**
DNA (100ng/µl)	1
TOTAL	**14**

Table 3.7 PCR volumes for *Xgwm130, Xgwm156, Xgwm233, Xgwm293, Xgwm304* and Barc133 markers when run individually.

Reagents	**Volume (µl)**
dH2O	5.75
Kapa® GreenMix	6.25
Primer-F	0.5
Primer-R	0.5
Total	**13**
DNA (100ng/µl)	1
TOTAL	**14**

The *Fusarium* multiplex (Table 3.8) cycle was as follows: 3 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C and 1 minutes at 72°C, and a final extension step of 10 minutes at 72°C. The PCR samples were ran on PAGE for visualization of markers.

Table 3.8 PCR volumes for the *Xgwm130, Xgwm156, Xgwm233, Xgwm293, Xgwm304* and Barc133 markers when run as a multiplex.

Reagents	**Volume (µl)**
2GFastMix	14.5
GWM304-F	0.5
GWM304-R	0.5
GWM293-F	0.5
GWM293-R	0.5
GWM233-F	0.5
GWM233-R	0.5
GWM130-F	0.5
GWM130-R	0.5
GWM156-F	0.5
GWM156-R	0.5
BARC133-F	0.5
BARC133-R	0.5
Total	**20.5**
DNA (100ng/µl)	1.2
TOTAL	**21.7**

Table 3.9 Wheat quality parameter markers used in the study.

Gene	Primers	Primer sequences (5' → 3')	Ta (°C)	Expected fragment size (bp)	References
Glu-Dx5	P1	F: GCCTAGCAACCTTCACAATC	63	450	Smith *et al*. (1994) Ahmad (2000)
	P2	R: GAAACCTGCTGCGGACAAG			
Glu-Dy10/ Glu-Dy12	P3	F: GTTGGCCGGTCGGCTGCCATG	63	576/ 612	Smith *et al*. (1994) Ahmad (2000)
	P4	R: TGGAGAAGTTGGATAGTACC			
GluA3	*Xpsp2999* F	F: TCCCGCCATGAGTCAATC	55	133-157	Manifesto *et al*. (2001)
	Xpsp2999 R	R: TTGGGAGACACATTGGCC			
Rht-B1b	BF	F: GGTAGGGAGGCGAGAGGCGAG	58	237	Ellis *et al*. (2002)
	MR1	R: CATCCCCATGGCCATCTCGAGCTA			
Rht-D1b	DF	F: CGCGCAATTATTGGCCAGAGATAG	58	254	
	MR2	R: CCCCATGGCCATCTCGAGCTGCTA			

The protein quality (Table 3.10) marker cycle was as follows: 5 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 63°C and 30 seconds at 72°C, and a final extension step of 5 minutes at 72°C. PCR samples were ran on a 1.8% (w/v) agarose gel with added 0.05g/l *EtBr* for staining and then visualized under UV-light using a Uvitec gel imaging system.

Table 3.10 PCR volumes for the *Glu-Dx5 and Glu-Dy10/Glu-Dy12* markers when run as a multiplex.

Reagents	Volume (µl)
dH2O	3.5
2GFastMix	12.5
P1	0.75
P2	0.75
P3	0.5
P4	0.5
Total	**18.5**
DNA	1.5
TOTAL	**20**

The *GluA3* (Table 3.11) cycle was as follows: 4 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 1.5 minutes at 65°C, and a final extension step of 3 minutes at 65°C. PCR samples were ran on PAGE for visualization of markers (see section 3.2.2.3 for details).

The *Rht-B1b* (Table 3.12) cycle was as follows: 5 minutes at 94°C, 7 cycles of 30 seconds at 92°C, 30 seconds at 30°C and 80 seconds at 72°C; followed by 30 cycles of 15 seconds at 94°C, 30 seconds at 60°C and 50 s at 72°C and a final extension step of 5 minutes at 72°C. The PCR samples were ran on a 1.8% (w/v) agarose gel with added 0.05g/l *EtBr* for staining then visualized under UV-light using a Uvitec gel imaging system.

Table 3.11 PCR volumes for the *GluA3* marker.

Reagents	Volume (µl)
dH2O	5
2GFastMix	6.25
Xpsp 2999F	0.25
Xpsp 2999R	0.25
Total	**11.75**
DNA	0.9
TOTAL	**12.65**

Table 3.12 PCR volumes for the *Rht-B1b* marker.

Reagents	Volume (µl)
dH2O	10
Kapa® GreenMix	12.5
BF	1
MR1	1
Total	**24.5**
DNA	1.2
TOTAL	**25.7**

The *Rht-D1b* (Table 3.13) cycle was as follows: 5 minutes at 94°C, 7 cycles of 30 seconds at 92°C, 30 seconds at 65°C and 80 seconds at 72°C; followed by 30 cycles of 15 seconds at 94°C, 30 seconds at 58°C and 50 seconds at 72°C and a final extension step of 5 minutes at 72°C. The PCR samples were ran on a 1.8% (w/v) agarose gel with added 0.05g/l *EtBr* for staining and then visualized under UV-light using a Uvitec gel imaging system.

Table 3.13 PCR volumes for the *Rht-D1b* marker.

Reagents	Volume (µl)
dH2O	5
2GFastMix	7.5
DF	0.75
MR2	0.75
Total	**14**
DNA	1
TOTAL	**15**

3.2.2.3. Polyacrylamide gel-electrophoresis (PAGE)

The Laemmli-SDS-PAGE protocol was used (as an alternative for marker visualization) and was used for SSR or microsatalite markers. The procedure followed is similar to the one described by He (2011) as was built on work reported by Laemmli (1970). The procedure was done in four steps: plate preparation, gel preparation, loading of samples and silver staining.

a) Plate preparation

A 500 µl volume of plate glue was diluted in a 1500 µl volume of 100% ethanol then 1740 µl of the diluted plate glue was mixed with 140 µl 10% acetic acid in a 2 ml micro centrifuge tube. The long and short gel-casting glass plates were thoroughly cleaned with 100% ethanol to remove any dirt or residues on the plates. Once both plates were cleaned and dried with tissue paper, the long glass plate was covered with a thin layer of Wynn's C-Thru (Wynn's, Johannesburg, South Africa), allowed to air-dry for three minutes, then any excess solution was dried off with tissue paper. The short glass plate on the other hand, was covered with a thin layer of plate glue mixture from the 2 ml micro centrifuge tube, allowed to stand to air-dry for 30 seconds, then any excess dried off with tissue paper. The short plate was placed on top of the long one with both cleaned surfaces of the plates facing each other and two 1 mm spacers between them. The edges of plates were properly aligned then inserted in a rubber boot to hold them together.

b) Gel preparation

From a foil-covered stock solution of 40% acrylamide (5.3 M acrylamide, 0.129 M bis-acrylamide and sufficient distilled water to bring to required volume), 6% sequencing gel mix was made by aliquoting 37.5 ml of the stock solution and mixing it with 6 M urea and 50 ml 5X TBE, then brought to 250 ml volume. A volume of 160 ml of the 6% sequencing gel mix was added into a 500 ml beaker, with 160 µl N, N, N‟, N‟-Tetramethylethylenediamine (TEMED) and 800 µl 10% ammonium persulfate (APS) solution (prepared by freshly dissolving 0.1 g ammonium persulfate in 1 ml distilled water) to make the final gel mix. As soon as the APS solution was added, the gel mix was stirred briskly and carefully poured on the glass plate assembly to avoid any bubbles forming, then a comb was placed at the top to develop loading wells and the mix was allowed to solidify for an hour.

c) Loading of samples

A volume of 10 µl loading dye (98% formamide, 10 mM EDTA, pH 8.0, 0.05% (w/v) bromo phenol blue and 0.05% (w/v) xylene cyanol FF) was added to each PCR sample (microsatellite products). The samples were denatured by incubating the PCR tubes for five minutes at 95°C and immediately placed on an ice bath, then loaded on the gel wells for electrophoresis. The gel was electrophoresed on a Life Technologies S2001 vertical gel electrophoresis system (United Scientific, Cape Town). The conditions use for running gels were at 65 Volts, 300 milli-ampere (mA) and 60 Watts; the running time was between 270 and 330 minutes depending on the specific PCR products being screened.

d) Silver Staining

Once electrophoresis was completed, the gel was removed and placed on ice for five minutes to ease the process of separating the two plates. The long plate was removed in one swift movement to avoid damaging the gel and then the gel-carrying short plate was put in a container with fixing solution (210 ml 100% ethanol with 1879.50 ml distilled water and 10.5 ml acetic acid added to the solution just before pouring it on the container). The container was placed on a GFL 3016 shaker (Gesellschaft für Labortechnik, Germany) and allowed to shake for 20 minutes then rinsed twice with 2 L distilled water for 5 minutes each time. Once rinsed, the gel was emmersed in staining solution (2.1 g of silver nitrate (AgNO3) dissolved in 2100 ml distilled water) and allowed to shake for another 20 minutes. Once staining was complete, the gel was rinsed in 2 L distilled water for 30 seconds. The gel was then placed in a container with developing solution (31.5 g of sodium hydroxide (NaOH) dissolved in 2100 ml distilled water with 8.505 ml of formaldehyde added to the solution just before use) and allowed to shake until discernable marker bands appeared. The gel was rinsed one last time then viewed on a white light screen to visualize the banding pattern.

3.2.3. Field trial

The 90 genotypes were planted in three different locations across the Western Cape of South Africa with three replications at each locality. These locations were Welgevallen (-33.943752, 18.864628), Mariendahl (-33.849247, 18.824712) and Napier (-34.469055, 19.911479). Randomized complete block design (RCBD) field layouts were planned and randomized using AgroBase Generation II Version 18.3.1 (Agronomix Software Inc, Winnipeg, Canada) and trials were planted alongside other wheat and triticale trials with a triticale boundary. Planting was done in the 3rd week of May in 2015 at the different localities.

3.2.3.1. Field preparation and planting

Field preparation began with ploughing of the field with a tractor to turn the soil as soon as adequate rainfall was received in the locations. Fertilizing followed being done with a quadbike mounted with a fertilizer Push Broadcast Spreader (Quad Master ATV Implements). Five hundred kilograms of 10:1:5 (31) Nitrophoska® fertilizer (ten 50 kg bags) was applied on the field equating to: 96.88 kg nitrogen, 9.69 kg phosphorus and 48.44 kg potassium in the field. The field was then levelled with a tractor-drawn rake, in the process removing excess debris from the previous season of field trials and incorporating the fertilizer into the soil. SAKURA®850 WG (active ingredient: Pyroxasulfone 850 g/kg) (Bayer CropScience), a pre-emergent herbicide was sprayed on the field (118 g/ha: 30 g dissolved in 70 litres of water).

Following the herbicide application, plots were measured out in the field. Each plot was 5 m long and 1.05 m wide, with eight rows spaced at 15 cm apart. Four plots were grouped together to form a block and the trial was 17 blocks long and four blocks wide with 270 plots in total. Planting was done with a Plotseed XXL planter (Wintersteiger, Delmas, South Africa) and the seeding rate was based on the individual genotype kernel weight to a sowing density of approximately 200 seeds/m^2.

3.2.3.2. Crop husbandry

Since the trial was set-up in a winter-rainfall area for dryland cropping, there was no supplementary irrigation and all water requirements for the trial were supplied by rainfall. Top dressing fertilizer was done according to recommendations from soil analyses executed before planting. Turbo 31 (Kynoch fertilizer) was used for top dressing which was broadcasted twice during the growth and development of the trial: at the stem elongation and grain-filling growth stage of the plants. A volume of 247 kg/ha of Turbo 31 was applied, supplying 48 kg/ha of nitrogen.

Regarding pest management, an intergrated pest management program (IPM) was followed to avoid any possible build-up of pest resistance to pesticides by pests prevalent to the localities. Herbicide spraying was done multiple times during the trial when there were weeds (mostly rye grass and broadleaf weeds such as clover and ramnas) growing between the plots to eliminate competition for resources, especially water. MCPA 400 SL [Greena®, active ingredient (4-chloro-2-methylphenoxy) acetic acid] and Axial® [Syngenta®, containing 100 g/l (9.8 % w/w) pinoxaden and 25 g/l (2.45 % w/w) cloquintocet-mexyl] were used to spray the field at rates of 2 L/ha and 778 ml/ha, respectively. Seven hundred mililitres of MCPA 400 SL and 273 ml Axial® were dissolved in 70 l of water and sprayed with a QM0422 – 100 l boomless sprayer. The insecticide and fungicide used were Chlophyrifos (active ingredient: 500 g/l Chlorpyrifos) sprayed at 750 ml/ha and Duett™ [BASF South Africa, active ingredient: 125 g/l epoxiconazole (DMI–fungicides) and 125 g/l carbendazim (benzimidazole)] sprayed at the recommended 1 l/ha.

3.2.3.3. Harvesting and data analysis

Harvesting was done in the third week of November 2015, (23, 27 and 30 November for Welgevallen, Mariendahl and Napier localities respectively), six months after planting. Harvesting was done with a plot combine harvester. Seeds were cleaned, conditioned and then weighed to determine the mass (kg) harvested in each plot. Grain yield per plot was converted to t/ha from plot masses. Samples were taken from the seeds for determination of moisture content, protein content and HLM using an Inframatic 9500 NIR Grain Analyzer

(Perten, Hägersten, Sweden). Data were entered into Microsoft Office Excel spreadsheets then uploaded into AgroBase Generation II Version 18.3.1 (Agronomix Software Inc, Winnipeg, Canada) for analyses. For individual localities, nearest-neighbour analyses (NNA) was done to check for spatial bias, then general linear model analyses of varience (GLM ANOVA) analyses. A RCBD ANOVA analyses was done for the overall MLFT.

3.2.4. Physiological trait study

Five of the best performing genotypes from the MLFT were selected based on their marker data and YRTs that were recorded and analysed. These genotypes were selected for a physiological trait study of YRTs in a controlled environment.

3.2.4.1. Hydroponic system set-up

A reticulation system was built and set-up at the start of this study from materials obtained from Agrimark (Stellenbosch, South Africa) (Table 3.14). The system was originially designed and assembled in-house. A pilot study was done and data was collected at different stages of growth development, as per the trait requirement. Data were analysed and the trial was replanted to validate results.

Table 3.14 Equipment required for reticulated hydroponic system set-up.

Description	Quantity
Addis Rough tote 45 l/Black storage box	2
X – Stream silica sand 40 kg	1
PVC running nipple 15 mm	4
Rubber insert ring 21 ID×37OD	8
PVC Backnut ½	8
6 m (radius 8 cm) PVC Pipe D/F SABS	1
Submersible/Water fall pump 600 l/h 1m head	1
Emjay filter inline 240 × 240	2
Raco tap adaptor female ½ (55214C)	5
Raco hose connecter STD ½ (55203C)	5
Hose clear reinforced PM 15 mm	1
PVC Compact ball valve size ¾	1
Electric weather box	1
TDT7 Top timer	1

A second planting was done which gave good results and a third follow-up trial was conducted to validate results of the second trial. The Addis Rough tote storage box was drilled to make two holes on opposite sides along its width. These holes were made at the central position at approximately 7 cm from the base of the box and they were made wide enough to fit 15 mm wide polyvinyl chloride (PVC) running nipples. Nipples were inserted to project equally on the inside and outside of the box [then rubber insert rings 21 ID×37OD were added with PVC backnut ½ fastened tight to ensure no leakage during irrigations].

The box was half-filled with loose stone and gravel (2.8 Mb–5-megapixel beach sharp pebbles) followed by a 3-5 cm layer of silica sand (Kaap Agri, Stellenbosch, South Africa). The 6 m PVC pipe was cut into 15 cm long pieces which were placed on top of the silica layer equidistant from each other in three rows of five. The pipes were pressed down in the sand to leave about 3 cm from the top of the box. More stones were added between and around the pipes until just over 5 cm from the top of the box for stability of the system. More sand was carefully added to the inside of the PVC pipes to serve as the growth media, leaving about 2 cm of space for planting and more silica sand to cover the seeds with.

The box was mounted on two plastic crates with a reticulation water system installed below it. Another 45 l storage box was used for the irrigation system, with two holes drilled in the lid. The holes were drilled on the same side along the width, approximately 10 cm apart and 5 cm from the edge of the lid width. Holes were made wide enough to fit 15 mm PVC running nipples, protruding equally on both side of the lid and secured with two rubber insert ring 21 ID × 37OD and two PVC backnut ½ in each hole. Raco tap adaptor females ½ (55214C) were screwed onto each of the 15 mm PVC running nipples on the exterior side (i.e. on both sides of each nipple assembly in the loose stone and sand containing box, as well as on the two assemblies above the lid for the irrigation system box) and an extra one attached below the lid to connect the water pumping mechanism to.

Raco hose connecters STD ½ (55203C) were attached on the corresponding female adapters on the one end and clear reinforced PM 15 mm hose pipe on the other. To the hydroponic system box (i.e. containing loose stones), one hose pipe of 1.5 m per box was connected towards the back hose connector, while on the front a clear hose pipe, measuring 15 cm, was connected. At the ends of these pipes, Emjay filters inline 240×240 were connected to filter the nutrient solution entering and exiting the hydroponic system box. The other ends of these filters were connected to more clear hose pipe fittings (20 cm long) which were in turn connected to the Raco hose connecters on the lid of irrigation system box to make a closed circuit system. On the lid, underneath the short pipe on the front end of the system, a 600 l/h 1 m head/submersible pump was connected and on the other side a PVC Compact ball valve size ¾ was connected for calibration of the system.

