Initiation of a pre-breeding effort for water stress resistance traits and yield improvement in wheat

by

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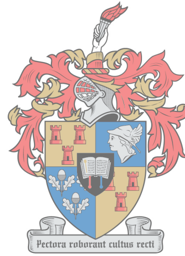

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December 2018

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Abstract

Wheat (*Triticum aestivum* L.) is an important crop produced in South Africa and across the world. Water stress and rust diseases (*Puccinia* spp.) are common factors hindering wheat growth and development. Leaf lifespan is reduced under water stress conditions from the leaf being infected by rust diseases. High-heritable Mendelian-inherited and quantitative traits as well as male sterility mediated marker assisted selection (MS-MARS) technique was utilised for water stress and rust disease resistant wheat characterisation. The aim of the study was initiation of a pre-breeding effort for water stress resistance traits and yield improvement in wheat

Sixty high-yielding genotypes and a female F1 1:1 male sterile and male fertile segregating population postulated to carry the leaf and stem rust resistance genes were screened for the presence of *Lr34*, *Sr2*, *Sr31*, *Sr24*, *Lr37*, *Sr26* and *Lr19* markers using a routinely standardised panel of markers used in the Stellenbosch University Plant Breeding Laboratory. Molecular characterisation of wheat lines was followed by cross-pollinations of a selected male sterile female and donor lines in the growth chamber using a reticulated hydroponic system (RHS) for the MS-MARS cycle scheme. Male fertile tillers were allowed to self-pollinate and were used for single-seed dehiscence.

Sixty genotypes were phenotypically screened using identified and selected target traits associated with water stress resistance. Five genotypes were selected and further screened for water stress resistance using added traits of interest. An RHS was utilised for screening of the target traits including excised-leaf water loss, leaf relative water content, specific leaf area, number of tillers (NT), number of leaves and length-related parameters such as root length (RL) and shoot length (SL). Fresh weight parameters included roots fresh weight (RFW), shoots fresh weight (SFW), leaves fresh weight (LFW) and total plant fresh weight (TPFW). Dry weight parameters included roots dry weight, shoots dry weight, leaves dry weight (LDW), above-ground dry weight and total plant dry weight (TPDW). Additional traits included chlorophyll content index (CCI), stomatal conductance, photosynthetic active radiation, leaf area index, radiation use efficiency, relative growth rate (RGR) and root-to-shoot ratio.

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Rust disease resistant genotypes were identified from the studied population. Molecular characterisation of the wheat genotypes for rust resistance genes showed increased allele frequencies in MS-MARS cycles 1 to 2 for both female and male lines, more specifically *Lr34* and *Sr2*. However, the male lines showed lower allele frequencies and absence of the *Lr19* marker in the population. Analysis of variance showed that water stress significantly influenced the growth and development of wheat genotypes for all the studied traits except RL and NT. The selected five genotypes showed better water stress resistance for all the traits studied. Genotypes were ranked as follows based on their performance under water stress conditions: 15HYLD-30, 15HYLD-22, 15HYLD-29, 15HYLD-18 and 15HYLD-26.

A strong positive association observed under water stress conditions from fresh weight components included LFW and RFW (r = 0,884), followed by TPFW with FW components such as RFW (r = 0,848), SFW (r = 0,922) and LFW (r = 0,920). A strong positive association was also recorded for SFW and SL (r = 0,832), CCI with SL (r = 0,835) and SFW (0,890) and lastly, TPDW with RGR (r = 0,879) and LDW (r = 0,872). A strong positive association was recorded under well-watered conditions namely TPFW showed a strong positive association with SFW (r = 0,872), LFW (r = 0,920), TPDW with SL (r = 0,877) and LDW (r = 0,841).

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Opsomming

Koring (*Triticum aestivum* L.) is 'n belangrike gewas wat in Suid-Afrika en wêreldwyd verbou word. Waterstres en roessiektes (*Puccinia* spp.) is algemene faktore wat die groei en ontwikkeling van koring belemmer. Blaarlewensduur word onder waterstresomstandighede verlaag deur blare wat met roessiektes besmet word. Hoë vererfbare Mendeliaanse oorgeërfde en kwantitatiewe eienskappe en die tegniek van manlike steriliteitsbemiddelde merkergeassisteerde seleksie (MS-MARS) is vir karakterisering van waterstres- en roessiekteweerstand onder koring gebruik. Die doel van die studie was die toepassing van 'n voorkwekingspoging gemik op eienskappe van waterstresweerstand vir die verhoging van koringopbrengste.

Sestig hoë-opbrengsgenotipes en vroulike F1 1:1 manlike steriele en manlike vrugbare geskeide populasies wat veronderstel is om die blaar- en stamroesweerstandgene te dra, is gesif vir die teenwoordigheid van *Lr34-, Sr2-, Sr31-, Sr24-, Lr37-, Sr26-* en *Lr19*-merkers met behulp van 'n roetine-gestandaardiseerde paneel merkers wat in die Universiteit Stellenbosch se planttelingslaboratorium (SU-PBL) gebruik word. Molekulêre karakterisering is opgevolg met kruisbestuiwings van seleksies manlike steriele en skenkerlyne in die groeikamer met gebruik van 'n hidroponiese stelsel vir die MS-MARS-siklusskema. Manlike vrugbare waterlote is toegelaat om te selfbestuif en is gebruik vir enkelsaad-oopspringing.

Sestig genotipes is fenotipies gesif met gebruik van geïdentifiseerde en gekose teikeneienskappe wat met waterstresweerstand geassosieer word. Vyf genotipes is gekies en verder gesif vir waterstresweerstand met behulp van bykomende belangwekkende eienskappe. 'n Geretikuleerde hidroponiese stelsel is gebruik vir die sifting van die teikeneienskappe, insluitende waterverliese van uitgesnyde blare, blare se relatiewe waterinhoud, spesifieke blaaroppervlakte, aantal waterlote en aantal blare, en lengteverwante parameters soos wortellengte (RL) en lootlengte (SL). Varsgewigparameters het ingesluit wortels se vars gewig (RFW), lote se vars gewig (SFW), blare se vars gewig (LFW) en die totale plant se vars gewig (TPFW). Droëgewigparameters het ingesluit wortels se droë gewig, lote se droë gewig, blare se droë gewig (LDW), bogrondse droë gewig en die totale plant se droë gewig

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(TPDW). Bykomende eienskappe het ingesluit chlorofilinhoud-indeks (CCI), stoma-konduktansie, fotosintetiese aktiewe straling, blaaroppervlakte-indeks, stralingsgebruikdoeltreffendheid, relatiewe groeitempo (RGR) en wortel-tot-loot-verhouding.

Roessiekteweerstand-genotipes is uit die bestudeerde populasie geïdentifiseer. Molekulêre karakterisering van die roesweerstandgene wat uit die koringgenotipes verkry is, het ʼn toename in die alleelfrekwensies in MS-MARS-siklusse een tot twee vir sowel vroulike as manlike lyne getoon, meer spesifiek *Lr34* en *Sr2*. Die manlike lyne het egter laer alleelfrekwensies en afwesigheid van die *Lr19*-merker in die populasie getoon. Die variansieontleding (ANOVA) het getoon dat waterstres die groei en -ontwikkeling van koringgenotipes vir al die bestudeerde eienskappe aanmerklik beïnvloed, behalwe die RL en NT. Die gekose vyf genotipes het die beste waterstresweerstand getoon van al die eienskappe wat bestudeer is. Die genotipes is op grond van hul prestasie onder waterstresomstandighede in die volgende rangorde geplaas: 15HYLD-30, 15HYLD-22, 15HYLD-29, 15HYLD-18 en 15HYLD-26.