Figure 3.2 Setting up of hydroponic system.

Forty litres of nutrient solution were added the irrigation system bucket. The water was allowed to be pumped into the hydroponic system with a timer to determine how long it takes for the hydroponic system to be filled with the solution. The pump was connected to a TDT7 Top timer to switch the pump on four times a day. Thus, irrigation water was supplied to plants for a time corresponding to the time it takes for system to be full, and then the solution was gravity-drained back to the irrigation bucket.

3.2.4.2. Planting and crop husbandry

The five genotypes selected for the trait study were: 15HYLD-21, 15HYLD-12, 15HYLD-07, 15HYLD-27, and 15HYLD-80 (Addendum 1). Three pipes/stations were allocated for each of the five genotypes, divided into three rows of replicates. Within each row or replicate, genotypes were randomized using the Microsoft Office Excel randomization tool to reduce any bias. Two seeds of the same genotype were planted in each pipe according to the planting design. Seeds were covered with a layer of sand to the brim of the PVC pipes. Seeds were hand irrigated for a week, three times a day: 08h00, 12h00, and 16h00.

After a week, seedlings were thinned out to remove the weaker seedling in each pipe and the reticulation system was started. Irrigations were done with nutrient solution made by mixing 164 g Sol-u-fert T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2 g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa), 77 ml potassium nitrate, 0.05% Jik (household detergent containing 3.5% sodium hypochlorite, Reckitt and Colman South Africa Pty Ltd, Elandsfontein, South Africa) and 100 l tap water. The solution had a final electrical conductivity (E.C.) concentration of 2.5 mS/cm, which was increased to 3.5 mS/cm during grain-filling. Irrigations were facilitated by the submersible water pumps four times a day. Irrigations were done at the following times: 08h00 to supply water to the plants at the start of the day, 12h00 and 14h00 to combat water lost during the point of maximum insolation and hottest parts of the day, respectively (Solar facts and advice, 2016). The last irrigation was at 18h00 at the end of day. Nutrient solution was changed twice a week to ensure a constant supply of nutrients to

plants and with each change, the system was recalibrated to ensure the times were still corresponding. Twice a month, the system was flushed out with running tap water to prevent algal growth in the system.

3.2.4.3. Data collection, harvesting and data analyses

Data on days to heading (DTH) were collected when spikes started to emerge and continued until all spikes had been formed. Thereafter, tiller number was recorded. Flag leaf area (FLA) was measured according to the Quarrie and Jones (1979), where, leaf area = length x breadth x 0.75 (Aldesuquy *et al*., 2014). Three flag leaves were selected in each plant (from the oldest three tillers) and their lengths and widths (at their widest point) were measured with a 30 cm ruler and then averaged. These measurements were made at the pollination stage when vegetative growth has completed (Zadoks 65).

Spike length was taken using a 30 cm ruler on three tillers, which were then averaged and this was done together with plant height when plants were at the hard dough stage (Zadoks 87). Measurements were taken from the base of the bottom spikelet (base of rachis) to the apex of the top spikelet, excluding the awns seeing that not all genotypes have awns on them (Cui *et al*., 2012).

Accordingly, spikelet number was taken from the three oldest tillers per plant, then averaged and rounded off for reporting. Spikelet number was taken at harvest according to Peel's (2000) method of counting, where the top and bottom spikelets are excluded from the total count since they have been shown to not significantly contribute to the yield.

Plant height was taken at harvest and measured against a white background with cm markings on it (Figure 3.3). Plants were cut at the base to remove the root system then laid flat on the measuring board with the spikes fully extended (since they tend to bend at maturity) to get the maximum height, excluding the awns (Cui *et al*., 2011).

After harvesting, individual plants were folded into brown bags and placed in an oven to remove any residual moisture in them (48 hours at 35^{o}C). After oven drying, plants were weighed in bags (mass of bag tarred off) to obtain overall plant biomass (g). Seeds were then thrashed from individual spikes, counted and weighed. Average grain number per spike was recorded from the mean grain number per individual spike. Grain weight (g) per spike was obtained in a similar manner. The masses of individual spikes were combined to give the overall grain mass per plant which was also used with plant biomass to calculate harvest index estimates (a unitless text).

Figure 3.3 Height measurements taken at harvest.

Overall traits studied in the greenhouse trial were: DTH, tiller number, plant height, flag leaf area (FLA), spike length, spikelet number per spike, plant biomass, grain mass, harvest index, grain number per spike and grain weight per spike (table 3.15). Data collected were entered into a Microsoft Office Excel (2013) spreadsheet and then uploaded into AgroBase Generation II Version 18.3.1 for analyses. A general linear model (GLM) ANOVA analysis was performed to determine the significances of differences between genotypes for each trait. After the first trial did not yield positive results, showing unexpected significant results between the replicates, a second trial was done in the same manner as described above. A follow-up and third trial was done to validate results of the second trial. A Pearson correlation coefficient table was also done using SAS (SAS Institute Inc., Cary, North Carolina, USA) to test for correlations between the various traits and to identify the possibility of indirect selection.

Table 3.15 Yield related traits used in the study and their parameters.

Traits	Stage of assessment	Unit	Organ	Destructive	How its measured	Length of assessment	Related traits	Quantitative	Marker/QTL	Field or GH	Reference
Flag leaf area	Zadoks 59	cm^2	Leaf	No	Ruler	Once	Leaf length and width	No	*Qfl.cau-2D, Qfl.cau-5A.1* and *Qfl.cau-5A.3*	Both	Fan *et al.* (2015)
Harvest Index (HI)	Harvest	-	Whole plant	Yes	Scale	Once	Grain yield and biomass	?	?	Greenhouse	-
Plant height	Zadoks 89	cm	Whole plant	No	Ruler	Once	Lodging	Yes	Dwarfing genes, mostly *Rht-B1b* and *Rht-D1b*	Both	Cui *et al.* (2011)
Tiller number	Zadoks 30	-	Whole plant	No	Count	Once	Grain number	No	*tin1, tin2* and *tin3* genes	Both	Xie *et al*. (2015)
Spike length	Zadoks 85	cm	Spikes	No	Ruler	Once	Grain number, Spikelet number	Yes	*HL1*	Both	Wu *et al*. (2014)
Spikelet no.	Zadoks 75	-	Spikes	No	Count	Once	Plant height, grain number	-	-	Both	Zhang *et al*. (2010)
Grain no./spike	Zadoks 87	-	Spikes	Yes	Count	Once	TKW	No	*FLOWERING LOCUS* (*FT*), *TaTEF*-7A, *TaGW2*	Greenhouse	Boden *et al*. (2015); Nadolska-Orczyk *et al*. (2017)
Grain mass/spike	Harvest	g	Spikes	Yes	Scale	Once	?	Yes	*TaSus1* and *TaSus2*	Greenhouse	Xie *et al*. (2016)
Stay green	Zadoks 87	-	Whole plant	No	LAUG	Continuous	Photoperiod, Stress tolerance, flag leaf	Yes	QSg.*bhu*-1A, QSg.bhu-3B, QSg.bhu-7D	Greenhouse	Kumar *et al*. (2010)

Traits	Stage of assessment	Unit	Organ	Destructive	How its measured	Length of assessment	Related traits	Quantitative	Marker/QTL	Field or GH	Reference
							area, biomass				
Days to heading	Zadoks 50	-	Spike	No	Observation	Once	Protein content	-	*Ppd-D1, Ppd-B1, Ppd-A1* genes. *Vrn-A1, Vrn-B1, Vrn-D1* genes	Both	Slafer, 2012
Biomass	Harvest	g	Whole plant	Yes	Scale	Once	Harvest Index, Plant height	Yes	-	Greenhouse	

3.3. Initiation of MS-MARS with a single seed descent breeding scheme

3.3.1. MS-MARS cycle I

Male and female germplasm was sourced from the pre-breeding base population nursery established in the SU-PBL in 1999 (Botes, 2001), using seed from the previous year's nursery. This population was established with the objective of creating pre-breeding populations, carrying multiple rust resistance genes for effective protection against rust pathogens due to their additive gene action. Male and female parents were space-planted under greenhouse conditions at Welgevallen Experimental Farm (Stellenbosch, South Africa) over a duration of two weeks with about three to four days between plantings. There were 200 male segregating lines, which were planted as replicates on the various planting dates, and a segregating population of female plants.

Six seeds were panted in nursery black bags (125 × 105 × 230 mm). After germination, the six seedlings were thinned out to four seedlings per nursery bag and four tables of 120 pots were allocated for the female lines as well as the same number (120) for male lines. Nutrients and water were supplied in the nutrient solution described in section 3.2.4.2 and gDNA extractions were conducted as soon as seedlings had reached heights of approximately 5 cm. Only female parents were screened and molecular markers from the rust resistance panel of markers were used (Table 3.1).

Since the female population was segregating 1:1 male sterile: male fertile plants, male sterile (female) plants were selected with great care to facilitate hybridization and avoid any possible selfing of male fertile plants. During flowering, female (sterile) tillers that had physiologically mature spikes were selected. These tillers were cut at the base from the plants, stripped off all their leaves except the flag leaf and then put in nutrient solution (section 3.2.4.2.) to prevent them dying from desiccation. Male fertile or pollen donor parents were selected from the male population and these were cut at a stage where anthers were mature and pollen shed would occur within a few hours to a few days i.e. having a very light green or pale yellow colour on them. These tillers stripped off all their leaves and put in a separate bucket containing nutrient solution. Once all viable tillers from both populations were cut, florets from the female parents were cut open to allow for maximum exposure to pollen shed from the male tillers. The tillers were cut at the bottom so that they have have uniform heights.

Custom-made galvanized steel troughs with black antifungal paint coatings on the inside and puncture holes on their tops, were used to hold the females during pollination and grain filling. The tops had dimensions of 60 cm × 45 cm and the punctured holes could hold 230 female spikes each. Female tillers were placed in steel trough tops in groups of 16 and tied together with two rows of these groups placed side by side. Pipes for air supply into the solution were also inserted in between the two rows and connected to a pump on the other end. The troughs were filled with standard nutrient solution to supply nutrients and water.

Male tillers were inserted at an angle into narrow and smaller galvanised troughs containing nutrient solution soaked oasis® (floral foam) inside and arranged into rows. The male tiller troughs were placed over the female trough on a metal stands. Pollen shed was allowed for a week with nutrient solution change on the male tillers half-way through the week. After pollen shed was completed, male tillers were discarded and the female tillers were allowed to complete grain-filling and mature.

Maintenance of the female tillers was done by changing nutrient solution every fortnight and trimming the tips of the tillers to remove dead cells, open up the phloem and xylem as well as avoid fungal growth. Seed physiological maturity took place six weeks after hybridization at which the tillers were removed from the troughs, placed inside brown paper bags and oven dried at 21°C for one week. Seeds were then thrashed by hand, counted to evaluate cross-pollination effectiveness and stored in brown envelopes for future plantings.

3.3.2. MS-MARS cycle II

The second cycle was conducted similar to the first cycle, with the exception of the male donor line sources. Segregating progeny seeds from the first cycle were planted as a source of male sterile (female) parental lines. Forty-four of the 90 high-yield field trial genotypes were selected based on their marker screening performance as pollen donors for the second cycle in order to introgress high yield genes (Column 4, Addendum 1). To these, eight new genotypes were added from previous studies at the SU-PBL as well four genotypes from a *Fusarium* introgression effort. These were again space-planted to stagger their maturing times. Progeny harvested from the second cycle was harvested and quickly replanted to initiate a single seed descent breeding scheme.

3.3.3. Single seed descent breeding scheme

MS-MARS cycle II progeny seeds were harvested and pooled into a brown envelope and mixed thoroughly in order to allow for random planting. The single seed descent population was planted in a glasshouse, with three seeds planted in a plastic planting bag. The plants were subjected to the standard husbandry conditions as previously described for the MS-

MARS cycle (section 3.3.1). When they were about 5 cm in height, leaf cuttings were harvested for gDNA extraction. The DNA samples were screened for the most important rust resistance markers: *Sr2* and *Lr34*, the dwarfing gene marker in the form of *Rht-B1b* and for all the *Fusarium* resistance markers (Table 3.5).

Plants were left to grow until they reached the flowering stage, at which the tillers were covered with plain, Canvasback® S38H waxed shoot bags (6.4 cm × 2.5 cm × 22 cm) (Seedburo, Chicago, USA) before pollination in order to ensure that self-pollination took place. The bags were kept on the wheat spikes until harvesting took place. During harvesting, male sterile plants were recorded and discarded since they had not formed any seeds and could not be used any further in the cycle. Data on plant height data, number of tillers as well as the health status of each individual plant was noted and recorded. Plant health status was based on visual assessment with scores of: 0 (healthy looking), 1 (slightly diseased), and 2 (heavily diseased). Spikes were thrashed individually and the number and mass of seeds was taken and recorded for selection purposes.

3.4. RPAS high throughput phenotyping platform pilot study

Using the material at the Welgevallen field trial (section 3.2.3), a pilot study into the possibility of establishing a technologically advanced phenotyping system was done. This was implemented with RPAS technology combined with cameras that capture wavelengths of different spectra.

3.4.1. RPAS technology and flights

At the beginning of the trial, a Y6 trooper multirotor RPAS (Figure 3.4) (3D Robotics, Berkerley, USA) was used to make data collection flights. Two multirotor quadcopters were later obtained to enable multiple flights to be done in a trial back to back, especially for localities that were not close to the pilot. Flights were automated using Tower APM planner (by Fredia Huya-Kouadio) a program for planning flight missions on an Android®-powered device. The flight plans were sent from the tablet to the RPAS with WiFi connection. Flights were done at 30 m above ground, using a speed of 2 m/s with a 65-75% overlap.

3.4.2. Camera technology

Images were initially taken using a Canon SX240 for visual mapping (normal RGB), plus another converted to collect NIR data. This allowed for a greater detail to be captured compare to one camera. This was later changed to GoPro cameras after experiencing some issues with the Canons. Pictures taken during the flight were uploaded to Dropbox with a program called AeroView to be downloaded by a service provider for processing.

The service provider compiled an orthomosaic image using Agisoft PhotoScan Pro 1.2. The image covered the whole locality based on the GPS data accompanying the images from the RPAS flight logs. The Agisoft program also generated point clouds from the 3D data which is a 2-dimensional array which store x, y and z coordinates in meters as well as the red, green and blue channels. The x and y coordinates were given relative to an arbitrary point in the image while the z coordinate was taken as the absolute height above sea level based on the RPAS's GPS sensor. Photogrammetry was then used by combining image positions and characteristics of the camera such as focal length to calculate the variations in height. Data obtained from the orthomosaic included NDVI data, percent coverage, hue, (all three unitless) average height (cm) and the volume of the individual plots (m^3).

Figure 3.4 3D Robotics Y6 Trooper multirotor RPAS armed with RGB and NIR cameras.

Height data captured by the camera was correlated to height data collected in the field at various times during the field trial. Correlations were done using Microsoft Excel 2013.

Chapter 4: Results and discussion

4.1. Yield and related traits' study

4.1.1. Molecular marker assessment

High yielding germplasm was screened using a modified version of the SU-PBL standard panel of markers (Wessels and Botes, 2014). The markers used were for rust resistance genes (*Lr34, Sr2, Sr31, Lr24/Sr24, Lr37/Sr38/Yr17, Sr26*, and *Lr19*) as well as the markers for baking quality (*Dy10, Dy12, Dx5*, and *GluA3*) and the dwarfing gene markers for height (*Rht-D1b* and *Rht-B1b*). The wheat quality markers were more prevalent than the rust markers and the highest number of markers possessed by an individual genotype was six and the least was two markers. The highest rust gene combination was only two markers which was contained in eight of the 60 genotypes. The most common combination was between *Lr24/Sr24* and *Lr37* found in two genotypes (Figure 4.1).

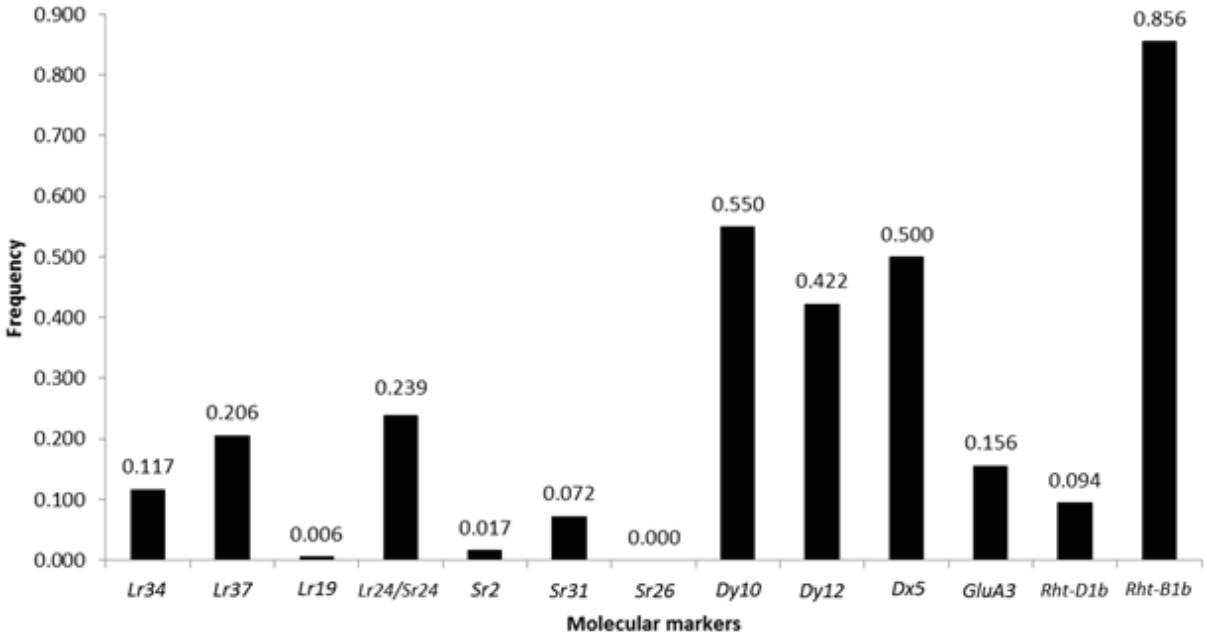

Figure 4.1 Marker frequencies for rust resistance and wheat quality markers based on the SU-PBL standard panel of markers.

Rust marker frequencies were much lower than expected in such a population of high yielding lines. The highest percentage of rust marker available was for the *Lr24/Sr24* gene complex at 23.9%, followed closely by *Lr37*. Gene frequencies for *Sr2, Sr31, Lr19* and *Sr26* were very low at below 10% with *Sr26* being 0%. Since the most effective control method against rust diseases is the use of resistance genes, it is hypothesised that resistance genes would be high in this population as early stages of pre-breeding should also account for germaplsm resistance. *Sr2* is a widely distributed marker across the world and the resultant

frequency of 0.017 was not expected. However, the marker has been previously reported to fail to detect the gene in some backgrounds so it is possible that it may not be validated in some South African liness (Pretorius *et al.*, 2012). This was confirmed by screening for the morphological marker in the field where pseudo-black chaff was observed in approximately 70% of the germplasm (Jia *et al*., 2018).

The low frequencies could possibly be due to the various breeding programs from where the germplasm was sourced using different resistance genes to those that form part of the standard panel of markers at SU-PBL (Wessels and Botes, 2014). Springfield (2014) investigated the possibility of adding *Sr35* and *Sr45* genes into the SU-PBL population. These genes may have already been successfully in the other programs since they are still effective against *Ug99* and they work well in a rust resistance pyramid genotype (Rouse *et al*., 2011). Other genes that have been recently added onto the SU-PBL nursery are *Lr54/Yr37*, *Lr56/Yr38*, and *Lr62/Yr42.* All these recently added genes will need to be part of the panel of markers and routinely screened for as they might be in the high yielding population already.

Some of the genes used in the standard panel of markers (*Sr31* and *Lr/Sr42*) have been overcome by new races of the respective pathogens they were previously resistant to (Pretorius and Kemp, 1990; Long *et al*., 1994; Das *et al*., 2006). Due to such issues, the markers may be in the process of being removed from some breeding programs. The SU-PBL still uses these genes because they are still effective when they have been combined with other genes in the same genotypes. For example, *Lr*24/*Sr*24 combines well with the *Lr9* gene to give good resistance (Pallavi *et al.*, 2015). Also, the program is in the process of incorporating a number of new resistance genes that have not yet been overcome by rust pathotypes. *Sr26* has been reported to generally have low frequencies among modern cultivars, being a gene that has not yet been widely deployed (Lowe and Soria, 2010). This explains the non-existance of the gene in the population. The low frequency of *Sr26* can also be attributed to the 9% yield penalty previously reported by The *et al*. (1988) which would make the gene undesirable in high yielding populations.

With regards to the quality and agronomy related traits the frequencies observed for these genes were much higher than the rust markers. There was a very low frequency of the *Rht-D1b* marker compared to the dwarfing gene on the *B-locus*. Type II resistance to FHB which prevents the spread of the fungi in the spike has been linked to QTLs on chromosomes 1B and 4B with the former being in the same region as the dwarfing gene (Prat *et al*., 2014).

Srinivasachary *et al*. (2009) reported that due to the close proximity of these two genes, germplasm carrying the *Rht-B1* gene in their trial were more susceptible to FHB with a larger portion of damaged seeds per spike. The use of this gene may need to be revised in this

population as there is an effort to increase resistance to *Fusarium* in the population as well (section 4.2.3). This is especially true since Type II resistance together with Type I resistance (resistance to initial infection) are the two major resistance types used in breeding for FHB resistance in wheat programs around the world (Xue *et al*., 2011). Furthermore, it was reported that Type II resistance was the most preferred due to an easier method of assessment and due to lack of germplasm with Type I resistance.

Markers associated with genes controlling protein content in wheat were mostly moderate to high with all the three markers associated with the *Glu-1D* locus (*Dx5, Dy10* and *Dy12*) having frequencies around 0.5. This shows the population has good baking qualities which is important as this contributes to human nutrition. The HMW glutenins, controlled by these genes determine end-use quality of wheat seed by controlling bread dough viscosity and elasticity (Koga *et al*., 2017).

A combination of *Dx5* and *Dy10* in the same genotype results in good baking quality, while *Dx2* with *Dy12* results in poor baking quality (Payne and Lawrence, 1983; Payne *et al.,* 1987). Liu *et al.* (2008) reported that due to the presence of *Dx5* and *Dy10*, the *Glu-1D* locus has the largest effect on baking quality. The *Dy12* subunit used in this population is not linked to *Dx2* so it is ideal for use in breeding programs.

The *GluA3* marker for the LMW glutenin subunit was very low in the population with a frequency of 0.156%. The gene has more than seven alleles which complicates using it in MAS (Zhang *et al*., 2004). This, coupled with the fact that LMW subunits have a less effect on grain quality, limits its adoption for use in breeding programs and explains why the frequencies are low (Koga *et al*., 2017). Weiser *et al*. (2000) also reported that the LMW subunits are an integral part of the gluten network as structural elements so they are most likely inherited together with HMW.

4.1.2. Field trial

All data analyses for the MLFT were done with AgroBase Generation II Version 18.3.1 (Agronomix Software Inc, Winnipeg, Canada). When initial analyses output revealed high coefficient of variation (CV) values (for example Addendum 2), data from the six genotypes which expressed a facultative winter type phenotype was excluded since they yielded much lower than the spring types (entries 66-71 Addendum 1). These very low yields were a result of these genotypes not receiving adequate vernalization temperatures that are required for proper growth and development (Evans *et al*., 1975).

General linear model (GLM) analyses of variance (ANOVA) was performed for the three individual locations (Addendums 3-5). Mariendahl had a mean yield of 0.63 t/ha with an

extremely high CV of 74.75%. The ANOVA table showed significant differences between replications (p≤0.0016) as well as between the genotypes (p≤0.0013). The coefficient of determination (R^2) value was moderate at 0.488. The Welgevallen trial on the other hand had a much higher mean yield than Mariendahl at 4.96 t/ha with an even better CV of 24.32%. Highly significant differences between genotypes and replications were found in the trial (p≤0.0000). The best yield was found at the Napier locality with a mean yield of 5.80 t/ha and the best CV of 18.37%. There were significant differences between genotypes but no significant differences were found between replications.

The year in which the trial was conducted (2015) was the first season of a drought period covering multiple seasons in South Africa. The drought effects were most pronounced at the Mariendahl locality which received a very small amount of rain in comparison to the other locations (daily mean rainfall: 1.81 mm, 1.90 mm and 2.62 mm for Mariendahl, Welgevallen and Elsenburg, respectively). Water stress was also intensified at this site bcause the soil is generally sandy and has poor water retention properties compared to the other two. Mariendahl was also affected by a few pests through the growing season but more so around grain-filling which also intensified the effects of water stress. Geese were a major problem at the germination stage at the Mariendahl trial site and also at Welgevallen where large sections of the field were almost wiped out (see Figure 4.2). Some weeds were also present as the Mariendahl trial was growing which was due to low plant populations as a result of geese damage, which meant that the plant canopy was not dense enough to prevent weed growth within the plots. A notable weed that was a problem is the broadleaf weed called Ramnas (*Raphanus raphanistrum*).

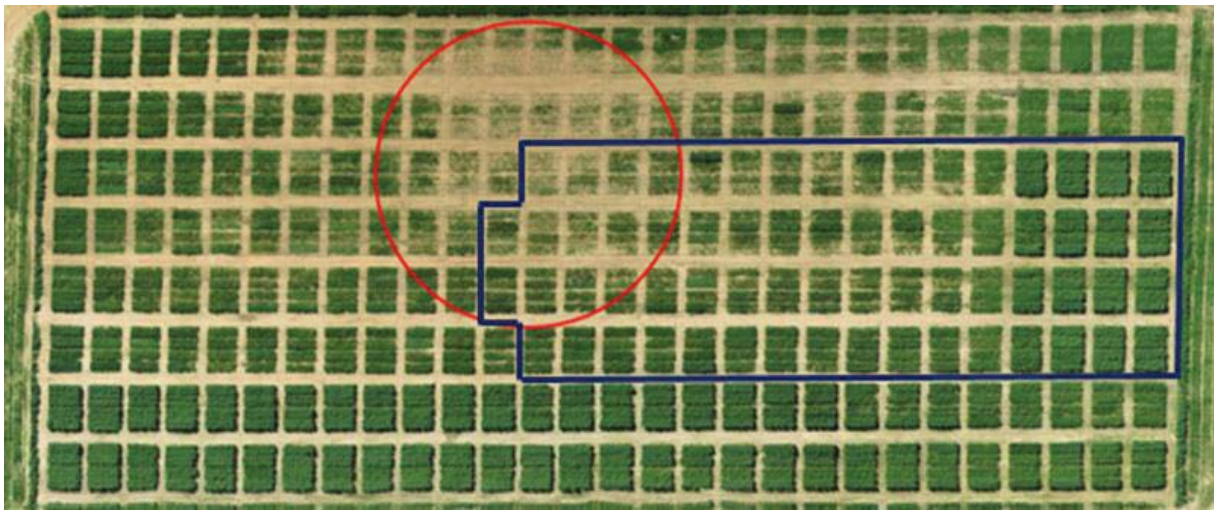

Figure 4.2 Arial view of overall Welgevallen trials with inserts of geese damage (red circle) and high yield field trial (blue).

Russian wheat aphids (RWA) (*Diuraphis noxia*) were a major insect pest in the late stages of plant growth. In countries such as Ethiopia and Pakistan, aphids are a major wheat pest

with direct yield losses of 40-68% caused by sucking plant sap, as well as indirect yield losses (20-80%) due to viral and fungal pathogen transmission (Aslam *et al.*, 2005; Araya, 2015). All these issues combined to reveal the significant differences between replications in the trial since stresses do not affect a field uniformly leading to these differences in yield. This phenomenon also explains the highly significant differences between the Welgevallen replicates where a section of the field was damaged by geese, mostly centralized into one replicate.

The ideal ANOVA results were those obtained at the Napier field trial where the differences were more or less what would be expected. In a population of high yielding genotypes, you would expect to see some differences between the yield attained as yield is affected by the environment and this will affect the genetic yield potential to varying degrees. Also, genetic potential of 90 genotypes from diverse breeding programs can never be the same. There were no significant differences between the replications on the Napier trial because the locality was less affected by stresses than the other two, thus the environment was fairly uniform and randomization eliminated bias in the trial. The Napier locality also had the closest value to the ideal CV for plant breeding trials of 13% or less (CV=18.37%).

A combined RCBD ANOVA for yield was performed for the overall trial using an Environment by Entry model. The initial ANOVA results are presented is Addendum 2 where the CV was 26.35% with a coefficient of determination value 0.9141. Removing the six genotypes with a winter type phenotype slightly reduced the CV to 25.51% while the coefficient of determination was reduced from the initial value, but it was still very good (R^2= 0.904) (Table 4.1). Significant differences were found between the entries or genotypes ($p \leq 0.0046$) while highly significant differences ($p \leq 0.0000$) were observed between the locations as well as in the entry by location (G×E) values.

The high CV (25.51%) for yield was due to the contrasting prevailing weather conditions at the different localities, especially with regards to the rainfall received. This resulted in the yield attained from genotypes to vary widely across the different localities. Mariendahl was under drought conditions, while Napier received good rainfall and Welgevallen was an intermediate of the two. Furthermore, the Southern Cape/Rûens region where Napier is found receives summer rainfall which ensures that at planting, there is higher soil moisture than in the Swartland region where the other two localities were (Mr Piet Lombrad – Department of Agriculture Western Cape, personal communication). This is further explained by the highly significant differences for entry by location as shown by the ANOVA table ($p=0.0000$) (Addendum 1).

Table 4.1 Analyses of variance (ANOVA) summary statistics for the multi-location field trial.

Locality	Trait	Pr>F	Grand mean	LSD (5%)	R^2	CV (%)	H^2
Overall MLFT	Yield	0.0046	3794.83 kg/ha	1170.98 kg/ha	0.9036	25.51%	0.414
Napier	Yield	0.0017	5795.07 kg/ha	1716.22 kg/ha	0.4629	18.37%	0.193
	Protein	0.0000	11.27%	1.83%	0.5263	10.10%	0.259
	HLM	0.2440	80.77 kg/hl	11.66 kg/hl	0.3725	8.96%	0.043
Welgevallen	Yield	0.0000	4957.58 kg/ha	1943.86 kg/ha	0.5573	24.32%	0.288
	Protein	0.1858	14.84%	3.08%	0.3791	12.87%	0.056
	HLM	0.0503	80.05 kg/hl	13.89 kg/hl	0.4073	10.76%	0.106
Mariendahl	Yield	0.0013	631.83 kg/ha	761.37 kg/ha	0.4878	74.75%	0.199
	Protein	0.6090	8.03%	11.87%	0.3810	97.71%	
	HLM	0.3298	74.39 kg/hl	50.25 kg/hl	0.4890	16.1%	0.027

The mean yield across localities obtained in the study was 3.79 t/ha which was lowered by the very low yield attained at Mariendahl. More than half of the genotypes (49) produced above average yields across the three localities. The yield averages of the overall trial, as well as the Napier and Welgevallen localities were much higher than the yield attained in the Western Cape province for the corresponding season. Ninety-eight percent of Western Cape wheat production is under dryland production and the province had a yield of 2.23 t/ha, while dryland production throughout the country was 2.04 t/ha in 2015 production season (SAGL, 2016). The differences in realized yield were due to the variation in the rain received at the different localities as well as pest infestation of geese and RWA at selected localities. The yield that all crops can potentially produce is limited by the amount of precipitation that is available during growth and development (McClellan *et al.,* 2012).

The best yielding genotype across all localities (15HYLD-80) was a SU-PBL entry, and it outperformed commercial checks. The 10 ten yielding genotypes are presented in Table 4.2, also showing the five genotypes selected for the hydroponic study of YRTs (section 4.1.3). All these genotypes except for 15HYLD-43 were in the top 10 yielding genotypes of at least one of the localities, and each of the localities had four genotypes in Table 4.2. There were no significant differences between the top 10 yielding genotypes (LSD = 1170.98 kg/ha).

The top two yielding genotypes were not only the best across all localities, but also the top two genotypes at Mariendahl. This means they not only perform well under good environments, but their genotypic potential gives good results under stress. While biotic stresses can be controlled with chemical products, abiotic stresses can only be limited by genetic yield potential. These genotypes have not been bred purely for high yield but for high genetic yield potential as well as yield stability across environments which should form the basis of abiotic stress breeding (Reynolds, 2012a; Gilliham *et al.,* 2017).

Table 4.2 Top 10 yielding genotypes from the yield trial based on combined data across localities, as well as the selection status for the yield trait study.

Rank	Yield (kg/ha)	Entry	GenCode	Hydroponic study
1	4751.35	80	15HYLD-80	Yes
2	4714.16	21	15HYLD-21	Yes
3	4488.62	12	15HYLD-12	Yes
4	4398.22	14	15HYLD-14	No
5	4362.75	7	15HYLD-07	Yes
6	4350.94	35	15HYLD-35	No
7	4334.73	27	15HYLD-27	Yes
8	4319.97	43	15HYLD-43	No
9	4318.01	15	15HYLD-15	No
10	4314.18	33	15HYLD-33	No

LSD (5%) = 1170.975

Grain protein content was 8.03%, 14.84% and 11.27% for Mariendahl, Welgevallen and Napier, respectively (Addedndum 3-5). Except at Mariendahl where the CV for the trait was 91.7%, grain protein was fairly constant at the other two localities with CV values of below 13%. There were no significant differences in genotype protein content at Mariendahl and Welgevallen but highly significant differences were reported at Napier. A number of genotypes had protein content of above 12%, more especially at the Welgevallen locality where less than 10 failed to do so. According to the latest South African wheat grading legislation, a minimum of 12% protein is required to classify wheat grain as Grade 1 wheat (SAGIS, 2016). The high percentage means the genotypes are suitable for human consumption as wheat is the highest source of protein in the world (Lucas, 2013). Contradictory results were obtained in this study where lower protein content was found in low-yielding environments where it is generally high (Slafer, 2012). This may be due to the negative relationship between grain yield and protein content (Tsilo *et al*., 2013).