Sterk positiewe assosiasie (SPA) wat onder waterstresomstandighede by vasgewigkomponente waargeneem is, het LFW en RFW (r = 0.884) ingesluit, gevolg deur TPFW met varsgewigkomponente soos RFW (r = 0.848), SFW (0.922) en LFW (r = 0.920). SPA is ook opgeteken vir SFW en SL (r = 0.832), CCI met SL (r = 0.835) en SFW (0.890) en, laastens, TPDW met RGR (r = 0.879) en LDW (r = 0.872). SPA opgeteken onder waterryke omstandighede, naamlik TPFW, het SPA met SFW (0.872), LFW (r = 0.920) en TPDW met SL (r = 0.877) en LDW (r = 0.841) getoon.

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List of abbreviations

%	percent
˚C	degrees Celsius
$^1O_2$	singlet oxygen
2n	diploid
µl	microlitre
µM	micromolar
ABA	abscisic acid
ABGB	aboveground biomass
ADC	arginine decarboxylase
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
ART	Addis Rough tote
ATP	adenosine triphosphate
B	Boron
bp	base pairs
Ca	Calcium
CAPS	cleaved amplified polymorphic sequence
CAT	catalase
CCI	chlorophyll content index
Chl	chlorophyll content
cM	centimorgan
cm	centimetre

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CMS	cytoplasmic male sterility
CO_2	Carbon dioxide
CS	Chinese Spring
CTAB N-Cetyl-N, N,	N-trimethyl Ammonium Bromide
Cu	Copper
DH	double haploid
dH_2O	distilled water
DNA	deoxyribonucleic Acid
dNTP	deoxyribonucleotidetriphosphate
DREB	DRE-Binding proteins
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
ELWL	excised leaf water loss
EtBr	ethidium Bromide
F	forward primer
F1	filial one
Fe	iron
Fe-S	iron sulfur clusters
FLS	flag leaf senescence
FW	fresh weight
g	gram
gDNA	genomic deoxyribonucleic acid
gg-1 d-1	gram per gram per decimetre

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GMS	genetic male sterility
GR	glutathione reductase
g_s	stomatal conductance
H^2	broad sense heritability
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HO	hydroxyl radical
HZ	Hertz
ICARDA	International Centre for Agricultural Research in the Dry Areas
ILDW	initial leaves dry weight
IRDW	initial roots dry weight
ISDW	initial shoots dry weight
K	potassium
kDA	kilodalton
LAE	leaf area
LAI	leaf area index
LDW	leaves dry weight
LEA	late embryogenic abundant
LED	light-emitting diode
LFW	leaves fresh weight
Lr	leaf rust resistance gene
LRWC	leaf relative water content

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LTN	leaf tip necrosis
M	molar
MAS	marker-assisted selection
Max	maximum
Mb	megabases
Mg	microgram
min	minutes
Min	minimum
ml	millilitre
mm	millimetre
Mm	millimolar
mmol m^{-2} s^{-1}	millimole per square millimetre per seconds
Mn	manganese
Mo	molybdenite
MS-MARS	male sterility-mediated marker-assisted recurrent selection
mtlD	mannitol-1-phosphate dehydrogenase
n	haploid
N	Nitrogen
NaCl	Sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
Ng	nanogram

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ng/µl	nanogram per microlitre
NL	number of leaves
NT	number of tillers
O^{2-}	superoxide radical
ODC	ornithine decarboxylase
P	Phosphorus
P5CR	pyrroline-5-carboxylate synthetase
PA	polyamine
PAR	photosynthesis active radiation
Pas	polyamines
PBC	pseudo-black chaff
PCC	positive correlation coefficiency
PCR	polymerase chain reaction
pH	Hydrogen ions concentration
PH	plant height
POX	peroxidase
PRO	proline
(Pty) Ltd	proprietary limited
PVC	polyvinyl chloride
QTL	quantitative trait locus
R	reverse primer
R: S ratio	root-to-shoot ratio
RAPD	random amplified polymorphic DNA

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RDW	roots dry weight
RFLP	restriction fragment length polymorphism
RFW	roots fresh weight
RGR	relative growth rate
RHS	reticulated hydroponic system
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RL	root length
RN	root number
RNA	ribonucleic acid
Rpm	revolutions per minute
RSA	Republic of South Africa
RUE	radiation use efficiency
RWC	relative water content
S	Sulphur
SAMDC	S-adenosylmethionine decarboxylase
SDS	Sodium dodecyl sulfate
SDW	shoots dry weight
sec	seconds
SFW	shoots fresh weight
SL	shoot length
spp.	species pluralis
Sr	stem rust resistance gene
SSD	single seed descent

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SSR	simple sequence repeat
SU	Stellenbosch University
SU-PBL	Stellenbosch University Plant Breeding Laboratory
TE	transpiration efficiency
TPIDW	total plant initial dry weight
TPDW	total plant dry weight
TPFW	total plant fresh weight
Tris-Cl	tris-chloride
U	unit
UV	ultraviolet
V	volt
Yr	stripe rust resistance gene

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CHAPTER 1: INTRODUCTION

Wheat is one of the major cereal crops produced in South Africa (SA) and worldwide. There are two major types of wheat species produced: *Triticum aestivum* L. and *Triticum durum*, commonly known as bread wheat and durum wheat, respectively. There are three major wheat-production provinces in SA: the Free State, the Western Cape and the Northern Cape (Esterhuizen, 2015). The annual average of wheat production in SA is 1.3 to 2 million tons (Esterhuizen, 2017). The production demand continues to escalate and is currently estimated to be 2.7 million tons, the result of a continuous increase in consumption of 1% every year (Esterhuizen, 2017). Production decreased by 50% in the country as a result of a reduction of hectares in the Free State caused by a major drought during the year of 2016 (Esterhuizen, 2017).

Several biotic and abiotic factors are hampering the efficient production of wheat. According to Ahmad *et al*. (2014), water stress is a major abiotic factor limiting the production of wheat (and other crops) and continues to be a challenge in crop production. Water stress severely affects about 50 and 70% of wheat production areas for both developing and developed countries (Nezhadahmadi *et al*., 2013). According to Nezhadahmadi *et al.* (2013) extremely dry conditions will result in a scarcity of water by 2025. Approximately 1.8 billion people will encounter severe water scarcity, and about 65% of the world's population will live under water-limited conditions (Nezhadahmadi *et al*., 2013).

Water stress and rust disease significantly affect the growth and development of wheat. Due to the ever-increasing world population, breeding for water stress and disease resistance is important to ensure food security (Ahmad *et al*., 2014; Simons *et al*., 2011). Genetic recombination, migration and mutation are important factors limiting the development of varieties with durable resistance (Todorovska *et al*., 2009). Variety development through gene pyramiding has been considered as the best option to ensure rust resistance and durability (Simons *et al*., 2011). According to Todorovska *et al*. (2009) multiple resistance genes limit rust disease by overcoming the pathogen.

Direct selection of the target traits from wheat crop plays a major role in yield improvement (Khakwani *et al*., 2011). Development of water stress resistance varieties remains a key objective in multiple plant breeding programmes. However,

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limited screening methods and germplasm sources to provide the genotypes showing noticeable stress responses to different stress conditions result in limited success (Mwadzingeni *et al*., 2016). According to Khakwani *et al*. (2011), development of varieties with improved yield and stress resistance requires an adequate source of genetic improvement to provide different traits and responses. A good understanding of the phenotypic traits that play a significant role in improved yield under water-limited conditions is important to understand the complexity of the genetic and physiological mechanisms that lead to variety acclimatisation (Pask *et al*., 2012). Selection criteria should be based not only on a single trait but also on the adaptive mechanism to optimise yield and improve integration of the variety (Mwadzingeni *et al*., 2016). Yield-based selection is very important, and proper calculations are required to support decision making and other factors such as the use and interpretation of the different drought indices (Mwadzingeni *et al*., 2016). Hence, allows to evaluate the yield response from a genotype under stress conditions. Data collection through use of newly available hand-held devices such as a ceptometer, leaf porometer and chlorophyll content meter can increase the efficiency of screening and selection. New technology can be used to optimise yield to support the use of molecular markers (Khakwani *et al*., 2011).