GLM ANOVA outputs for HLM values were 74.39 kg/hl (CV=16.1%), 80.05 kg/ hl (CV=10.76%) and 80.78 kg/hl (CV=8.96%) for Mariendahl, Welgevallen and Napier, respectively (Addendum 3-5). There were no significant differences between genotypes or between replications for all three localities (Table 4.1). All mean hectolitre values were above the minimum value required by the local processing industry which is 74 kg/hl (Miles *et al*., 2013). Makgoba (2013) reported that HLM is mostly affected by the prevalent environment during grain-filling, especially rainfall. The same results were observed in this study with the HLM values following the rainfall trend where Mariendahl had the lowest values and Napier was highest. Correlation between grain yield and HLM in the trial was found to be 0.12, which is slightly lower than the expected range of 0.32-0.82 (Engelbecht, 2008).

4.1.3. Yield-related traits

From the top 10 yielding genotypes from the field trial, five genotypes were selected for a greenhouse hydroponic study on yield attributing traits. The top three yielding genotypes were selected while the fourth and sixth were disgarded due to poor rust resistance marker performance as well as plant height being outside of the ideal range. ANOVA results of the first hydroponic system had low CV values for all traits and these were within the acceptable range, i.e. less than 13%. There were significant differences between genotypes for all of the traits except for plant height (Table 4.3). No significant differences were reported between replications for most traits except for grain yield ($p \leq 0.0225$) and plant biomass ($p \leq 0.0118$). The significant differences between the replications in grain yield and plant biomass were indicators that there may be bias in the results as the expected outcome in a randomized experiment such as this.

Table 4.3 Summary of nearest neighbour analyses analyses of variance (ANOVA) results for the first hydroponic study (full results Addendum 6).

Trait	Grand mean	CV (%)	Variety p-value	Replication p-value	Heritability (h^2)
Tiller number	4.47	11.917	0.0711	0.1031	0.433
Days to heading	79.67	3.637	0.0031	0.2091	0.919
Flag leaf area (cm^2)	3.98	5.549	0.0011	0.9064	0.909
Plant height (cm)	76.07	4.889	0.8244	0.4178	0.303
Spike length (cm)	9.35	4.619	0.0191	0.0500	0.878
Spikelet number	17.18	4.616	0.0120	0.0771	0.651
Plant biomass (g)	19.79	8.763	0.0610	**0.0118**	0.905
Grain mass (g)	10.60	9.554	0.0465	**0.0225**	0.923
Grain number/spike	53.93	10.372	0.0675	0.1971	0.797
Grain mass/spike	2.41	7.442	0.0186	0.1107	0.936
Harvest Index (HI)	0.53	1.277	0.0055	0.8438	0.985

The significant differences between genotypes for almost all the YRTs indicate that high yield potential in the five genotypes is not attributed by the exact same traits at equal degrees. Plant height on the other hand shows no significant differences because of the extensive use of dwarfing genes in wheat, which generally contributes to high yields (Jamali and Ali, 2008). Figure 4.1 showed that in the high yielding population, these five genotypes were a part of, the *Rht-B1b* gene is predominant and as such, plant height is more or less similar in these five lines. Lack of significant differences between replications were due to the trial being in a controlled environment to ensure reliable data without bias. Very low heritability estimates were found for the various traits because of a lack of genetic variability and the very small population size.

The repeat trial (Addendum 7) had contrasting results to the first where all traits in the second trial had no significant differences between entries and a number of them also had high CV values exceeding 13% (Table 4.4). There were also no significant differences between replications which is the ideal outcome. Heritability estimates remained low and below 1%. A third and final trial (Addendum 8) was done to validate results of the first two trials and see if there was any consistence in the results. Results of the third experiment revealed no significant differences between genotypes for most traits (Table 4.5). The CV values were also higher than the desired range in a number of the traits including grain mass, plant biomass, and grain number per spike. There were also no significant differences between the replicates for all traits and heritability estimates were still low.

Table 4.4 Summary of nearest neighbour analyses analyses of variance (ANOVA) results for the second hydroponic study (full results Addendum 7).

Trait	Grand mean	CV (%)	Variety P-value	Replication P-value	Heritability (h^2)
Tiller number	3.67	15.347	0.0551	0.8145	0.472
Days to heading	80.67	13.611	0.0676	0.5170	0.441
Flag leaf area (cm^2)	4.15	13.062	0.5510	0.7779	0.067
Plant height (cm)	72.00	7.739	0.2329	0.9150	0.626
Spike length (cm)	8.19	9.538	0.5521	0.7017	-0.067
Spikelet number	15.49	10.328	0.6861	0.9997	0.163
Plant biomass (g)	14.26	30.370	0.3365	0.7574	0.612
Grain mass (g)	7.52	27.761	0.1709	0.6902	0.271
Grain number/spike	39.66	19.126	0.1705	0.5911	0.271
Grain mass/spike	1.99	16.945	0.1146	0.6417	0.770
Harvest Index (HI)	0.53	4.944	0.4687	0.7318	0.591

Table 4.5 Summary of nearest neighbour analyses analyses of variance (ANOVA) results for the third hydroponic study (full results Addendum 8).

Trait	Grand mean	CV (%)	Variety P-value	Replication P-value	Heritability (h^2)
Tiller number	3.60	8.603	0.3564	0.0915	0.681
Days to heading	82.00	5.608	0.0536	0.1401	0.784
Flag leaf area (cm^2)	3.35	10.431	0.7227	0.3832	0.350
Plant height (cm)	70.67	7.303	0.4416	0.3168	0.015
Spike length (cm)	8.68	11.105	0.6978	0.8767	-0.171
Spikelet number	15.61	9.074	0.6024	0.8266	0.568
Plant biomass (g)	13.42	23.895	0.9630	0.5668	-0.403
Grain mass (g)	7.45	23.483	0.9169	0.7793	0.275
Grain number/spike	45.79	20.048	0.7674	0.7883	0.222
Grain mass/spike	2.12	18.305	0.7489	0.8123	-0.209
Harvest Index (HI)	0.56	6.690	0.6526	0.1044	-0.139

The lack of significant differences between genotypes for the different traits was consistent in the second and third experiments of this trial while there were significant differences in the first one. This lack of significant differences between genotypes is due to the genotypes being high yielding and perhaps having some of the traits optimized in the various breeding programs where the lines were developed. Significant differences between genotypes in the first trial could be due to a lack of uniformity and homogeneity in the setting up of the hydroponic system because of human error causing bias towards some of the planting stations or replications. Liu *et al.* (2014b) reported that cultivated wheat has a narrow genetic base due to founder effect during crop domestication leading to few successes in yield improvement. This has sparked the interest in pre-breeding programs to make crosses between cultivated wheat and its wild relatives in the primary and secondary gene pool to widen its genetic variability.

Heritability estimates were low across all the hydroponic experiments due to a small sample size and reduced variability. The trial will have to be repeated with a larger sample of varieties with some genotypes that had moderate to low yield included to increase variability in the YRTs and improve heritability. Improving heritability for traits will establish new selection criteria of traits that generally have higher heritability than yield per se. Spike length revealed negative heritability estimates in the second and third hydroponic experiments whilst the first the results were positive. Negative heritability estimates sometimes occur in experimental data analyses (especially when sample sizes are small) and some researchers attribute it to error in analyses models but they should generally be taken as zero percent (Brown, 2016).

Pearson correlation coefficients analysis was conducted for all the traits from the five genotypes using the data from all three hydroponic systems replicated three times individually (Table 4.6). Traits included in the correlation table are: days to heading, number of tillers, plant height, flag leaf area, spike length, spikelet number, plant biomass, overall grain mass, harvest index, grain number per spike and grain weight per spike. A majority of the traits were positively associated with other traits meaning indirect selections could be made for multiple traits at the same time. Days to heading and harvest index were the only traits that expressed a negative association with at least five other traits meaning that positive selection for one of these traits will result in unintentional negative selections for a number of other traits.

Table 4.6 Multiple trait correlation table detailing the inter-relationships amongst traits studied and their significance ($p<0.05$) to each other.

	Days to heading	Number of tillers	Plant height	Flag Leaf Area	Spike length	Spikelet number	Plant biomass	Grain mass	Harvest index (HI)	Grain No/Spike
Days to heading										
Number of tillers	-0.2183 0.1500									
Plant height	-0.1767 0.2455	0.4149 0.0046								
Flag leaf area	-0.1448 0.3428	0.0575 0.7075	0.3448 0.0204							
Spike length	-0.0490 0.7492	0.4514 0.0019	0.6149 <.0001	0.1631 0.2844						
Spikelet number	0.1064 0.4866	0.5345 0.0002	0.5822 <.0001	0.2126 0.1609	0.8556 <.0001					
Plant biomass	-0.1947 0.2001	0.8055 <.0001	0.6637 <.0001	0.2820 0.0606	0.6835 <.0001	0.7541 <.0001				
Grain mass	-0.2629 0.0810	0.7574 <.0001	0.6609 <.0001	0.3090 0.0389	0.6999 <.0001	0.7447 <.0001	0.9780 <.0001			
Harvest Index (HI)	-0.3316 0.0261	-0.2054 0.1760	-0.0237 0.8770	0.0534 0.7278	0.0530 0.7296	-0.0388 0.8002	-0.1098 0.4729	0.0926 0.5451		
Grain No/Spike	-0.0792 0.6049	0.3918 0.0078	0.5772 <.0001	0.1527 0.3167	0.7673 <.0001	0.7711 <.0001	0.7559 <.0001	0.7892 <.0001	0.1478 0.3326	
Grain mass/Spike	-0.0813 0.5956	0.1634 0.2835	0.5719 <.0001	0.3212 0.0314	0.6381 <.0001	0.6283 <.0001	0.6616 <.0001	0.7290 <.0001	0.2818 0.0608	0.8812 <.0001

DTH was negatively correlated to all traits except for spikelet number (r=0.1064, p≤0.4866). Most of the correlations were not significant (i.e. p>0.05) except for the DTH correlation with HI which was significant (p≤0.0261). DTH is an important trait and allows for the flexible nature of wheat, and lead to its adaptability to a wide range of environments (Slafer, 2012). Negative correlations between the trait and the rest is due to a lack of common QTL regions between DTH and other traits. Liang *et al.* (2018) reported that DTH QTLs were in similar regions as those controlling protein content and leaf senescence. Spikelet number is one of the very first spike-related traits to be determined and since DTH has an effect on spike emergence, the two traits may be determined at the same time in wheat phenology (early floral initiation to terminal spikelet initiation) leading to the positive association (Ferrante *et al.*, 2017).

Number of tillers was positively correlated with all traits except for harvest index (r=-0.2054, p≤0.1760) which was not a significant correlation. The lowest positive correlation that the trait had was with flag leaf area (r=0.0575) and the strongest relationship was with plant biomass (0.8055) which was also highly significant (p≤0.0001). Another relationship that was highly significant was its relationship with grain mass (r=0.7574). Tiller number determines grain yield potential in wheat by determining the number of grain-bearing spikes (Xie *et al.*, 2015). Borràs-Gelonch *et al*. (2012) reported a positive relationship between the trait and DTH, and in the current study the relationship was negative and not significant. The positive relationship between tiller number and grain number was low, but significant which is in line with tiller number increasing grain yield by improving grain number (Xie *et al.*, 2015).

Harvest index was negatively correlated to most traits except for flag leaf area, spike length, grain number per spike, grain mass per spike and grain mass. Correlations were low to moderate, ranging from r=-0.0237 for plant height to r=-0.3316 for DTH. Days to heading was also the only trait that had a significant correlation with HI (p≤0.0261) while the rest of the correlations were not significant at 95% confidence. As expected in a high yield population, biomass partitioning to grain was above the acceptable HI range for spring-type wheat (0.45-0.50) with the overall mean being 0.54 in this study (Xie *et al*., 2016). This however is still 10% below the potential HI ceiling of 0.64 meaning that in these genotypes the trait could still be improved (Peltonen-Sainio *et al.*, 2008; Foulkes *et al*., 2011; Reynolds *et al*., 2012b).

HI is an important trait for yield determination and has a positive relationship with grain mass and grain number as was the case in this study (r=0.0923 and r=0.1478, respectively) (Peltonen-Sainio *et al.*, 2008). Low CV values were found across all three studies, because the trait is generally fixed in genotypes unless there is an event of stress during the growing season (Dai *et al*., 2016). Peltonen-Sainio *et al.* (2008) and Xie *et al*. (2016) have also reported

a negative relationship between plant height and harvest index which was also found in the present study.

Plant height was positively correlated with all traits except for DTH and HI. Correlations were moderate to high, ranging from r=0.3448 (FLA) to around 0.66 for both plant biomass and grain mass respectively. Correlations were also highly significant (p≤0.0001) for most of the traits. Since plant height in wheat is comprised of the internode lengths as well as spike length, a positive relationship between the trait and spike length can always be expected (Cui *et al.,* 2011). While other studies have reported this correlation to be weak, in this study the relationship was moderately high (r=0.6149) and highly significant. Positive and highly significant correlations were found in plant height with spikelet number per spike (r=0.5822), grain number per spike (r=0.5772) and grain mass per spike (r=0.5719), which were also reported in another study (Jamali and Ali, 2008).

Flag leaf area generally had positive correlations with other traits (except with DTH) and most of these were not statistically significant. Grain mass and grain mass per spike both had significant positive relationships with flag leaf area, (r=0.3090 and r=0.3212, respectively). The positive relationship between flag leaf area and grain mass is well documented with as much as 75% of grain-filling assimilates (50% overall) coming from the flag leaf (Al-Tahir, 2014). Positive relationships with flag leaf area have also been reported for spike length and spikelet number which are in line with results obtained in the current study (Wang *et al*. 2011; Xue *et al*. 2013; Fan *et al.*, 2015; Wu *et al*., 2016).

Days to heading was the only trait negatively correlated with spike length. Besides DTH, correlations to spike length were mostly high with the least being r=0.6149 for plant height and the highest being r=0.8556 for spikelet number. Most of the correlations were highly significant except (p≤0.0001) for number of tillers which was significant (p=0.0019), while the HI (p≤0.7296) and flag leaf area (p≤0.2844) relationship were not significant. Spike length is very important to yield determination because green immature spikes will contribute to photosynthesis, especially under limited water stress conditions (Maydup *et al*., 2010). Positive correlation between spike length and traits like plant biomass and grain yield have been reported as was the case in the current study where correlations were also positive and significant (Moghaddam *et al*. 1997; Donmez *et al*., 2001).

Correlations of spikelet number with traits were almost all positive, except for harvest index which had a low (r=-0.0388) and insignificant correlation. They were also very high with the highest being the spike length correlation (r=0.8556) mentioned in the previous paragraph. Days to heading's positive correlation was low (r=0.1064) and not significant. Highly significant correlations were obtained between spikelet number and the traits reported by Savii and

Nadelea (2012). As was the case in their study, correlations between spikelet number and grain number per spike were higher (r=0.7711, p≤0.0001) than correlations with grain mass per spike (r=0.6283, p≤0.0001).

Plant biomass, had positive correlations with all traits, except for DTH and harvest index which were both not significant. The trait was generally highly related to the traits (r>0.65) and the strongest and highly significant relationship was with grain mass (r=0.9780). Other highly significant relationships were found with grain mass per spike, grain number per spike, spikelet number, spike length, plant height and the number of tillers. Unlike in this study, Sandaña and Pinochet (2011) found a low and non-significant relationship between biomass and grain weight per spike, however, they also found a strong positive relationship with grain number which was in line with results obtained in the current study. Quintero *et al*. (2018) also found a positive relationship between grain yield and biomass, and reported that genetic gains in wheat grain yield can be driven by increased biomass. Correlation with plant height was moderately high (r=0.6637) and not as strong as in other studies (Townsend *et al.,* 2017). The high correlation with grain mass means that there is a chance that selecting for plant biomass may be one of the best options for improving yield (Reynolds *et al*., 2012b).

Grain mass per spike was positively correlated to all traits except for DTH which also had a non-significant relationship. Correlations ranged from low to moderate with the lowest correlation being with number of tillers (r=0.1634) and the highest correlation with grain yield. Correlations were highly significant except with harvest index, tiller number, flag leaf area and DTH. Peng *et al*. (2011) reported that seed size was an important trait in the domestication of wheat and still remains one of the most important traits in some programs. The high and highly significant correlation of grain number per spike with grain mass per spike (r=0.8812) are in line with the idea that grain weight per spike is determined by TKW and the number of grains (Xie *et al*., 2016).