To improve wheat genetic material and production of viable hybrid seeds, effective fertility restoration techniques and proper pollination controls may be useful for successful plant breeding programmes (Singh *et al*., 2015). Recurrent selection can be utilised as a valuable tool to improve the required allele frequencies of a specific characteristic from the germplasm. According to Stuthman *et al*. (2007), genetic male sterility can be used to facilitate crossing in wheat, thereby improving the population through use of the recurrent mass selection scheme. The established recurrent mass selection method used in the Stellenbosch University Plant Breeding Laboratory (SU-PBL) for self-pollinated crops such as wheat was implemented based on the *Ms3* gene (Marais *et al*., 2000). According to Marais *et al*. (2000) the method involves the use of a hydroponic system developed to make crosses whereby F1 male sterile females tillers are selected and crossed with donors, thereby producing more hybrid seeds.

The SU-PBL has a set of primers routinely used for screening material in wheat nurseries (Smit, 2013). The set of primers includes stem rust (*Sr*), leaf rust (*Lr*) and

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yellow/stripe rust (*Yr*) resistance gene markers. The markers are used to characterise the F1 base population in the nurseries, thereby identifying rust disease resistance and susceptible genotypes prior to field evaluation (Marais *et al*., 2000). Molecular markers play a key role in screening the material and selection of the target traits.

The aim of the study was to initiate a pre-breeding effort for water stress resistance and yield improvement in wheat. Selection of high-heritability Mendelian-inherited and quantitative traits for screening wheat genotypes utilising statistical analysis to improve selection. In order to achieve the aim, the following objectives were identified:

(a) Screening of genotypes for water stress resistance. Phenotypic screening of 60 genotypes sourced from the SU-PBL and collaborators was done for water stress resistance. Thirty genotypes selected through statistical analysis and ranks were further screened to determine the top five. Mendelian-inherited and quantitative traits were assessed from different stages of growth using a reticulated hydroponic system (RHS). Molecular screening was done for rust resistance genes through male sterility-mediated marker-assisted recurrent selection (MS-MARS) from the SU-PBL and collaborators using an RHS. All plants were screened using standardised SU-PBL molecular markers.

(b) To develop MS-MARS cycles 1 and 2. The F1 1:1 male sterile female segregating population was crossed with donor lines sourced from the SU-PBL nursery and collaborators. Validation of mendelian-inherited and quantitative traits for water stress resistance was done from five selected males using an RHS. The five selected males screened for water stress resistance were crossed with an SU-PBL nursery female segregating population (from MS-MARS Cycle 1) screened for rust resistance genes. Crosses were done to introduce the traits of interest and/or novel germplasm into the SU-PBL breeding population. Molecular screening of five males (screened for water stress) for rust resistance genes was done.

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CHAPTER 2: LITERATURE REVIEW

2.1 Domestication of wheat

The *Triticum* (genus) consists of six different wheat plant species: *T. monococcum* L., *T. turgidum*, *T. aestivum* L., *T. urartu*, *T. timopheevii* and *T. zhukovskyi* (Dvorak & Akhunov, 2005). Wheat (*T. aestivum* L.) is a member of the grass family (*Poaeceae*), which includes rice (*Oryza sativa*) and maize (*Zea mays*), together considered as staple crops. Domestication of wheat can be traced back 8 000 to 12 000 years (Figure 2.1) in Southwest Asia. Ancient people survived through hunting and gathering, followed by a gradual transition to cultivated crops. The human lifestyle changed drastically through evolution; this led to domestication of major cereal crops that are nowadays a staple food. Human societal transition was marked by the domestication of barley, wild emmer and einkorn (Harlan & Zohary *et al*., 1966). Domestication of crops resulted in replication of genetic material from crops (Figure 2.1).

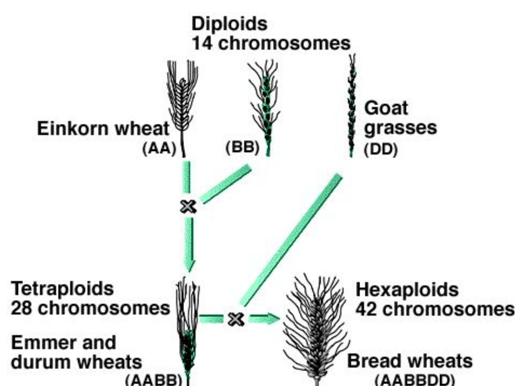

Figure 2.1: Domesticated wheat evolution

Source: Adapted from Levetin & McMahon (1996).

The evolution of domesticated wheat involved natural cross-pollination of *T. monococcum* (diploid [2n] = 14, AA) with possible wild grass species *A. speltoides* (2n = 14BB). Through an evolutionary process, replication of genetic material followed and resulted in many cultivated species and wild forms of tetraploid (2n = 28, AABB). Tetraploid (*dicoccum and T. durum*) species, more precisely *dicoccum*, were

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repeatedly and naturally hybridised by weeds species *A. squarrosa* (2n = 14, DD), which resulted in (2n = 42, AABBDD) new hexaploid species (Akhunov *et al*., 2010). Wheat has three ploidy levels: diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) and hexaploid (2n = 6x = 42). Ploidy levels collectively gave rise to allopolyploid series (Akhunov *et al*., 2010). These series of allopolyploids can further be classified into three major series of categories, namely *monococcon*, *dicoccoidea* and *Triticum*, with the subsections being the ploidy levels such as diploid, tetraploid and hexaploid, respectively, with a corresponding number of chromosomes (Table 2.1) (Matsuoka, 2011).

Wheat varieties are classified into cultivated and wild varieties or both (Levetin & McMahon, 1996). *Triticum turgidum* and *T. timopheevii* are classified as wild species under *dicoccoides*, often shortened to *T. dicoccoides*, with genome formula AABB and AAGG, respectively. However, *T. timopheevii* occurs in both wild and cultivated forms (Matsuoka, 2011). Natural hybridisation of a wild species *T. urartu* (genome formula AA) and unidentified or extinct species in the lineage and gave rise to tetraploid species namely, the wild emmer and durum wheat. Apparently, unidentified or extinct species is a close relative of *Aegilops speltoides* (genome formula BB) that can be traced back 0.2 to 0.5 Ma years (Matsuoka, 2011). *Aegilops speltoides* is a goat grass family of a genome formula SS, with S being closely related to wheat genome B, which could not be identical (Akhunov *et al*., 2010).

Common bread wheat (*T. aestivum*) and *T. zhukovskyi* exist in a form of cultivated species and constitute genome formulas AABBDD and AAAAGG, respectively. However, dicoccoidea (*T. turgidum* and *T. timopheevii*) and monococcon (*T. monococcum*) occur as domesticated and wild form species (Matsuoka, 2011). *Triticum aestivum* originated about 8 500 years ago through natural hybridisation of *T. turgidum* with a diploid *Ae. tauschii* of a genome formula DD (McFadden & Sears, 1946). According to Nesbitt and Samuel (1996, cited by Dvorak & Akhunov, 2005) archaeological records reveal that *T. aestivum* originated approximately 8 000 years ago, but the period of origin of *T. turgidum ssp*. is indeterminate. In contrast, Harlan and Zohary *et al*. (1966) reported that wheat was domesticated 8 000 to 12 000 years ago in Southwest Asia. According to Peng *et al*. (2011), *T. aestivum* was domesticated approximately 9 000 years ago in the same region. Thus, the time of origin is uncertain

but nonetheless ranges between 8 000 and 12 000 years back. *Triticum Urartu* and *T. momococcum* are diploid species of wheat that diverged at most one Ma back (Huang *et al*., 2002). According to Matsuoka (2011), all the species of *Triticum* originated in the Fertile Crescent, named after its crescent shape. As the name implies, this fertile region is rich in wetlands (Matsuoka, 2011). *Triticum* species originated in the Near East part of this region, which covers Transcaucasia, the northern and western parts of Iran, the southern and eastern parts of Turkey and the eastern part of the Mediterranean (Figure 2.2). *Triticum zhukovskyi* evolved by hybridisation of two cultivated diploid varieties of wheat (*T. monococcum* and *T. timopheevii*), and their lineage consists of inadequate distribution of *T. zhukovskyi* and *T. timopheevii* native to Transcaucasia. According to Matsuoka (2011), a limited number of research projects have been conducted on the evolution and diversification; therefore, its domestication remains unknown. However, *T. aestivum* L. and subspecies of *T. aestivum* and cultivated varieties of the *T. turgidum* lineage are now found everywhere and are produced worldwide (Peng *et al.*, 2011). The genome formula of *T. durum* and wild emmer wheat species is considered as the core of domestication simply because of their similarities. Common bread wheat inherited two genomes from these species of wheat (Peng *et al.*, 2011).