Grain number per spike was negatively correlated with DTH, which was also not a significant relationship (r=-0.0792, p≤0.6049). Flag leaf area and harvest index had low correlations with the trait and they were both not significant. All other traits had moderate to high correlations with grain number per spike, the lowest of these being with number of tillers (r=0.3918) and the highest being grain weight per spike (r=0.8812). These correlations were also highly significant. However, the highest positive correlation that grain number per spike has is with grain mass per spike which has been previously reported to be a negative relationship (Tahmasebi *et al*., 2017). Grain number is considered to be the main determinant of grain yield in wheat, and is an ideal trait because it is flexible and adaptable to different environments (Xie *et al*., 2016).

Grain mass had a positive relationship with all traits except DTH which was a low correlation and that was not significant. The correlations were mostly high (r>0.65) except for flag leaf area which was low (r=0.3090) but significant (r=0.0389) and harvest index which was not significant. Correlations were highly significant except for the two already mentioned (FLA and HI). The high correlations between grain mass and other traits were expected as these traits form part of the universal yield-related traits in wheat (Liu *et al.*, 2014b). Physiological traits like these are postulated to be the means by which future gains in yield through plant breeding will be made without looking to transgenic technology. Using yield components as part of selection criteria simplifies selection for yield and for them to be used successfully they also need to have high correlations with yield as is the case in this study (Savii and Nedelea, 2012; Xie *et al*., 2016). Traits like grain mass per spike, tiller number and plant biomass had higher heritability estimates in this study and will result in higher genetic gains when selected for, which will lead to yield improvement.

4.2. MS-MARS

4.2.1. Cycle I

Rust resistance genes from the Male sterile-marker assisted recurrent selection (MS-MARS) population initial cycle were screened using their associated markers and presented in Figure 4.3. The highest frequency was observed for the stem and leaf rust resistance gene combination in *Lr24/Sr24* with almost all the female parents (457 in total) having this gene. This confirms that the gene is almost fixed in the population, due to the latter version of the gene with a shortened *Agropyron elongatum* segment that does not have the linkage to red kernels (Sears, 1973). *Lr24/Sr24* is unique because it is active from the seedling stage and also works as an APR, making it an ideal candidate for pyramiding (Pallavi *et al.*, 2015). Although the gene has been overcome by the two rust pathogens, it is still used in breeding programs as shown here. This is mainly because when *Lr24/Sr24* is combined with other resistance genes, the combinations are normally effective which will increase the longevity of genes that have yet to be overcome by rusts (Pallavi *et al.*, 2015).

The *Sr26* gene frequency was very low in the population (9.5%). *Sr26* has been reported to have a negative association with grain yield in wheat, and it is low due to indirect selection against the gene (The *et al.,* 1988). The gene will be removed from the population after a few more cycles of MS-MARS, to possibly be replaced with another gene or the shorter *Ag. elongatum* gene segment which does not have any yield penalty (Dundas *et al*., 2001).

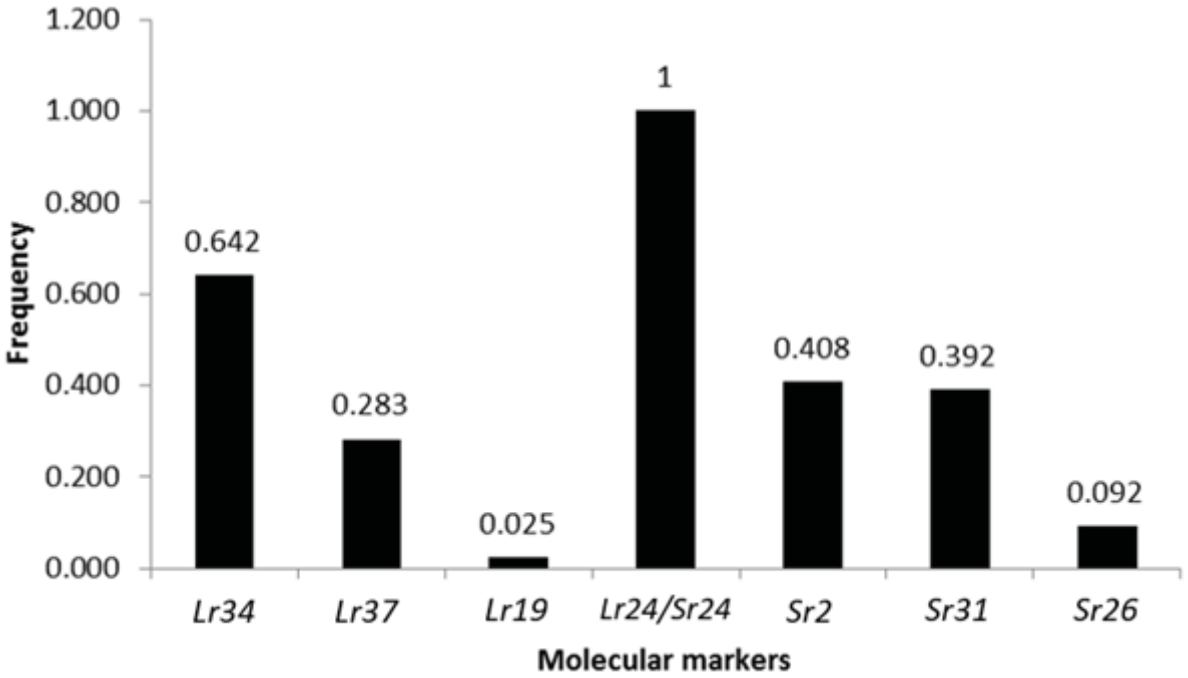

Figure 4.3 Marker frequencies for rust resistance in the MS-MARS cycle one female population.

The frequencies obtained in this cycle were compared to those reported in the previous year reported by Springfield (2014), who had screen the same nursery material in her study. The chi-square test was used because it is robust in checking for significant differences between the frequencies observed for one or more categories (McHugh, 2013). Since that study had only used four of the seven markers used in the current study, the other three were excluded as they had not been reported before in the nursery and there was nothing to compare to. The chi-square test (Table 4.7) reported a chi-square value of 0.0218 (rounded) which was much less than the table chi-square value for 3 degrees of freedom at $\alpha = 5\%$ (7.81). Since the chi-square value obtained was smaller, there were no significant differences in the frequencies observed in the two studies. The small differences observed are due to chance and the differences in the population size used.

Table 4.7 Chi-square test to test for differences between MS-MARS I rust gene frequencies and the previous cycle.

Markers	**Observed**	**Expected**	**X^2**
Lr34	0.64	0.63	0.00023
Sr2	0.41	0.38	0.00211
Sr26	0.09	0	-
Lr24/Sr24	1.00	0.87	0.01943
Total			**0.02175**

At the times of harvest, the segregation ratios of the population were investigated to determine if the *Ms3* gene was still intact in the population. A total of 457 plants of the female population were successfully grown to maturity, of which 242 were male sterile and 215 were

male fertile. The chi-square test was again used to see if there were significant differences between the obtained values and the expected values of 228.5 plants for each category (i.e. 228 and 229 since there is no fraction of a plant). The obtained chi-square value from calculations was 1.60 and the one obtained from the table was 3.84 (α = 5%, 1 df) therefore one could conclude that there were no significant differences between the values obtained in the count and those expected. This means that the *Ms3* gene in the population segregates as expected (Zhai and Liu, 2009).

4.2.2. Cycle II

Gene frequencies from the populations of the two MS-MARS cycles remained fairly similar, following the same trend with the highest gene frequency remaining as the top and hierarchy maintained (Figure 4.4). The very low frequencies for *Lr19* and *Sr31* are the result of deliberate selection against these specific genes from the population since both of these resistance genes been overcome by their respective virulent rust pathotypes (Smit *et al*., 2013). At the time of the discovery of *Ug99*, *Sr31* and *Sr24* resistance genes were widely distributed in commercial wheat cultivars and this new race of stem rust was discovered in its virulence against these two genes (Pretorius *et al.*, 2000). The *Lr19* gene is being selected against because it is the older and longer segment that has tight linkage with the *Y* gene for yellow pigmentation in flour (Knott, 1968). Modified translocations of *Lr19-149-299* and $Sr31_{38.9}$ (concluded in 2012) are in the process of being intergrated into the nursery populations as the old ones are being phased out.

The *Lr37* gene was the third least frequent gene, above *Sr26* and *Lr19* (Figure 4.4). This gene is very important since it is part of a complex obtained from *T. ventricosum* and is linked with other very important resistance genes. The gene is part of a long chromosome fragment that carries resistance genes for powdery mildew, stem rust, leaf rust, stripe rust, eyespot as well as resistance genes against cereal cyst nematodes and Hessian fly (Seah *et al*., 2000). The frequency of this gene will need to be increased in this population by positively selecting for individual plants that carry this gene. With it being a chromosome fragment that does not recombine with wheat chromosomes, an increase in leaf rust resistance will increase resistance to the other biotic agents. This is especially important as nematodes are becoming increasingly important in local cereal production. Some fields have reportedly been abandoned due to continued decreases in yields due to nematode damage as they have a wide host range (Fourie *et al.*, 2009).

Comparisons were made between the two MS-MARS cycles in terms of their gene frequencies to ascertain if there were differences in them. An observed trend was that the gene frequencies were lower in the second cycle than those reported in the first cycle; with the

exception of *Sr2* (Figure 4.4). While this was a welcome result for the genes being selected against for removal in the population, the rest need to remain constant or increase. The goal of using recurrent selection is to improve self-pollinating crops by concentrating desirable alleles in a population (Acquaah, 2007).

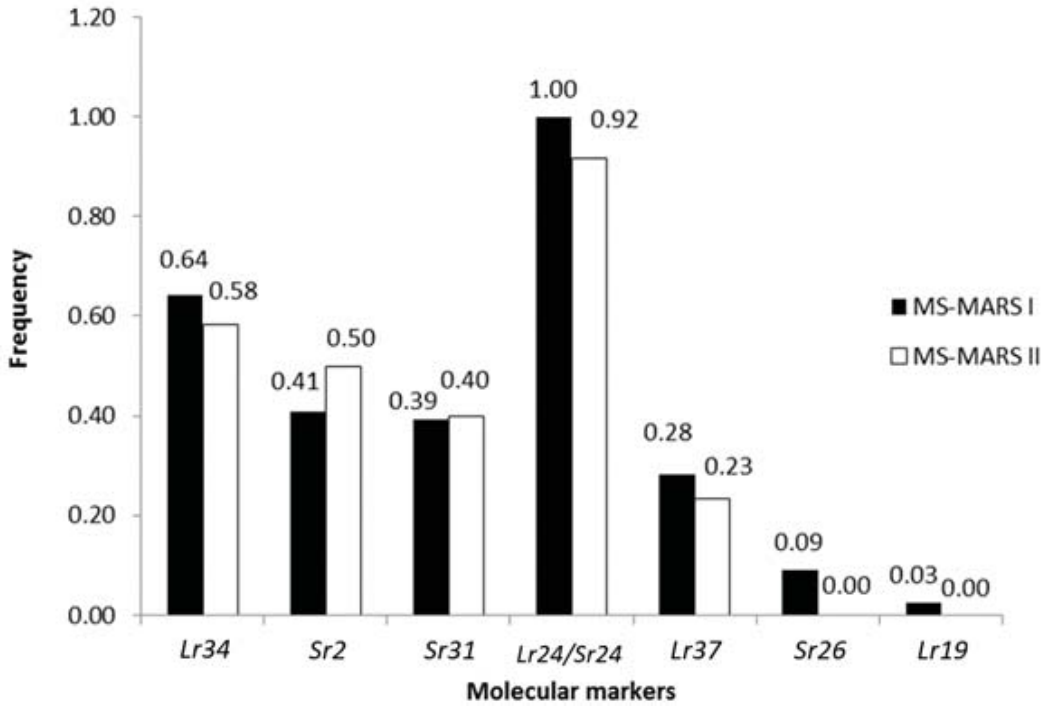

Figure 4.4 Marker frequencies for rust resistance in the MS-MARS cycle two female population, compared to the first cycle.

The chi-square test (Table 4.8) revealed no significant differences between the two cycles (α=5% and 6 df; 12.59). This indicates that even though the observed frequencies in the second cycle were lower, the gene frequencies between the two cycles were not significantly different.

Table 4.8 Chi-square test to test for differences between MS-MARS I and MS-MARS II rust gene frequencies.

Markers	**Observed**	**Expected**	**χ2**
Lr34	0.5833	0.6417	0.00530
Sr2	0.5	0.4083	0.02058
Sr31	0.4	0.3917	0.00018
Lr24/Sr24	0.9167	1	0.00694
Lr37	0.2333	0.2833	0.00882
Sr26	0	0.0917	0.09167
Lr19	0	0.025	0.025
Total			**0.15849**

4.2.3. Single seed descent

The progeny from the second MS-MARS cycle were planted to be used as the first cycle of single seed descent. The total number of seeds that germinated into mature plants was 129 and of these, only 60 were male fertile and formed seeds (Addendum 9). This was in line with the expected segregation ratio of 1:1 when GMS systems such as the *Ms3* gene are used (0.31 calculated chi square against 3.84, therefore no significant differences). When heterozygoes male sterile (Ms3*ms3*) plants are pollinated by homozygous recessive male fertile plants (*ms3ms3*) the progeny is 50% sterile and the rest fertile (Zhai and Liu, 2009). With the removal of the heterozygote male sterile progeny, the population is now completely male fertile. This shows how easy it is to remove dominant genes in a population while recessive alleles can never be fully removed in a population because their expression is always masked by dominant alleles in diploid species (Sleper and Poehlman, 2006; Singh *et al*., 2016b). At harvest, the single seed descent selection population was kept intact to increase the population before selections could begin. This was done so variability could be maintained as the population size increases from the current population of 60 plants in total.

Four principal traits were studied in the population as shown in the summary statistics table (Table 4.9). Very high variance values were observed for the plant height and number of seeds obtained in each plant. These variations were a result of some plants germinating faster than others, giving them an advantage early on which resulted in shadowing effects at a later stage. The plants that germinated later did not receive enough light and that made them less competitive and to produce very small biomass which in turn affected the number of seeds produced due to a weak source strength. The delay in germination is due to differences in seed size and possibly non-uniformity in the growth media density which led to some pots draining nutrient solution slowly, reducing the amount of air available in the soil.

Table 4.9 Summary statistics of agronomic traits assessed in single seed descent population.

Trait	Number of tillers	Plant height (cm)	Number of Seeds	Seed mass (g)
Mean	7.304	70.696	101.441	4.055
Variance	19.779	242.288	7686.147	15.134
Standard deviation	4.447	15.566	87.671	3.890
CV	60.9%	22.0%	86.4%	95.9%

The average height (70.70 cm) was in the desired range of 70-100 cm which enables adequate biomass without any yield penalties due to lodging observed in tall plants (Richards, 1992). The height in the population is controlled by *Rht-B1b* dwarfing genes which have been introgressed in the program and stable for a long time. Since there is an excess of 25 *Rht* genes that have been characterized and named, other genes could be considered for use in

this population to avoid the interference of *Rht-B1b* on the *Fhb4* gene (Srinivasachary *et al*., 2009). However, the benefit of changing the dwarfing gene would have to be a substantial one as this would be a time-consuming task as the *Rht-B1b* gene would need to be removed first before adding a new gene to avoid double dwarfism.

Only two genes for resistance to rust pathogens were screened in this initial single seed population since the *Fusarium* resistance genes were mostly screened by PAGE which is time consuming and more work still needed to be done. Only *Sr2* and *Lr34* were screened to save time and they were chosen because they are both major slow rusting genes for their respective pathogens, and also harbour resistance genes to other pests (McIntosh *et al.,* 1995; Keller *et* al., 2013). The gene frequencies in the single seed descent population were slightly lower (0.317 and 0.417 for the *Lr34* and *Sr2* genes respectively) than those observed in cycle II females of MS-MARS. However, this was not a significant decrease (χ^2 test).

This reduction in the marker frequencies is due to a reduced population size, with the single seed population of plants being only a fraction of its parental MS-MARS population. Additionally, the male parents used in the second MS-MARS cycle was not from the nursery as in the first cycle where the male population has a pyramid of rust resistance genes already in it. The male parents used in this cycle was comprised mostly of the high yield population germplasm, with 12 additional lines. The high yield population's rust resistance marker frequencies were low (Figure 4.1) which would bring down the high frequencies observed in female parents to the resultant progeny.

The *Sr2* gene is important for rust resistance in wheat, more especially because it not only provides resistance against stem rust, but within this complex there is additional resistance against leaf and stripe rusts as well as powdery mildew (McIntosh *et al.,* 1995). The resistance of *Sr2* has been used for successfully in breeding programs for over 80 years (Jia *et al*., 2018). *Lr34* is also part of a complex with resistance against stripe rust (*Yr18*), powdery mildew (*Pm38*) and increased tolerance to barley yellow dwarf virus (*Bdv1*) (Keller *et al.*, 2013). The resistance gene also has added benefits for flour protein content which is important in impoverished populations that depend on wheat as a staple ensuring they get adequate proteins (Labuschagne *et al*., 2002). This makes this initial single seed descent population a good start for the future selections that will have to be made, since selections were not be made at the end of the first cycle to maintain variability in the population.