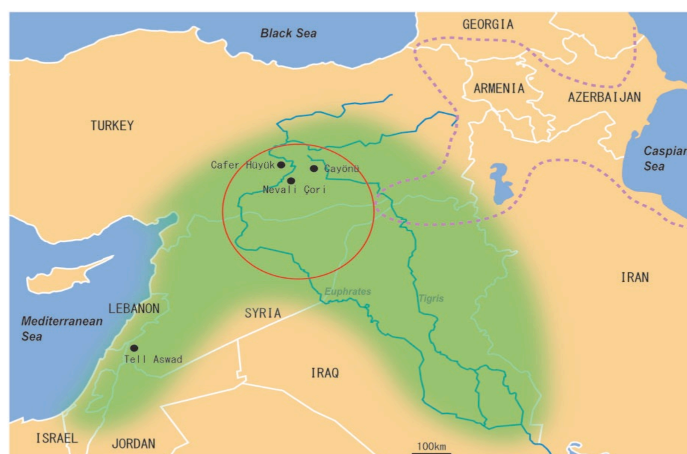

Figure 2.2. Diagram of the Fertile Crescent

Source: Matsuoka (2011).

The green-shaded region (Figure 2.2) indicates the borders of the Fertile Crescent. The solid red line and the dotted purple line indicate the central region of domestication. It is believed to be the region where agriculture emerged (Matsuoka,

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2011). *Aegilops tauschii* species' distribution range was measured at the edge of the western region, the region believed to be the most probable region of *T. aestivum* allopolyploid speciation (Matsuoka, 2011).

The domestication of wheat resulted in transition of key features such as rachis, diverting from brittle (*Br*) to non-brittle (non-*Br*). Such features are considered as empirical evidence of domestication (Peng *et al*., 2011). The principal concern was the loss of yield through shuttering of major cereal crops, but modification of *Br* to non-*Br* overcame the problem, realising considerable yield. According to Salamini *et al.* (2002), the quantitative trait loci (QTLs) associated with the *Br* gene in wheat was mapped in Group 3 of the homologous chromosomes. Domesticated wild emmer wheat has a non-*Br* character (Matsuoka, 2011). Contrasting with their wild progenitor, in domesticated varieties, development of a fracture zone is suppressed by tough glume and delayed until harvest (Peng *et al*., 2011). According to Salamini *et al.* (2002), due to the agricultural and biological importance of the *Br* character, several studies have been conducted to examine its genetic basis. These studies sought to explain the methods involved in genetic control of the non-*Br* trait. Some research reported that recessive alleles were responsible for controlling the non-*Br* trait mapped in Group 3 A and B of the short arm chromosomal region (Matsuoka, 2011; Nalam *et al*., 2006). Comparing the results of molecular work shows that *Br A1* and *B1*, commonly known as *Br2* and *Br3*, respectively, are responsible for controlling this trait. Recent multiple research studies reported that the traits were being controlled by several genetic pathways and that shattering was controlled by diverse genetic origin of loci in polyploid species (Salamani *et al.*, 2002).

Glume tenacity is one of the important traits modified during domestication of wheat (Gill *et al*., 2007). It is closely linked to free-threshing ability. Glumes are used to distinguish between cultivated and wild varieties of wheat (Villareal *et al*., 1996). Domesticated varieties are covered by soft glume (free threshing) whereas wild varieties are covered by tough glumes (difficult to thresh) (Gill *et al*., 2007). Two genes controlling the free-threshing trait evolved through domestication (Villareal *et al*., 1996). Several QTLs linked to the free-threshing trait were mapped in chromosome locations 2A, 2B, 2D, 5A, 6A, 6D and 7B. Nonetheless, there are partially recessive alleles at tenacious glume (*Tg*) loci and partial dominant allele at loci *Q* on

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chromosomes 2DS and 5AL. Hence, they have been found predominantly controlling free-threshing trait(s) (Peng *et al.*, 2011). According to Matsuoka (2011), research studies showed that the *T. momococcum* soft glume (*sog*) gene was found in chromosome location 2AS chromosomal arm near the centromere. Furthermore, the tenacious gene (*Tg*) in common bread wheat was mapped in the same chromosomal arm but in the most distal region of chromosome location 2DS chromosomal arm (Matsuoka, 2011). The different locations mapped in chromosomes indicate different evolutionary origins in mutation for free-threshing ability. In recent times, the exact location of *Tg1* was mapped on 2DS (Matsuoka, 2011).

Ancient wheat cultivars consisted of hulled seeds, and for them to be winnowed from the chaff, they needed to dry out (Zhang *et al*., 2014). During domestication, farmers selected cultivars with low glume tenacity, fragile rachis and free-threshing ability; consequently, harvesting was more convenient. The free-threshing trait allowed easy removal of naked kernels following harvesting; therefore, the kernels were ready for milling (Matsuoka, 2011). The free-threshing ability of common cultivars of *T. aestivum* L. and *T. durum* indicates the ultimate stage of domestication. QTLs associated with free-threshing ability were found to also influence speltoid character, glume tenacity and rachis fragility (Jantasuriyarat *et al*., 2004). These QTLs were mapped together with *Q* gene(s) in chromosome 5AL chromosomal arm.

The interaction of *Tg* and *Q* loci was found to have a major influence on spike morphology (Matsuoka, 2011). The *Tg* gene regulates glume toughness because of its epistatic effect on the gene locus *Q*. However, the *Q* gene has a major influence on many traits such as glume shape and toughness, plant length, spike length and spike development duration (Jantasuriyarat *et al*., 2004). The *Tg* allele has an epistatic effect on the free-threshing ability of the wild wheat varieties, caused by genetic interaction (Zhang *et al*., 2014). However, the *Tg* allele was recently found to have no effect on domesticated varieties due to the presence of the dominant *Q* allele with a genotype formula *QQTgTg*; therefore, it had no effect on the free-threshing ability of wheat (Matsuoka, 2011). Wild varieties carried genome formula *qqTgTg* that was associated with non-free-threshing ability (Figure 2.3). Domestication resulted in genetic changes from *qqTgTg* to *QQtgtg*, which played a major role in the existence of the free-threshing phenotype (Zhang *et al*., 2014; Matsuoka, 2011).