Frequencies of the markers linked to FHB resistance genes were low due to this being the first progeny of a recurrent selection where germplasm with resistance was added as male parents (Figure 4.5). The highest marker frequency observed was for the *Xgwm304* marker linked to the *Fhb5* gene on the short arm of chromosome 5A. However, the frequency values

themselves are not of much importance unless flanking markers are scored on the same plant to show that the gene has been successfully transferred. Six of the 60 plants had scored positive for the two markers flanking the *Fhb1* gene (*Xgwm533* and *Xgwm493*) located on chromosome 3BS. This indicated that the gene was successfully transferred to 10% of the population. The other flanking marker for this gene was *Barc133* which had a much lower frequency than the other two linked to the gene. This might be due to this marker producing a fragment size of 125 bp which is almost the same size as the *Xgwm130* marker of 126 bp causing an interference with the scoring of the marker.

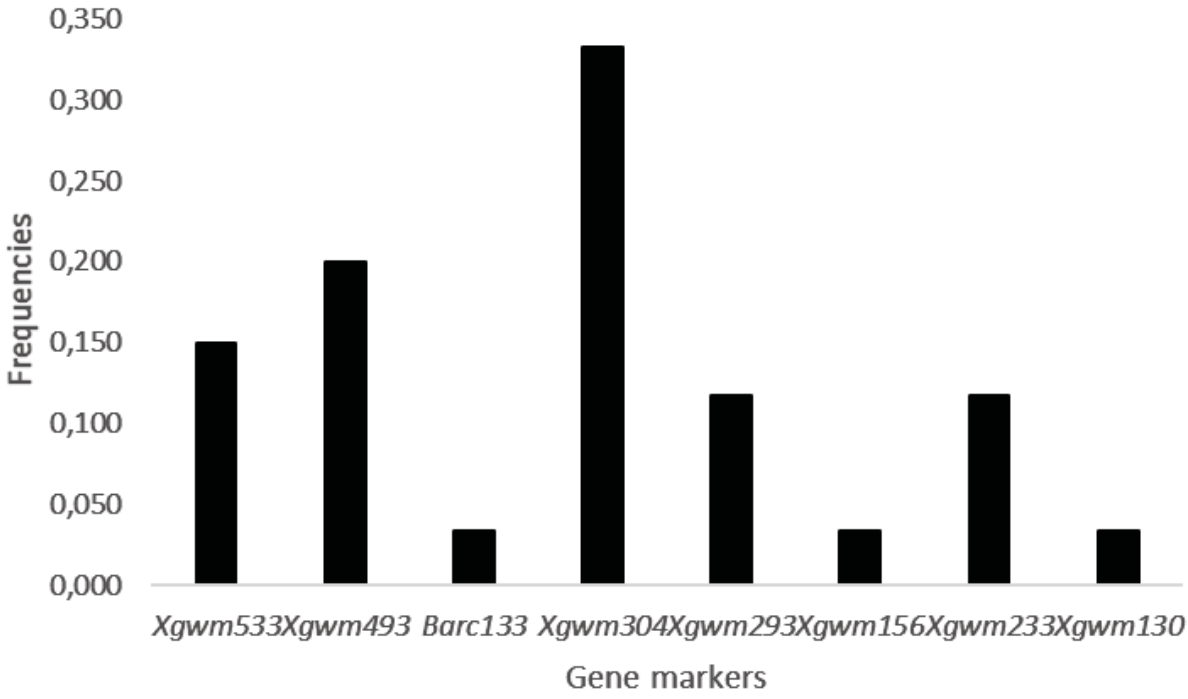

Figure 4.5 Relative prevalence of *Fusarium* resistance gene QTLs after one cycle of crossing.

Markers linked to the *Fhb5* gene were also out of proportion with the marker above the gene (*Xgwm304*) having a frequency of 33.3% while the other flanking at the bottom (*Xgwm293*) was only scored in 11.7% of the plants. The discrepancy in the two marker frequency values is possibly due to recombination events taking place resulting in one segment of the marker interval being passed on much more than the other. The distance between the two markers is 2.5 cM, which is much longer than the interval where the *Fhb5* gene was mapped onto. Xue *et al*. (2011) mapped the location of the gene to a 0.3 cM interval flanked by *Xgwm304* and a different marker named *Xgwm415*. Lin *et al*. (2006) reported a tight linkage between the gene and this *Xgwm415* marker so future screening for the gene will need to utilize this marker to ensure more reliable results.

Only three plants had both flanking markers used for the gene in our genotyping, and using *Xgwm415* could possibly raise the proportion of plants having the gene if recombination events did indeed take place in the 2.5 cM interval. Marker *Xgwm156* is linked to another QTL on chromosome 5A named *Qfhs.ifa-5A-2*. The flanking marker (*Barc197-2*) for this QTL was not used because in previous local studies it could not be successfully used (Sydenham, 2014). There were only two plants that had the *Xgwm156* which is a low frequency, but due to the lack of a flanking marker, the successful transfer of this QTL could not be validated. The three QTLs related to FHB resisatnce on chromosome 5A could be scored together and a genotype with all three could be taken as carrying both QTLs (Sydenham, 2014).

The biggest marker interval between flanking markers used in the study was for the chromosome 7A QTL. The distance between *Xgwm130* and *Xgwm233* was shown to be 75.2 cM. While there were only two plants with both flanking markers, the distance is long enough to allow for a number of recombination events to occur during meiosis since crossing over is a function of the distance between genes (McClean, 1997). However, Kumar *et al*. (2007b) reported a very lengthy QTL of 39.6 cM in this chromosome, placing it close to the centromere. They also found a very tight linkage between the 7A QTL and marker *Xbarc121* where they used interval regression analysis which consistently showed a peak in the marker region over three environments. Mardi *et al*. (2006) also used marker *Xgwm233* to confirm the presence of this 7A chromosome marker in the population, which was the same marker used in our study. The QTL location needs to be fine mapped and the markers that closely flank it or are tightly linked to it be reported and used for future genotyping exercises.

4.3. Establishment of phenotyping platform

4.3.1. Height data collection

This objective was undertaken as a pilot study to investigate if the feasibility of a RPAS-based phenotyping platform. A few flights were taken at various stages during the growth and development cycle of plants. Images that were eventually analysed for the study were taken on the 6th of October 2015 (140 days after planting). The biggest factor leading to this was the delayed roll-out of legislation and regulations for the use of RPAS in South Africa by the South African Civil Aviation Authority (SACAA). The minister of transport's ammendements to the 2011 Civil Aviation Regulations only came into operation on the 1st of July 2015 and the regulations workshop to educate Western Cape users about these was only held on 3rd of August 2015 (CAA, 2017).

Correlations were made with a big sample size representing all the plots used in the Welgevallen field trial, i.e. 90 genotypes, three replications totaling 270 plots. Field data had

low correlations with orthomosaic generated height data. The mean plant height of each plot from the orthomosaic was used as the original height. Initial correlation with this height was very low at r=0.334 with a coefficient of determination (R^2) of 0.230 (Figure 4.6). The very low coefficient of determination (R^2) lead us to re-evaluate the data output from the program.

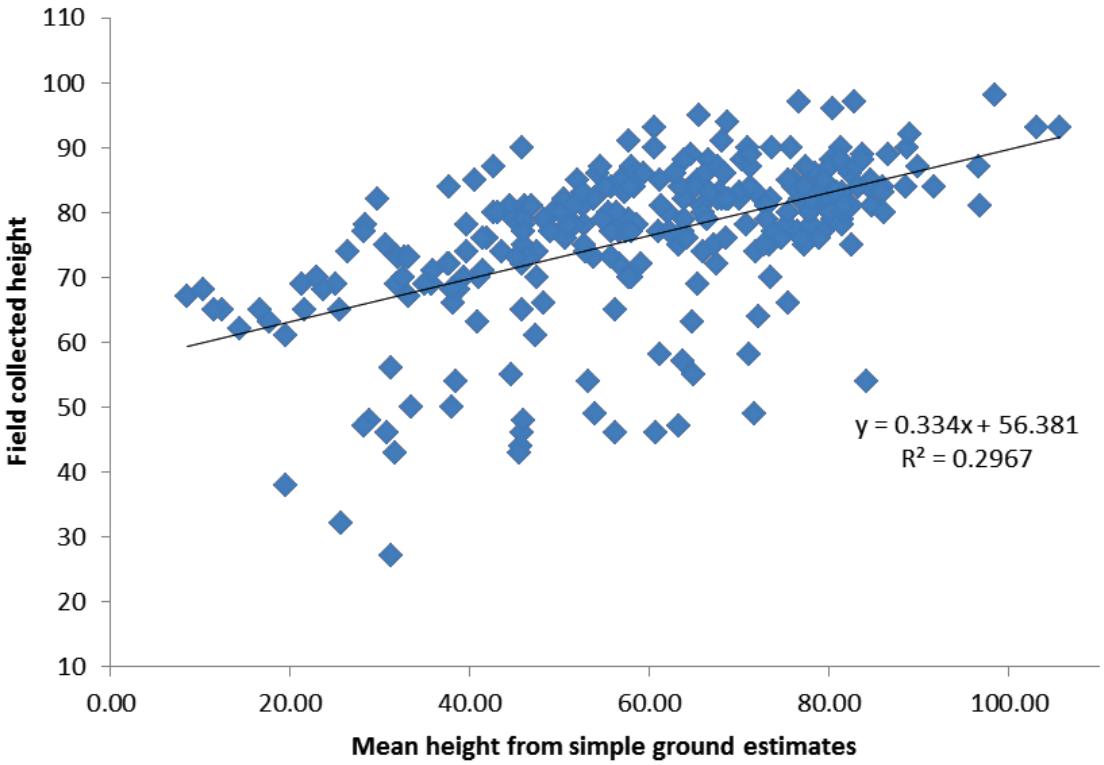

Figure 4.6 Initial correlation results between field collected data and the mean height from the simple ground estimates.

The first step was to compare different statistical models based on the median height value, as well as the 10th, 1st and ½th percentile values. These changes in the initial model were done to determine if any improvements could be made on the output data and correlations. None of the new models improved the correlation values with values lowering from the initial value (0.334) to 0.309, 0.314, 0.305 and 0.304 for the 10th, 1st and ½th percentile correlations, respectively (Table 4.10). The R^2 values were still very low, meaning that the resultant correlations explained only a very small proportion of the data points.

The next step was to reconfigure the ground estimation aspect of the program and see if that would improve the correlation in the data set. This was because the RPAS height measurements are very sensitive to the accuracy of the ground plane. The initial ground estimating procedure was a simple ground estimate which looked at the points around each block of plots in the orthomosaic, then placed a flat plane through the lowest point to estimate the ground.

The next ground estimate procedure was the mean ground estimate, where a flat plane was put through the mean height of four points located at a small distance outside of the four corners of each block. The mean ground estimate resulted in notable increases in the correlation values between the two data sets, as well as improved the coefficient of determination values. The lowest correlation was with the median height values which was r=0.520 (R^2=0.440) and the other four height statistics were not too far from each other in terms of their correlations (Table 4.10 and Figure 4.7). The R^2 values from this ground estimate procedure were still very low, and the resulting correlation formula could not better explain the relationship between the two data sets. Results from this output were still unusable and this was an indication that the output data from the RPAS was not realistic, and very different to the data collected from the plants in the field.

Table 4.10 Correlation values with field collected data using various ground data estimates and selecting varying RPAS statistics of RPAS -output data.

Ground Data Estimate	Mean	Median	10th Percentile	1st Percentile	Half Percentile
Simple (R^2)	0.3340 (0.2967)	0.3086 (0.3126)	0.3137 (0.2984)	0.3046 (0.2730)	0.3040 (0.2696)
Mean (R^2)	0.6274 (0.4584)	0.5201 (0.4396)	0.6373 (0.4971)	0.6481 (0.4701)	0.6505 (0.4656)
Plane of Best Fit (R^2)	0.6923 (0.4800)	0.5542 (0.4534)	0.8293 (0.5965)	0.8898 (0.5828)	0.8980 (0.5782)

The last ground estimate procedure configured on the height estimation programme was the plane of best fit which uses a slightly more accurate plane compared to the others. This was the first ground estimate to yield correlations above r=0.8 and with coefficients of determination of above R^2=0.5, thus half of the correlations could be explained by the trendline. Tenth percentile, first percentile and half percentile heights had the best correlations overall in the project at r=0.829 (R^2=0.597), r=0.890 (R^2=0.583) and r=0.898 (R^2=0.578), respectively (Table 4.10, Figure 4.7, and Figure 4.8).

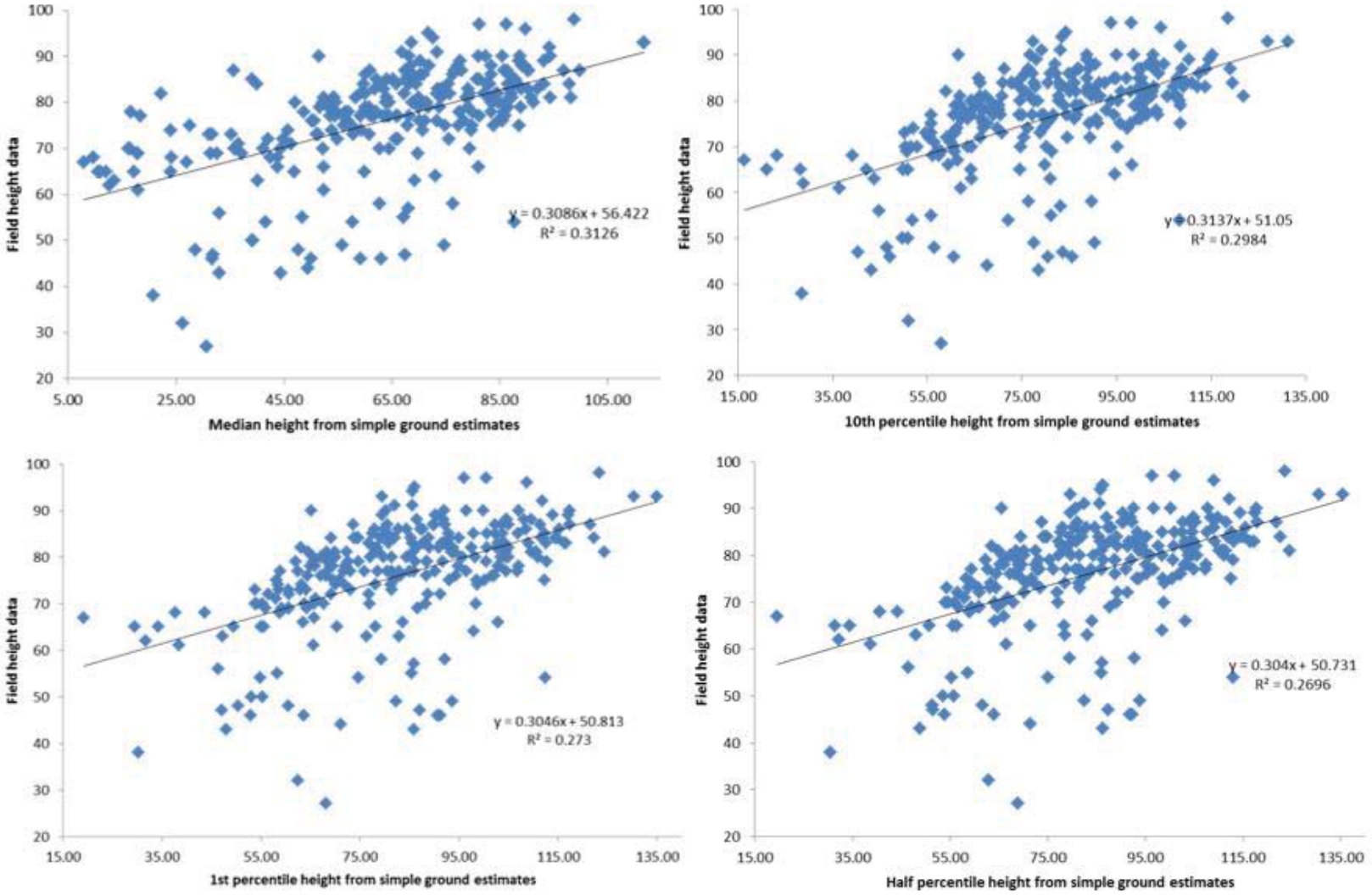

Figure 4.7 Selected correlation graphs to show the relationship between field collected height data and RPAS-generated heights using simple ground estimates.

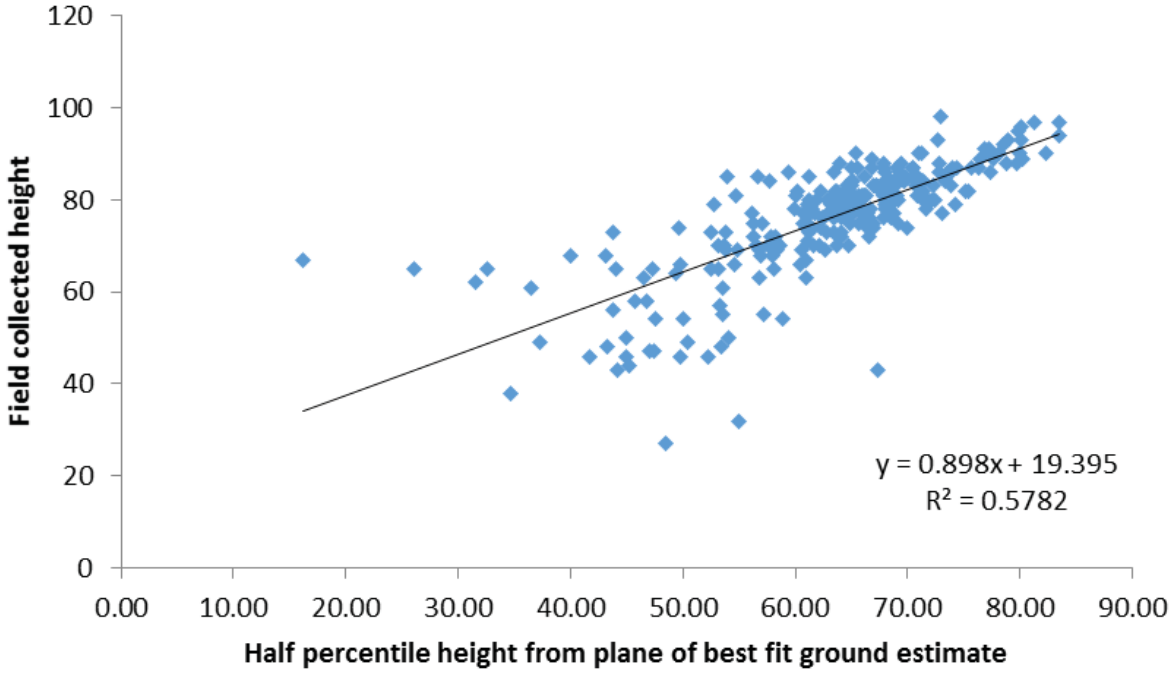

Figure 4.8 The best correlation fit between field-collected data and the half percentile RPAS-generated height data obtained using the best-fit ground estimate.

Kozak *et al*. (2012) reported that in instances where a correlation coefficient is expected to be very high (i.e. close to 1), and if the realized correlation coefficient value is around 0.7 or 0.8, the association should not be considered as strong. In the current study, a very high coefficient correlation (≥0.9) with an equally high coefficient of determination value is required to confirm the suitability of the RPAS as an accurate phenotyping platform. Also, the best height statistic to be used in the data collection process should be the mean height of the plot as that takes into account all the height data points in each plot unlike the percentile statistics which only take into account small proportions.

The best explanation for the low correlations between the two height variables is the developmental stage at which the RPAS flights were conducted to obtain the plant height data. At 140 DAS, the wheat plants are physiologically mature and have undergone anthesis and grain filling. During grain filling, there is an increase in head mass for the plants which leads to the spikes bending over at this stage (Reynolds *et al*., 2012). To obtain accurate field height, plants are usually straightened which is not possible with RPAS-collected plant heights. To establish the RPAS flights as a suitable platform for remotely collecting height data, the flights will need to be done much earlier in the plant development cycle, i.e. before anthesis, to see if there is a significant change in the correlations. Torres-Sánchez *et al.* (2014) reported very high accuracy measures in their study which was conducted in early season wheat from 35-75 DAS.

4.3.2. Troubleshooting during the study

As this was a pilot study, there were challenges that were encountered, some of which were solved and some will need to be improved moving forward. Initial RPAS flights were affected by the use of low capacity batteries that only lasted for part of the overall flight before being drained and needing to be recharged. Batteries were recharged overnight which meant that the images taken on separate days could not be used for scientific use. This issue was rectified by changing the battery type to ones with a higher capacity which were able to cover the specified field area. Another issue was the choice of imaging systems. The Canon SX240 cameras used in the initial stages of the trial were not designed for this sort of work and were negatively affected by the dust particles raised by the copter blades at take-off and landing. This led to them losing function, slightly delaying the project as we looked to getting an improved visual mapping system in a GoPro camera.

The GoPro system cameras were a great improvement to the former because they were lighter and when housed in their case they were dust and crash proof. The initial images taken with these cameras showed a fisheye lens effect, which produced strong visual distortions on the images. These images could not be used for the orthomosaic procedure, so the cameras had to be first calibrated before they could eventually be used. RPAS flights were also limited by prevalent weather conditions. The best days for conducting RPAS flights are sunny days with minimal cloud cover and little or no prevalent winds. Windy conditions will not only affect the RPAS while in flight, but also bend the plants resulting in inaccurate height being recorded.

Chapter 5: Conclusions

Having the MLFT conducted in a year of drought brought about a number of challenges but as climate change effects continue to make their presence felt, this will become a regular problem. There was a positive aspect to it in that the study revealed that not only were the top two yielding genotypes best in good environments, but they still outperformed the rest in a locality that was heavily afflicted by drought. Outside of the scope of this project, the field trial was rerun in the following year with a reduced population where the genotypes expressing the winter type phenotype were removed from the population. Other additional genotypes were removed from the population due to poor performance in this trial reducing the population from 90 genotypes to 65.

Observations from the greenhouse hydroponic trial revealed reduced viariability in the small sample studied which was made of genotypes that produced yield in the top 10 across all localities in MLFT. The lack of variability resulted in very low heritability estimates for the different traits under study, with some even giving negative heritabilities (which were taken as zero). Results from this part of the project revealed highly significant correlations between grain mass with a number of traits including: number of tillers, plant height, spike length, spikelet number, plant biomass as well as grain traits (grain number and weight per spike). This study should be conducted within the field trial where the traits are interacting with the environment as that will likely improve heritability estimates. A field study will also improve the negative correlations that were obtained between days to heading and grain mass. Including genotypes across the yield potential spectrum will help increase variability as well.

Other important traits such as the number of tillers, have been reported to be intergral in crop survival during water limited conditions. In addition, a field trial including sites with varying water availability will confirm if these results can be replicated locally. Future work regarding this objective of a field trial will be to include molecular markers linked to the yield related traits. Adding markers on a trait study will increase reliability of results and correlations could be made between field phenotype and marker data. In addition, markers are not affected by the environment, giving reliable results. Using markers early in the field trial stages will narrow the population.

Regarding markers that are already in the standard marker panel for the program, the ones that are being removed were shown to be almost completely out in the nursery populations. These can now be replaced with functional genes in the case of those that have been overcome by virulence of new pathogen pahotypes or replaced with shorter segments where there is linkage drag. Some markers may need to be replaced with markers that are

easier and quicker to use. For instance, using the STS marker linked to *Glu*-A3 could make marker identification easier if PCR products is run as on agarose gel, rather than on PAGE.

The male sterility-marker assisted recurrent selection (MS-MARS) program was also successfully completed. Differences in gene frequencies between the four cycles (previous cycle, the two cycles in the current project and the progeny/single seed descent population) were not significantly different from each other which can be expected because recurrent selection increases frequencies of desired alleles steadily over time. Where there were changes, these were attributed to chance as different sized populations were used in the various stages. The *Ms3* gene was successfully used and maintained in MS-MARS program with the expected frequencies of male fertile and male sterile genotypes observed at harvest.

The initiation of a SSD effort will add value into the program for a number of reasons. The starting populations were made up of a female nursery population that carries rust resistance genes and a male parent population that is high-yielding. Additional *Fusarium* male parents were added into the cross as male parents and these introduce a new criteria on the platform. The single seed descent method is ideal for such activities because it fixes genes in breeding populations in a relatively short space of time so in a few years and with careful selections, the effort will give a good line(s) for use in breeding programs.

The high throughput phenotyping platform aspect of the study was a pilot study and as such, a number of problems arose in the course of the project which needed trouble-shooting. More work still needs to be done in improving the data that is obtained from the RPAS work but once the issues have been sorted out, this will be an important tool to not only this program but to the whole local plant breeding field. Measuring height data with the RPAS in the late stages of the project also hampered progress and future work with the RPAS will need to start very early in the growing cycle, should be peridodically done (e.g. every two weeks) and correlations constantly being performed with field data.

At the onset of the project the objectives set out were firstly to asses yield and YRTs in both a field and greenhouse hydroponic study in order to identify crossing parents. Secondly it was to to initate a MS-MARS nursery and a single seed descent population with yield and *Fusarium* head blight resistance as additional goals to the rust resistance focus. The third objective was to investigate the feasibility of a high throughput phenotyping platform based on RPAS imagery for use in collecting agronomic trait data from field trials. Objectives were successfully achieved with 44 parents identified from the first objective and used to initiate the single seed descent population. Regardles of challenges, RPAS phenotyping still gave good results to warrant further investigation within the program. The overall aim of the project was therefore achieved in the study and new channels for research have been opened.

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Addendum 1: Germplasm used in study

Entry	GenCode	Pedigree	MS-MARS II
1	15HYLD-01	UNKNOWN	
2	15HYLD-02	UNKNOWN	
3	15HYLD-03	UNKNOWN	
4	15HYLD-04	SST57/SST38//SST55	
5	15HYLD-05	SST57/SST825//SST825	
6	15HYLD-06	SST55/SST57//35F*6-19/SST57	
7	**15HYLD-07**	**INIA66/VGA1//VGAI*2/INIA66**	
8	15HYLD-08	ALPHA/SST825*3//SST886	
9	15HYLD-09	ALPHA/SST825*3//SST886	
10	15HYLD-10	ALPHA/SST825*3//SST886	
11	15HYLD-11	UNKNOWN	
12	**15HYLD-12**	**UNKNOWN**	
13	15HYLD-13	UNKNOWN	
14	15HYLD-14	UNKNOWN	
15	15HYLD-15	09US071	
16	15HYLD-16	01W20728_3	
17	15HYLD-17	04W40343	
18	15HYLD-18	02W50274_1	
19	15HYLD-19	06W31582	
20	15HYLD-20	BABAX/LR42//BABAX/3/BAVIACORA	
21	**15HYLD-21**	**UNKNOWN**	
22	15HYLD-22	UNKNOWN	
23	15HYLD-23	UNKNOWN	
24	15HYLD-24	B-BPT12-04	
25	15HYLD-25	B-BPT12-08	
26	15HYLD-26	C-BPT12-01	
27	**15HYLD-27**	**C-BPT12-08**	
28	15HYLD-28	WAXWING*2/HEILO	
29	15HYLD-29	CRN826/3/ALPHA/SST825*2//SST885	
30	15HYLD-30	CRN826//DUZI/CRN826*	
31	15HYLD-31	INIA66/VGA1//VGA1*2/INIA66/7/CRN826/6/CRN826/5/SST3*//SCOUT*5/AG/3/KAST/PY487(PALMIET)/4/SST23/3/YDING"S"/BLUETIT"S"//KAL/BB	
32	15HYLD-32	CRN826/GROOTAAR-2	
33	15HYLD-33	UNKNOWN	
34	15HYLD-34	CN079/PF70354/MUS/3/PASTOR/4/BABAX/5/BAVIACORA/6/FRAME/BUCHIN	
35	15HYLD-35	ALPHA/SST825*2//SST885/3/INIA66/VGA1//VGA1*2/INIA66	

Entry	GenCode	Pedigree	MS-MARS II
36	15HYLD-36	ALPHA/SST825*2//SST885/3/PROINTA_FEDERAL	
37	15HYLD-37	CRN826/3/INIA66/VGA1//VGA1*2/INIA66	
38	15HYLD-38	CRN826/3/INIA66/VGA1//VGA1*2/INIA66	
39	15HYLD-39	CRN826/3/INIA66/VGA1//VGA1*2/INIA66	
40	15HYLD-40	CRN826/4/FRONTANA/SST876//SST876/3/SST876	
41	15HYLD-41	CRN826/4/FRONTANA/SST876//SST876/3/SST876	
42	15HYLD-42	CRN826/3/W98-6/NC96BGTA-3//CRN826	
43	15HYLD-43	06W30025	
44	15HYLD-44	06W31413	
45	15HYLD-45	PROINTA_FEDERAL/SST88	
46	15HYLD-46	2011 USPBL-057	
47	15HYLD-47	E3A11_16	
48	15HYLD-48	E3B11_05	
49	15HYLD-49	B-BPT09-10	
50	15HYLD-50	MAKHATINI2011(MM ARE)_337	
51	15HYLD-51	BSY 200	
52	15HYLD-52	RSM13_01 [KRONSTAD"S" (F2004//PBW65/2*SERI.1B)]	
53	15HYLD-53	RSM13_02 [BABAX/LR42//BABAX/3/BERKUT/5/CN079//RF70354/ M45/3/PASTOR/BABAX]	
54	15HYLD-54	RSM13_03 [RSM MATCHET]	
55	15HYLD-55	RSM13_04 [RSMW-135]	
56	15HYLD-56	RSM13_05 [03W10068R]	
57	15HYLD-57	DON MARIO13_07	
58	15HYLD-58	DON MARIO13_21	
59	15HYLD-59	DON MARIO13_36	
60	15HYLD-60	DON MARIO13_50	
61	15HYLD-61	KWS MOMONT 1 (MH 11.13)	
62	15HYLD-62	KWS MOMONT 2 (MH 11.30)	
63	15HYLD-63	KWS MOMONT 3 (BASMATI)	
64	15HYLD-64	KWS MOMONT 4 (MH 11.11)	
65	15HYLD-65	KWS MOMONT 5 (MH 11.07)	
66	15HYLD-66	KWS-CORIDALE	
67	15HYLD-67	KWS-STERLING	
68	15HYLD-68	KWS-TARGET	
69	15HYLD-69	KWS-BELUGA	
70	15HYLD-70	KWS-SANTAGO	
71	15HYLD-71	KWS-GRAFTON	
72	15HYLD-72	UNKNOWN	
73	15HYLD-73	UNKNOWN	
74	15HYLD-74	UNKNOWN	

Entry	GenCode	Pedigree	MS-MARS II
75	15HYLD-75	UNKNOWN	
76	15HYLD-76	UNKNOWN	
77	15HYLD-77	UNKNOWN	
78	15HYLD-78	UNKNOWN	
79	15HYLD-79	UNKNOWN	
80	**15HYLD-80**	**UNKNOWN**	
81	15HYLD-81	UNKNOWN	
82	15HYLD-82	UNKNOWN	
83	15HYLD-83	UNKNOWN	
84	15HYLD-84	UNKNOWN	
85	15HYLD-85	UNKNOWN	
86	15HYLD-86	UNKNOWN	
87	15HYLD-87	UNKNOWN	
88	15HYLD-88	UNKNOWN	
89	15HYLD-89	UNKNOWN	
90	15HYLD-90	UNKNOWN	

** **Bold** – Genotypes used in YRT study

Addendum 2: MLFT Yield ANOVA

Overall initial RCBD ANOVA for yield (kg/ha)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	89	806637490.8	9063342.6	3.15	0.0046
Entry x location	178	512853420.3	2881199.0	3.28	0.0000
Locations	2	3643364061.8	1821682030.9	332.05	0.0000
Reps within locs.	6	32917150.3	5486191.7		
Residual	534	469557933.048	879321.972		
Total	809	5465330056.2			

Grand Mean = 3559.343 kg/ha R-Squared = 0.9141 CV = 26.35

Overall revised RCBD ANOVA for yield

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	198021214.5	2385797.8	1.51	0.0046
Entry x location	166	262763305.5	1582911.5	1.69	0.0000
Locations	2	3870101488.0	1935050744.0	286.58	0.0000
Reps within locs.	6	40513836.9	6752306.2		
Residual	498	466541158.894	936829.636		
Total	755	4837941003.7			

Grand mean = 3794.827 kg/ha R^2 = 0.9036 CV = 25.51% LSD (5%) = 1170.975 kg/ha

DF – Degrees of freedom CV – Coeffcient of variation LSD – Least significant differences Locs – Locations RCBD – Randomized complete block design

Addendum 3: Mariendahl yield trial ANOVA tables

General Linear Models ANOVA

Mariendahl yield

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	32278834.829	388901.624	1.74	0.0013
Replications	2	2989350.324	1494675.162	6.70	0.0016
Residual	166	37028298.525	223062.039		
Total	251	72296483.677			

Grand mean = 631.828 kg/ha R^2 = 0.4878 CV = 74.75% Heritability = 0.199

General Linear Models ANOVA

Mariendahl protein

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	4247.645	51.176	0.94	0.6090
Replications	2	1288.622	644.311	11.89	0.0000
Residual	166	8993.105	54.175		
Total	251	14529.372			

Grand mean = 8.027% R^2 = 0.3810 CV = 91.70%

General Linear Models ANOVA

Mariendahl hectolitre mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	87320.223	1052.051	1.08	0.3298
Replications	2	67003.650	33501.825	34.48	0.7294
Residual	166	161282.783	971.583		
Total	251	315606.657			

Grand mean = 74.388 kg/hl R^2 = 0.4890 CV = 16.1% Heritability = 0.027

Addendum 4: Napier yield trial ANOVA tables

General Linear Models ANOVA

Napier yield

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	161363658.9	1944140.5	1.72	0.0017
Replications	2	797134.4	398567.2	0.35	0.7040
Residual	166	188146051.243	1133409.947		
Total	251	350306844.6			