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Table 2.1: Wheat (*Triticum*) species (Matsuoka, 2011)

Monococcon		
Species	Type of genome	Ordinary name
Triticum monococcum L.	AA	
Subspecies		
Aegilopoides		
Monococcum		Wild einkorn
Dicoccoidea		
Species	Type of genome	Ordinary names
Triticum turgidum L.	AABB	
Subspecies		
Dicoccon		Cultivated emmer
Dicoccoides		Wild emmer
Polonicum		Polish wheat
Durum		Durum/macaroni
Turgidum		Rivet wheat
Turanicum		Khorassan
Paleocolchicum		Georgian wheat
Carthlicum		Persian wheat
Armeniacum	AAGG	Wild timopheevii
Timopheevii		Cultivated timopheevii
Triticum timopheevii		
Triticum		
Species	Type of genome	Ordinary name
Triticum aestivum L.	AABBDD	Common wheat
Subspecies		
Sphaerococcum		Indian dwarf wheat
Aestivum		Bread wheat
Compactum		Club wheat
Spelta (L.)		Spelt
Macha		
Triticum zhukovskyi	AAAAGG	

From the genetic perspective, the notion of genetic mutation of the *Q* allele during domestication can be explained by its pleiotropic nature whereby transcription factor properties are believed to be manipulated through the substitution of an amino acid (Nezhadahmadi *et al*., 2013). The genetic expression of free threshing and non-shattering of the seeds are found only in polyploids. Therefore, modifications caused by polyploidisation and interaction amongst homeoalleles and genetic constituents are also important (Zhang *et al*., 2014). The size of a genome of the most famous domesticated bread wheat (*T. aestivum*. L) was found to be almost twice as big as that of a human genome (Brenchley *et al*., 2012). The size of a genome is 17 000 MB, and it is composed of a repetitive DNA sequence up to 80%, which are generally

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retrotransposons. According to Brenchley *et al.* (2012), bread wheat genome studies showed that over 94 000 genes were found from three chromosomal location A, B and D. Genetic diversity and domestication are sophisticated parameters of evolution; understanding the mechanisms involved in domestication, evolutional trend, genetic drift and mutational forces could assist the progress of plant breeding programmes (Brenchley *et al.*, 2012).

Figure 2.3. Comparison of Q and q genes of hexaploid wheat
Source: Zhang *et al*. (2014).

Multiple research studies can be conducted utilising the wild ancestors of wheat to understand the genetic modification involved, species transformation and genetic forces. Hence, more knowledge can be gained regarding species' adaptation and their mode of action (Brenchley *et al.*, 2012). Genetic forces interaction plays a key role in species diversity that occurs in the wild. Once evolutionary processes are understood, one might understand the modern breeding approaches for successful variety improvement under stress conditions. A thorough understanding of plant behaviour and genetic responses under stress provides a better chance to improve genotypes using newly available technologies and molecular markers.

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2.2 Modern breeding approaches

Classical breeding techniques incorporated stress tolerant traits that were not well established due to complexity of the traits into crops. Gene-pyramiding of these traits provides the best alternative for incorporating the genes and allows rapid improvement of the target regions (Brenchley *et al.*, 2012). According to Breseghello (2013), current approaches used in genetic engineering largely depend on genetic transfer of the encoded gene(s) through signalling endpoint and/or biochemical pathways. This plays a key role in directly or indirectly protecting the plants against unfavourable environmental conditions.

Water stress was found to be a cause of high yield loss due to reduced plant growth and development, followed by yield reduction (Nezhadahmadi *et al*., 2013). Environmental stresses and/or cold-inducible traits are very broad; plants need to be exposed to very low temperatures for their expression. According to Breseghello (2013), modern approaches such as molecular markers enable the screening and tagging of low-temperature QTLs. Consequently, hardy plants that are resistant to low temperatures can be selected without conducting frost experiments and subjecting plants to frost.

2.3 Molecular markers in plant breeding

The most recent developments involving technological innovation such as the use of molecular markers have increased the chances for success in plant breeding programmes (Nezhadahmadi *et al*., 2013). Molecular markers are widely used by several plant breeding programmes to map QTLs associated with important traits. Numerous molecular markers are available to detect wheat QTLs and genes and to carry out gene tagging of various important traits for the marker-assisted breeding method in water-limited environments (Collard *et al*., 2005). Marker-assisted breeding is utilised to develop novel wheat varieties tolerant to water stress from several plant species.

2.3.1 Marker-assisted selection

Marker-assisted selection (MAS) has been utilised in plant breeding for more than a decade. MAS can be described as genetic selection of desirable traits using markers from the germplasm (Collard *et al*., 2005). This is a principle whereby morphological characteristics (phenotype) are selected based on the genetic material (genotype) of

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the marker. However, genetic mapping of identified markers for previous studies has almost never been suitable for MAS. This means that available markers should be continuously developed and that they must be further tested or validated for reliable results (Collard *et al*., 2005). For quality assurance and efficient use of molecular markers, validation prior to use is recommended for certainty of the results. Usually, MAS is associated with a series of steps essential for its development, such as high-resolution mapping, validation of markers and marker conversion (Collard *et al*., 2005).

According to Ribaut and Betran (1999), variety selection and development goals for target traits in plant breeding programmes involve selection of varieties with multiple resistance. Usually, plant breeders work with several sites, many fields and large populations. Therefore, MAS in plant breeding offers an opportunity to effectively select plants from a large population. The presence of molecular markers in modern plant breeding programmes has provided a great opportunity to screen the material and select the traits of interest prior to field evaluation (Ribaut *et al*., 1997).

2.3.2 Advantages of marker-assisted selection

The establishment of molecular markers in modern plant breeding programmes was a great achievement associated with several advantages (Collard *et al*., 2005):

- The use of markers in the laboratory allows the elimination of complex trials in the field, thereby saving time.
- Due to environmental effects, phenotypic evaluation carried out in the field brings uncertainty; this is eliminated by molecular work.
- It allows screening of the material at an early stage of growth, such as the seedling stage.
- It allows combination of various genes (gene pyramiding).
- It offers the opportunity to select less heritable traits.
- Important in situations where phenotypic evaluation cannot be applied (for example, quarantine restrictions might forbid inoculation with foreign pathogens).
- Unwanted genes, such as deleterious genes, and undesirable traits can be easily eliminated.

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The use of DNA markers enables mapping of QTLs for water stress tolerance and other traits (Nezhadahmadi *et al*., 2013). The molecular markers utilised for mapping are said to be directly or closely linked to the genes of interest or QTLs. The use of molecular linkage maps is a remarkable method utilised to improve water stress resistance in wheat crops (Nezhadahmadi *et al*., 2013). Successful mapping of the traits provides an opportunity to utilise closely linked markers to quickly screen several samples to detect genotypes with target traits. According to Rana *et al*. (2011), the use of MAS provides an opportunity to select the traits of interest at genetic level instead of phenotypic level. MAS was found to be an effective method used to accelerate improvement of cultivated wheat varieties.

In pyramiding genes for various stress tolerance, MAS plays a vital role in differentiating among genes with the same characteristics and in improving several cultivated varieties, thereby effecting durable resistance to stress. William *et al*. (2007) reported the decline of heritable traits that were inversely proportional to MAS, which was regarded as beneficial. A threshold can be reached by the less heritable traits, caused by escalated QTL complexity (Nezhadahmadi *et al*., 2013). A threshold may also be reached when the environment and the QTLs interact and negatively affect efficiency of the markers, resulting in unreliable markers. MAS is the best option when one needs to analyse large quantities of seed, which is not common in plant breeding (William *et al*., 2007). When this technique focuses on protein profiles and/or DNA-based markers, it can be implemented based on initiating early selection.

According to Rutkoski *et al*. (2011), nowadays MAS is a generally accepted method continuously utilised by commercial breeding programmes and various breeding approaches, thereby enhancing gain per unit of selection. Interestingly, breeding approaches enabling the use of molecular markers offer an opportunity to estimate the value of a single characteristic for selection and to backcross traits/alleles of interest into novel and elite germplasm whereby a donor plant transfers a gene to the recipient (Rana *et al*., 2011). In such a context, the molecular markers are utilised to monitor and accelerate the presence of a trait of interest by targeting lines with minimum donor chromatin; possibly, this can be carried by linkage maps. Hospital (2009) suggests that MAS appears to be the greatest collaboration of conventional breeding methods and molecular markers utilised in modern breeding approaches.