Grand mean = 5795.074 kg/ha R^2 = 0.4629 CV = 18.37% Heritability = 0.193

General Linear Models ANOVA

Napier protein

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	220.033	2.651	2.05	0.0000
Replications	2	18.905	9.453	7.30	0.0009
Residual	166	215.048	1.295		
Total	251	453.987			

Grand mean = 11.270% R^2 = 0.5263 CV = 10.10% Heritability = 0.259

General Linear Models ANOVA

Napier hectolitre mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	4932.332	59.426	1.14	0.2440
Replications	2	223.360	111.680	2.13	0.1216
Residual	166	8686.100	52.326		
Total	251	13841.792			

Grand mean = 80.777 kg/hl R^2 = 0.3725 CV = 8.96% Heritability = 0.043

Addendum 5: Welgevallen yield trial ANOVA tables

General Linear Models ANOVA

Welgevallen yield

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	267142027.4	3218578.6	2.21	0.0000
Replications	2	36727352.1	18363676.1	12.63	0.0000
Residual	166	241366807.920	1454016.915		
Total	251	545236187.4			

Grand mean = 4957.580 kg/ha R^2 = 0.5573 CV = 24.32% Heritability = 0.288

General Linear Models ANOVA

Welgevallen protein

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	356.879	4.300	1.18	0.1858
Replications	2	12.647	6.324	1.73	0.1797
Residual	166	605.326	3.647		
Total	251	974.853			

Grand mean = 14.842% R^2 = 0.3791 CV = 12.87% Heritability = 0.056

General Linear Models ANOVA

Welgevallen hectolitre mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entry	83	8348.154	100.580	1.36	0. 0503
Replications	2	116.764	58.382	0.79	0.4570
Residual	166	12318.069	74.205		
Total	251	20782.987			

Grand mean = 80.047 kg/hl R^2 = 0.4073 CV = 10.76% Heritability = 0.106

Addendum 6: ANOVA tables for first YRT study

Days to heading (DTH)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	370.6667	92.6667	10.2773	0.0031
Replications	2	34.5333	17.2667	1.9150	0.2091
Residual	8	72.1333	9.0167		
Total	14	477.3333			

CV = 3.637% Heritability = 0.919%

Flag leaf area (FLA)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	2.8351	0.7088	13.9123	0.0011
Replications	2	0.0101	0.0051	0.0995	0.9064
Residual	8	0.4076	0.0509		
Total	14	3.2528			

CV = 5.549% Heritability = 0.909%

Grain mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	42.5994	10.6498	3.9561	0.0465
Replications	2	34.0975	17.0487	6.3331	0.0225
Residual	8	21.5359	2.6920		
Total	14	98.2328			

CV = 9.554% Heritability = 0.923%

Grain number/Spike

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	456.7983	114.1996	3.3715	0.0675
Reps	2	135.7243	67.8622	2.0035	0.1971
Residual	8	270.9785	33.8723		
Total	14	863.5011			

CV = 10.372% Heritability = 0.797%

Grain mass/Spike

Source	*DF*	*Sum of Squares*	*Mean Square*	*F-Value*	*Pr>F*
Entries	4	1.5578	0.3894	5.6412	0.0186
Replications	2	0.4051	0.2026	2.9342	0.1107
Residual	8	0.5523	0.0690		
Total	14	2.5152			

CV = 7.442% Heritability = 0.936%

Plant height

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	24.9333	6.2333	0.3692	0.8244
Replications	2	32.9333	16.4667	0.9753	0.4178
Residual	8	135.0667	16.8833		
Total	14	192.9333			

CV = 4.889% Heritability = 0.303%

Harvest index (HI)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	0.0105	0.0026	8.5505	0.0055
Replications	2	0.0001	0.0001	0.1735	0.8438
Residual	8	0.0024	0.0003		
Total	14	0.0130			

CV = 1.277% Heritability = 0.985%

Spike length

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	5.1562	1.2890	5.5832	0.0191
Replications	2	2.0590	1.0295	4.4590	0.0500
Residual	8	1.8470	0.2309		
Total	14	9.0622			

CV = 4.619% Heritability = 0.878%

Plant biomass

Source	*DF*	*Sum of Squares*	*Mean Square*	*F-Value*	*Pr>F*
Entries	4	106.9507	26.7377	3.5245	0.0610
Replications	2	123.2573	61.6287	8.1238	0.0118
Residual	8	60.6893	7.5862		
Total	14	290.8973			

CV = 8.763% Heritability = 0.905%

Spikelet Number

Source	*DF*	*Sum of Squares*	*Mean Square*	*F-Value*	*Pr>F*
Entries	4	16.5773	4.1443	6.5905	0.0120
Replications	2	4.5160	2.2580	3.5908	0.0771
Residual	8	5.0307	0.6288		
Total	14	26.1240			

CV = 4.616% Heritability = 0.651%

Number of tillers

Source	*DF*	*Sum of Squares*	*Mean Square*	*F-Value*	*Pr>F*
Entries	4	3.7333	0.9333	3.2941	0.0711
Replications	2	1.7333	0.8667	3.0588	0.1031
Residual	8	2.2667	0.2833		
Total	14	7.7333			

CV = 11.917% Heritability = 0.433%

Addendum 7: ANOVA tables for second YRT study

Days to heading (DTH)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	1624.0000	406.0000	3.3679	0.0676
Replications	2	172.9333	86.4667	0.7173	0.5170
Residual	8	964.4000	120.5500		
Total	14	2761.3333			

CV = 13.611% Heritability = 0.441%

Flag leaf area (FLA)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	0.9563	0.2391	0.8129	0.5510
Replications	2	0.1524	0.0762	0.2592	**0.7779**
Residual	8	2.3529	0.2941		
Total	14	3.4616			

CV = 13.062% Heritability = -0.067%

Grain mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	36.7931	9.1983	2.1125	0.1709
Replications	2	3.3829	1.6914	0.3885	0.6902
Residual	8	34.8335	4.3542		
Total	14	75.0095			

CV = 27.761% Heritability = 0.271%

Grain number per spike

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	486.8273	121.7068	2.1153	0.1705
Replications	2	64.6630	32.3315	0.5619	0.5911
Residual	8	460.2935	57.5367		
Total	14	1011.7838			

CV = 19.126% Heritability = 0.271%

Grain mass per spike

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	1.3790	0.3448	2.6223	0.1146
Replications	2	0.1234	0.0617	0.4692	0.6417
Residual	8	1.0518	0.1315		
Total	14	2.5541			

CV = 16.945% Heritability = 0.770%

Plant height

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	248.6667	62.1667	1.7454	0.2329
Replications	2	6.4000	3.2000	0.0898	0.9150
Residual	8	284.9333	35.6167		
Total	14	540.0000			

CV = 7.739% Heritability = 0.626%

Harvest Index

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	0.0049	0.0012	0.9824	0.4687
Replications	2	0.0008	0.0004	0.3248	0.7318
Residual	8	0.0100	0.0012		
Total	14	0.0157			

CV = 4.944% Heritability = 0.591%

Spike length

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	1.9786	0.4947	0.8108	0.5521
Replications	2	0.4519	0.2259	0.3703	0.7017
Residual	8	4.8808	0.6101		
Total	14	7.3113			

CV = 9.538% Heritability = -0.067%

Plant biomass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	103.5560	25.8890	1.3347	0.3365
Replications	2	11.1640	5.5820	0.2878	0.7574
Residual	8	155.1760	19.3970		
Total	14	269.8960			

CV = 30.370% Heritability = 0.612%

Spikelet number

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	5.9307	1.4827	0.5796	0.6861
Replications	2	0.0013	0.0007	0.0003	0.9997
Residual	8	20.4653	2.5582		
Total	14	26.3973			

CV = 10.328% Heritability = -0.163%

Number of Tillers

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	4.6667	1.1667	3.6842	0.0551
Replications	2	0.1333	0.0667	0.2105	0.8145
Residual	8	2.5333	0.3167		
Total	14	7.3333			

CV = 15.347% Heritability = 0.472%

Addendum 8: ANOVA tables for third YRT study

Days to heading (DTH)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	320.6667	80.1667	3.7258	0.0536
Replications	2	109.2000	54.6000	2.5376	0.1401
Residual	8	172.1333	21.5167		
Total	14	602.0000			

CV = 5.608% Heritability = 0.784%

Flag leaf area (FLA)

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	0.2684	0.0671	0.5223	0.7227
Replications	2	0.2784	0.1392	1.0838	0.3832
Residual	8	1.0275	0.1284		
Total	14	1.5743			

CV = 10.431% Heritability = 0.350%

Grain mass

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	2.9363	0.7341	0.2250	0.9169
Replications	2	1.6787	0.8393	0.2573	0.7793
Residual	8	26.0958	3.2620		
Total	14	30.7108			

CV = 23.483% Heritability = 0.275%

Grain number per spike

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	153.2004	38.3001	0.4544	0.7674
Replications	2	41.3125	20.6563	0.2451	0.7883
Residual	8	674.3114	84.2889		
Total	14	868.8244			

CV = 20.048% Heritability = -0.222%

Grain mass per spike

Source	*DF*	*Sum of squares*	*Mean square*	*F-Value*	*Pr>F*
Entries	4	0.2897	0.0724	0.4824	0.7489
Replications	2	0.0641	0.0320	0.2133	0.8123
Residual	8	1.2010	0.1501		
Total	14	1.5547			

CV = 18.305% Heritability = -0.209%

Plant height

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	111.3333	27.8333	1.0451	0.4416
Replications	2	70.9333	35.4667	1.3317	0.3168
Residual	8	213.0667	26.6333		
Total	14	395.3333			

CV = 7.303% Heritability = 0.015%

Harvest Index (HI)

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	0.0037	0.0009	0.6338	0.6526
Replications	2	0.0090	0.0045	3.0363	0.1044
Residual	8	0.0118	0.0015		
Total	14	0.0245			

CV = 6.900% Heritability = -0.139%

Spikelet Length

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	2.0844	0.5211	0.5611	0.6978
Replications	2	0.2485	0.1243	0.1338	0.8767
Residual	8	7.4297	0.9287		
Total	14	9.7626			

CV = 11.105% Heritability = -0.171%

Plant biomass

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	5.7173	1.4293	0.1390	0.9630
Replications	2	12.5440	6.2720	0.6099	0.5668
Residual	8	82.2627	10.2828		
Total	14	100.5240			

CV = 23.895% Heritability = -0.403%

Spikelet number

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	7.5027	1.8757	0.7190	0.6024
Replications	2	1.0173	0.5087	0.1950	0.8266
Residual	8	20.8693	2.6087		
Total	14	29.3893			

CV = 9.074% Heritability = 0.568%

Number of tillers

Source	DF	Sum of squares	Mean square	F-Value	Pr>F
Entries	4	0.9333	0.2333	1.2727	0.3564
Replications	2	1.2000	0.6000	3.2727	0.0915
Residual	8	1.4667	0.1833		
Total	14	3.6000			

CV = 8.603% Heritability = 0.681%

Addendum 9: Single seed descent germplasm performance

Plant	Fertile	Tillers	Height (cm)	Health	No. of Seeds	Seed mass	Lr34	Sr2	Rht-B1b
01A	1	9	69	0	123	3.831	0	0	1
01B	1	3	52	1	43	1.182	1	0	1
01C	1	6	60	0	22	1.57	H	1	1
02A	1	5	65	0	113	5.022	H	0	1
02B	1	4	48	1	46	1.137	H	0	1
02C	0	5	65				0	1	1
03A	1	7	75	0	175	8.108	1	0	1
03B	0	6	50				0	0	1
03C	0	5	40				0	1	1
04A	1	2	63	1	60	1.782	0	1	1
04B	0	3	43				0	0	1
04C	1	22	87	0	100	5.296	1	1	1
05A	1	7	50	0	43	2.098	1	0	1
05B	1	17	120	0	492	21.49	0	1	1
05C	0	4	47				H	1	1
06A	1	4	66	1	54	1.512	H	0	1
06B	1	7	79	0	23	1.078	1	0	1
06C	0	7	75				0	0	1
7A	0	12	63				H	0	1
08A	0	16	75				0	1	1
08B	1	9	85	1	175	5.941	0	1	1
08C	-99	-99	-99	-99			H	0	1
09A	1	12	75	1	153	5.787	0	1	1
09B	0	12	80				0	1	1
09C	0	6	76				0	1	1
10A	1	6	60	1	153	3.624	H	1	1
10B	1	3	93	0	219	10.664		0	1
10C	0	9	61				H	0	1
11A	0	3	47				H	0	1
11B	1	12	95	1	94	4.188	0	1	1
11C	0	13	73				H	1	1
12A	1	16	88	1	148	8.671	1	1	1

Plant	Fertile	Tillers	Height (cm)	Health	No. of Seeds	Seed mass	Lr34	Sr2	Rht-B1b
12B	1	9	73	0	74	3.628	0	0	1
12C	1	8	68	0	104	2.149		0	1
13A	1	14	80	0	143	6.667	H	0	1
13B	1	6	57	1	75	2.968	0	0	1
13C	1	6	71	2	82	2.89	H	0	1
14A	0	10	75				1	0	1
14B	1	7	58	2	104	3.054	0	0	1
15A	0	6	60				0	1	1
15B	0	9	80				1	0	1
15C	0	11	62				0	0	1
16A	0	6	69				1	0	1
16B	1	4	45	0	6	0.416	H	0	1
16C	0	26	113				1	1	0
17A	1	5	55	0	69	2.634		1	1
17B	0	13	83				H	0	1
17C	1	8	85	0	175	8.494		1	1
18A	1	4	66	1	46	1.956	0	0	1
18B	0	6	25				1	0	1
18C	0	5	56				0	1	1
19A	1	7	94	0	294	12.413	0	0	1
19B	-	-	-	-			H	1	1
19C	0	11	85				0	1	1
20A	0	21	88				1	1	1
20B	1	13	64	2	81	2.551	H	0	1
21A	0	5	66					1	1
21B	1	9	84	0	228	10.722	1	1	1
21C	0	16	42				H	0	1
22A	0	3	30				H	0	1
22B	1	6	77	1	120	3.136	0	1	1
22C	1	15	88	0	228	8.692	0	0	1
23A	0	3	55				0	0	1
23B	1	17	103	2	268	11.254	0	1	1
23C	0	9	80				1	1	1
24A	0	12	85				0	1	1
24B	1	3	55	1	19	0.46	0	1	1
24C	0	11	65				H	1	1

Plant	Fertile	Tillers	Height (cm)	Health	No. of Seeds	Seed mass	Lr34	Sr2	Rht-B1b
25A	1	2	55	0	17	0.58	H	1	1
25B	0	6	68				H	0	1
25C	0	5	70				H	1	1
26A	0	7	60				H	0	1
26B	1	13	92	0	203	9.551	H	0	1
26C	0	9	65				0	0	1
27A	0	3	64				0	0	1
27B	1	7	78	1	80	4.104	1	0	1
27C	0	6	65					0	1
28A	1	2	46	0	8	0.414	0	1	1
28B	1	10	76	0	201	6.338	H	1	1
28C	0	14	84				1	1	1
29A	0	9	79				0	0	1
29B	1	3	62	0	38	1.178	H	0	1
29C	0	13	80					1	1
30A	0	9	70				H	1	1
30B	1	8	74	1	101	3.96	H	0	1
30C	1	3	60	1	23	0.756	0	1	1
31A	1	10	85	0	218	7.593	0	1	1
31B	0	5	65				0	0	1
31C	1	4	73	0	45	1.477	H	0	1
32A	1	2	45	1	44	0.96	0	0	1
32B	0	9	85				H	0	1
32C	1	3	70	1	29	1.263	0	1	1
33A	1	4	55	1	51	1.814	0	1	1
33B	1	11	73	1	64	4.048	0	0	1
33C	0	7	50				0	1	1
34A	1	5	64	0	50	1.943	H	0	1
34B	0	16	75				0	0	1
34C	0	6	80				1	1	1
35A	0						H	0	1
35C	0							0	1
36A	0						0	0	1
36B	0			2			H	0	1
36C	0						0	1	1
37A				0	498	21.984	H	1	1

Plant	Fertile	Tillers	Height (cm)	Health	No. of Seeds	Seed mass	Lr34	Sr2	Rht-B1b
37B							1	0	1
37C	1			0	12	0.294	1	0	1
38A	1			1	59	1.807	H	0	1
38B	1			0	66	3.369	0	0	1
38C	0						0	0	1
39A	0						H	1	1
39B	1			0	25	1.399	H	0	1
40A					104	2.566	0	0	1
40B					38	1.771	0	0	1
41A	1	3	50	0	64	1.636	0	0	1
41B	0	11	95				0	0	1
42A	1	5	65	1	76	0.712	0	1	1
42B	1	5	63	0	19	2.237	H	1	1
43A	0	8	95				0	0	1
43B	1	7	75	1	94	2.274	0	0	1
44A	-	-	-	-			H	0	1
44B	0	9	70				0	1	1
45A	0	6	70				H	0	1
45B	0	5	70				0	0	1
46A	1	5	75	1	46	2.496	1	1	1
46B	0	7	75				H	0	1
47A	0	11	77				0	1	1
47B	0	5	48				1	0	1
48A	1	5	70	1			H	0	1
48B	0	13	83				1	1	1