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2.3.3 Molecular marker selection

2.3.3.1 Criteria for marker selection

Proper selection of molecular markers that results in successful plant breeding programmes may consider the following factors (Mohan *et al*., 1997): -

- A reliable and precise genetic map together with molecular markers linked to QTLs or target genes is needed.
- There must be a solid combination of markers and target genes/QTLs. Markers should be positioned in the most appropriate location and often needs presence of the major gene(s) cloned. A genetic distance of 1 cm should be considered when markers are linked to the major or lesser genes, thereby minimising linkage drag (unwanted genes can be dragged and linked with the target gene) (Mohan *et al*., 1997). Moreover, special markers such as polymorphic markers and necessary genetic recombinants should be better flanked to the required QTLs and be between QTL region.
- The degree of polymorphisms (some genotypes can discriminate others and/or genetic variation).
- The chances of simultaneously managing multiple populations in a cost-effective manner should be considered. Considerable continuous production without complications is required at a fast rate.

Nezhadahmadi *et al*. (2013) investigated use of molecular markers such as amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) in winter wheat crops for detection of target gene or QTL for flag leaf senescence (FLS) under optimum and water-deficit conditions. According to Verma *et al*. (2004), the gene-controlling FLS was mapped and described, and the QTL was identified in chromosome 2D, responsible for increased tolerance under water stress conditions. According to Quarrie *et al*. (2005), molecular research work utilised several DNA markers including AFLP, SSR and restriction fragment length polymorphism (RFLP) in water-scarce environments to tag QTLs in wheat crops. Molecular markers such as sodium dodecyl sulfate (SDS) proteins, isozymes and DNA sequences have made a major contribution and have been extensively utilised for the last few decades in the selection of QTLs from plants subjected to dehydration. Russell *et al*. (1997) reported extensive use of these markers in wheat for identification of genotypes, gene mapping

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and evaluation of genetic diversity. Molecular markers can be linked to a specific trait; for example, a study conducted on durum wheat showed the presence of few markers linked to crop yield (grain) and morpho-physiological traits in water stress environments (Davila *et al*., 1999).

Ashraf *et al.* (2008) investigated several DNA markers, including SSR, single nucleotide polymorphism, random amplified polymorphic DNA (RAPD), RFLP, cleaved amplified polymorphic sequence (CAPS), AFLP, polymerase chain reaction (PCR) indels and sequences of DNA, thereby estimating the inheritance of stress resistance. RAPD markers have been thoroughly utilised in wheat by making use of DNA primers (Milad *et al*., 2011). Microsatellite molecular markers were found to be extensively utilised for genetic mapping of cereal crops. RAPD together with microsatellite markers were observed to be associated with FLS genes in wheat under water-scarce conditions. In addition, RAPD markers in hexaploid wheat assist to mark genes. An added advantage of MAS includes correlation of selected stress resistance target traits and molecular markers that they are greater than the heritability of the traits (Nezhadahmadi *et al*., 2013). Therefore, it can be concluded that molecular markers are very important in improving stress tolerance in wheat under water stress conditions.

2.4 Male sterility

To improve wheat genetic material and production of viable hybrid seeds, effective fertility restoration techniques and proper pollination control may be useful for successful plant breeding programmes (Singh *et al*., 2015).

2.4.1 Genetic male sterility

Genetic male sterility (GMS) occurs on a large scale in plants, and about 11 genes with the potential to induce GMS in wheat have already been discovered (Singh *et al*., 2015; Rao *et al*., 1993) (Table 2.2). Some of these QTLs have been identified and are responsible for recessive or dominant GMS in wheat. Among the GMS genes, the *Ms3* dominant gene is commonly used to induce male sterility in plants. The *Ms1* gene cannot be used simply because it cannot provide adequate male sterility in plant breeding programmes to facilitate crosses (Whitford *et al.*, 2013). According to Zhang *et al*. (2014), a promising dominant *Ms2* gene was found in a heterozygous state in nature through cross-pollinations with the recessive pollen from homozygous varieties.

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This allowed continuous segregation from the progenies of male sterile and fertile plants, and as a result longer plant height could not be distinguished. *Ms2* was commonly used GMS and later crucial discovery of *Ms3* gene. Development of the *Ms3* rectified the GMS problem, followed by early-stage screening of plants using molecular markers at a later stage (Cao *et al*., 2009).

The dominant *Ms3* gene could be easily transferred to the progeny and produce male sterile and male fertile progenies (Singh *et al*., 2015). This was followed by identification of a marker *WG341* linked to the *Ms3* gene and used for preliminary-stage screening of the plants. However, *Ms3* can only be used under greenhouse conditions, which led to development of a hydroponic system. Higher temperatures in the field cause instability; therefore, temperatures of 18 °C to 22 °C are required in the growth rooms (Singh *et al*., 2015). GMS systems seek to sustain genetic variation and enhance desirable allele frequencies in recurrent selection programmes (Singh *et al*., 2015). The alternative was to come up with a newly developed hydroponic system. The system enables massive cross-pollination of male sterile females from F1 1:1 segregating female plants and donor lines of interest. According to Marais & Botes (2009), cross-pollination between Inia 66 spring wheat and KS87UP9 (male sterile) resulted in F1 male sterile progeny displaying spring growth behaviour. After introduction of the *Ms3* gene to the F1 progenies, several further cross-pollinations were performed with seven spring wheat genotypes. A series of crosses made from diverse disease resistance plants which included crosses between male sterile F1 and randomly selected lines through creation of diversity in the breeding population (Marais & Botes, 2009).

MAS assists from early screening of the resistant plants to stress before introducing them into the germplasm. Continuous pyramiding of genes of interest in the population may enhance allele frequencies and facilitate several genetic resistances. The hydroponic system currently used in the SU-PBL termed MS-MARS cycle was developed (Figure 2.4). Variety improvement through GMS may lead to progress in variety development. A good source of genetic improvement can provide genotypes with improved yield on less available land for production. Sufficient production may lead to economic development through meeting the demand for consumption and

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export of wheat as a staple crop in SA. Increasing wheat production is the key objective in the SA production industry.

Table 2.2: Genetic male sterility genes

GMS	Location	Allele	Reference
Ms1	4BS	Recessive	Endo *et al*. (1991)
Ms2	4DS	Dominant	McIntosh *et al*. (1998)
Ms3	5AS	Dominant	McIntosh *et al*. (1998)
Ms4	4BS	Dominant	Klindworth et al. (2002)
Ms5	3AL	Recessive	Klindworth *et al*. (2002)
	***Ms1* (mutants)**		
Pugsley's (*Ms1a*)	4BS	Recessive	Suneson (1962)
Probus (*Ms1b*)	4BS	Recessive	Fossati and Ingold (1970)
Cornerstone (*Ms1c*)	4BS	Recessive	Driscoll (1987)
FS2 (*Ms1d*)	4BS	Recessive	Klindworth *et al*. (2002)
FS3 (*Ms1e*)	4BS	Recessive	Klindworth *et al*. (2002)
FS24 (*Ms1f*)	4BS	Recessive	Klindworth *et al*. (2002)

2.5 Wheat production

There are three major wheat-producing provinces in SA: the Western Cape, the Northern Cape and the Free State (Esterhuizen, 2013). Together, these three provinces produce about 85% of the wheat produced in SA. Production increased from year 2016 by 50%, 75% and 14% in the Western Cape, the Free State and the Northern Cape, respectively. About 1.1 m tons were produced by the Western Cape, followed by 308 000 tons by the Free State and 266 000 tons by the Northern Cape from 2016 to 2017 (Esterhuizen, 2017). The massive increase in wheat production by the Free State was caused by an increase of the area planted as a result of a major drought from 2016 to 2017. Farmers were directed to consider wheat as an alternative to maize (*Zea mays*) because the area used for maize production was affected by drought (Esterhuizen, 2017). Nevertheless, the region used for wheat production continuously decreased every year with a proportional increase of consumption by 1% every year for the past decade (Esterhuizen, 2017).

For the past two decades, wheat production has been uneven in SA (Esterhuizen, 2017) (Figure 2.4). There has been a steady decrease in production from 2011 to

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2016. This was caused by a lack of cheaper available alternatives such as rice and maize. Other staple crops (combined) available for consumption has doubled the price as a result of drought for the last two years whereas the demand escalated. Wheat consumption is expected to increase every year in line with the 1% increase of the previous years (Esterhuizen, 2017).

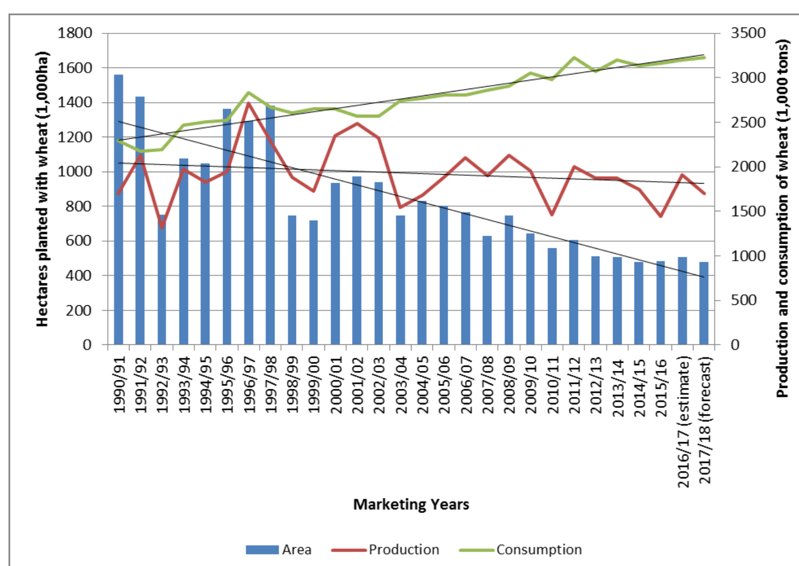

Figure 2.4: Production trends, area used for planting and consumption of wheat in SA for the past four decades

Source: Esterhuizen (2017).

The wheat production industry endeavours to find any possible solution to revive the industry, including high-yield varieties (Esterhuizen, 2017). However, there are many biotic and abiotic factors such as rust diseases and water stress affecting wheat, thereby limiting high yield gains. Rust is a devastating fungal disease that can result in significant yield loss through hampering grain formation in the spike (Ellis *et al*., 2014).

2.6 Wheat rust disease

Wheat growth and development can be affected by several biotic and abiotic factors. Rust is one of the primary biotic factors affecting wheat and is caused by *Puccinia* species. Stem (*P. graminis*), leaf (*P. triticina*) and stripe (*P. striiformis*) rust are prominent diseases in wheat associated with yield loss (Ellis *et al*., 2014). Rust originates from the phylum Basidiomycetes, consisting of 6 000 species. According to Cuomo *et al*. (2013), *P. triticina* has a larger genome size of 135.34 Mb in relation to

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P. graminis with a size of 88.64 Mb and other fungi in the family. *Puccinia* species is parasitic and relies on the host for nutrients. It forms specialised structures to infect the host, thereby extracting available nutrients from the host plant. The pathogen can hamper the defence mechanism of the host through secretion of effector protein clusters.

Rust reproduces both sexually and asexually (Ellis *et al*., 2014). Reproduction varies among host plants; asexual reproduction occurs in wheat and sexual reproduction in other host plants such as meadow rue and barberry. Rust disease develops by inoculating the plant using spores termed 'aeciospores' or 'urediniospores' (Cuomo *et al*., 2013). The rust-like colour of urediniospores is produced from the wheat plant (stem or leaf organ) thereby protruding on the surface through busting the epidermis. The spores are primarily airborne, and this may lead to reinfection of the same plant. Black teliospores are produced at plant maturity, and this designates the fungus' overwintering stage; the fungus remains dormant through the winter season. According to Singh *et al*. (2002), when optimum conditions prevail, each cell can grow and produce single-haploid basidiospores. Spores are carried by the wind and infect the new host through the stomata or the vectors that carry the spores.

Rust can be controlled in two ways, namely host plant resistance and chemical control (not within the scope of the study) in cereal crops. Oliver (2014) states that genetic resistance is widely used and highly recommended due to the economic and environmental perspective, and resistance against fungicide developed by the pathogen. In general, two types of genetic resistance are utilised by plant breeding programmes for rust, namely adult plant resistance and pathogen race-specific resistance genes, based on phenotype level. According to Ellis *et al*. (2014), the adult plant resistance gene is only expressed in adult plants and pathogen race-specific resistance genes are expressed from an early stage of growth up to adulthood. These two genetic divisions of rust resistance genes are classified as leaf rust (*Lr*) and stem rust (*Sr*) disease resistance.

2.6.1 *Lr34*

More than 60 QTLs and leaf rust resistance genes have been identified in wheat. The greater proportion of these genes is race specific, and many are being utilised in variety improvement programmes by plant breeders (Ellis *et al*., 2014). The resistance

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lifespan of these genes may not be long simply because *P. triticina* (pathogen causing leaf rust) endlessly evolves into new races and gain counter-virulence (Cuomo *et al*., 2013). Gene-pyramiding of genetic complexes such as slow rusting may provide adequate rust resistance under intensive infestation; however, slow-rusting effectiveness largely relies on environmental conditions (Singh *et al*., 2003). Race-specific genes can be effectively utilised by plant breeders and by supplementing with slow-rusting genes.

Molecular characterisation using markers facilitates gene pyramiding technique. Leaf rust genes such as *Lr34* and *Lr*46 are categorised under a small group named slow-rust genes (Singh *et al*., 2003; Martínez *et al*., 2001). This group consist of durable yield and non-race specific adult resistant genes. Moreover, non-race specific genetic resistance is relatively lower than that of race specific resistance. About a decade after *Lr34* had been cloned, but similarities were observed with the stripe rust adult resistance gene *Yr18*, together with the *Pm38* gene that provides resistance to powdery mildew and, finally, the *Ltn1* gene responsible for leaf tip necrosis (LTN) (Krattinger *et al*., 2009). These clusters of genes are generally known as coding adenosine triphosphate (ATP)-binding cassette transporters. *Lr46* and *Yr29* were reported to have a pleiotropic effect between them and to be associated with stripe rust slow-rusting genes. *Lr34* was described about 51 years ago from a cultivated variety, Frontana (Dyck *et al*., 1966). The chromosomal location of *Lr34* was mapped in 7D, short arm, within the vicinity of the marker *Xgwm295*. The phenotypic resistance characteristics exhibited by this gene may involve small size of uridina, fewer uridina and a longer latent period. According to Schnurbusch *et al.* (2004), a strong genetic linkage was reported between *Lr34* and LTN loci; moreover, this association may result in a pleiotropic effect in the LTN phenotype.

2.6.2 *Sr2*

Black rust, commonly known as stem rust, is a common disease in wheat, and the *Sr2* gene provides genetic rust resistance in the adult plant (Martínez *et al*., 2001). Application of the *Sr2* gene in breeding programmes to provide broad-spectrum resistance and durability in wheat was traced back more than six decades (McNeil *et al*., 2008). This may involve *Ug99* resistance to a challenging wheat fungus rust strain and related isolates. The *Sr2* gene was isolated from Yaroslav emmer wheat species

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and introduced to hexaploid wheat. This gene was mapped in chromosome 3B, at the short arm of the chromosome. According to Spielmeyer *et al*. (2003), the *Sr2* gene exists in a recessive state and provides partial self-reliant resistance. However, the *Sr2* complex assists to provide noticeable rust resistance in accordance with recessive *Sr2* genes (Singh *et al*., 2004). The homologous stage of the *Sr2* gene has been associated with setbacks such as phenotypic expression being only noticeable in adult plants that are most likely to be influenced by the environment and genetic components (Singh *et al*., 2004). Pseudo-black chaff (PBC) is a morphological marker found to be associated with *Sr2*; this marker provides partially dominant in plants. PBC appears at the bottom of the internodes, peduncle and from the glumes as dark pigment (Mago *et al*., 2011b).

However, PBC can be expressed at different levels based on genetic material and environmental conditions. According to Mago *et al*. (2011a), microsatellite markers such as *gwm533* and *csSr*2 (CAPS) and other markers tightly linked to *Sr*2 have been widely used in many plant breeding programmes for stem rust resistance in wheat. Marker *Xgwm533* is tightly linked to *Sr*2 in different wheat genotypes and is determined by the band size of the 120 base pair (bp) (Spielmeyer *et al*., 2003). However, in chromosome 3B, two separate markers of *Xgwm533* were identified, which led to contradiction because lines that did not carry *Sr2* also expressed the presence of marker *Xgwm533* and possessed a different sequence (Spielmeyer *et al*., 2003). However, this was soon rectified by Hayden *et al*. (2004) through development of sequence-tagged microsatellite markers, which played a significant role in distinguishing between the markers.

Furthermore, by using bacterial artificial chromosome, new SSR loci were identified by Hayden *et al*. (2004) that were even closer than the *Xgwm533* RSS marker to *Sr2*. Nonetheless, they existed as polymorphic between the lines and they would be either presence as a resistance gene or absent. Such lines can be utilised in breeding programmes; however, examination of parental lines may be required. In addition, Mago *et al*. (2011a) reported another marker even closer than *Xgwm533*, namely the *csSr2* marker. Malik *et al*. (2013) estimated an increased level of molecular marker precision through simultaneous use in rust resistance genetic screening.

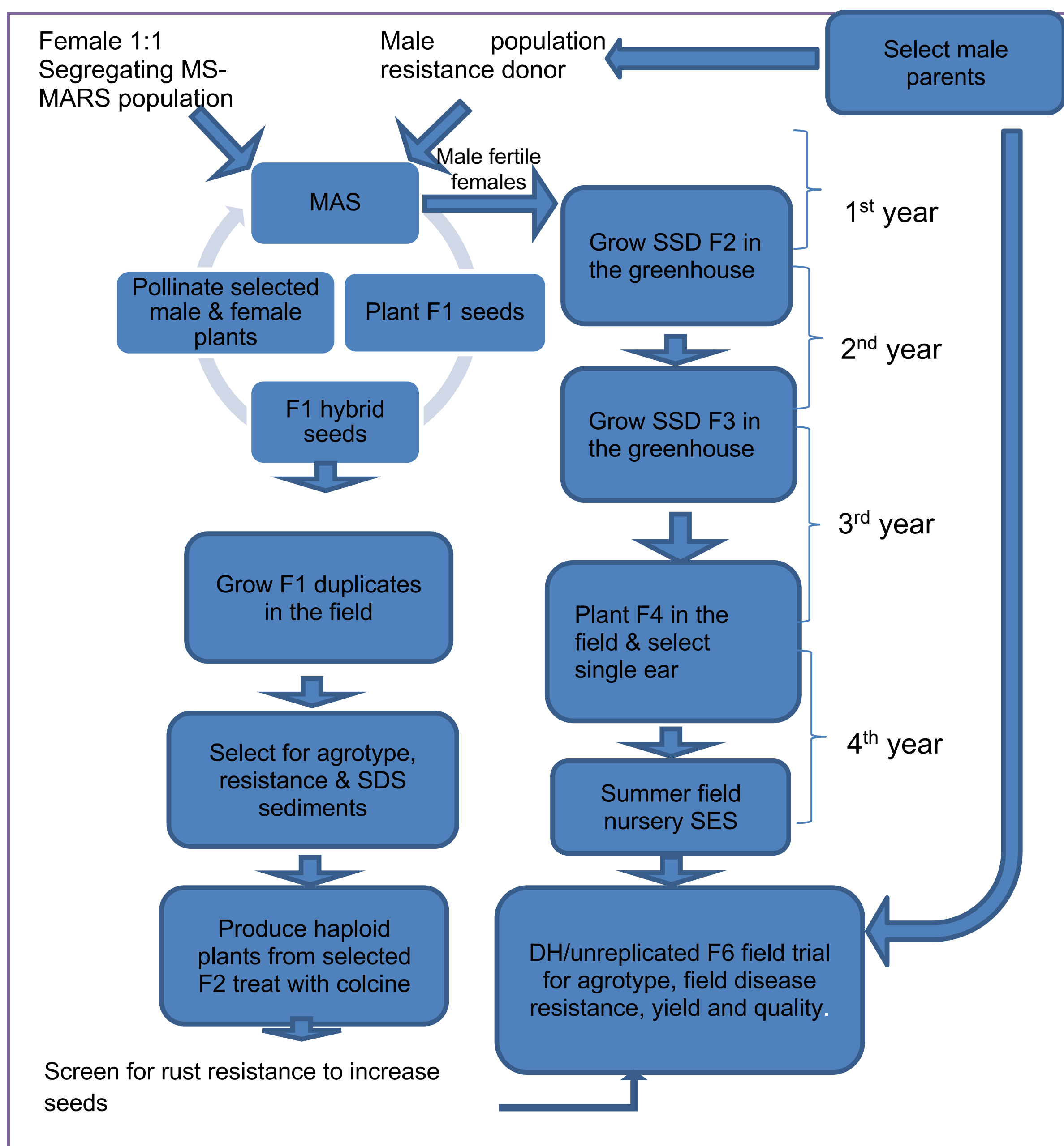

Figure 2.5: MS-MARS cycle scheme flow chart

Source: (Marais & Botes, 2009).

Environmental stresses are one of the devastating stresses affecting wheat growth and development. Added abiotic stress such as water stress from the infected leaves was reported to significantly reduce the leaf lifespan in barley (Aym & Zadors, 1979). Fungal diseases were found to be increased under water-limited conditions (Desprez-Loustau *et al*., 2007).

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2.7 Environmental stress resistance

Classical breeding techniques incorporated stress tolerant traits that were not well established due to complexity of traits involved (Nezhadahmadi *et al*., 2013). Gene-pyramiding of these traits provides the best way to incorporate the genes and to allow rapid improvement of the varieties from the target regions. According to Breseghello (2013), current approaches used in genetic engineering largely depend on genetic transfer of the encoded gene(s) through signalling endpoint and/or biochemical pathways. This plays a key role in directly or indirectly protecting plants against unfavourable environmental conditions.

2.8 Water stress resistance genetic improvement

The following three key strategies are utilised to improve water stress resistance lines in minimally productive areas (Fleury *et al*., 2010):

a) Characterisation and phenotypic screening of parental lines with improved yield under water-limited environments. This classical approach has been extensively used, and modern varieties are empirical evidence of the programmes' success. However, the demand for food constantly escalates and production has plummeted for the past few decades. Consequently, the demand cannot be met because the demand is greater than supply (Tester & Langridge, 2010).
b) Richards *et al*. (2010) proposed a strategy to describe physiological ideotypes according to the concept conceived by Donald (1968) for improved yield in dry areas. Ideotype plants are expected to acclimatise and perform exceptionally well in the target environments and produce considerable yield. Therefore, the ideotype method of breeding is based on improving cultivars' genetic material for exceptional yield gains under water stress conditions. This method predicts improved variety performance, and crops are anticipated to perform better in dry areas and serve to establish new varieties with traceable record of descendent. Despite intensive application of these methods, they have been associated with little success. Richards *et al*. (2010) claim that selection for water use efficiency utilising the carbon isotope discrimination trait for screening could be more successful in the development of new varieties.

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c) MAS in accordance with screening for traits of interest at QTLs for water stress resistance. Nevertheless, the intensive use of molecular markers and several publications of articles on water stress resistance *QTLs*. Gupta *et al.* (2001) reported less success in solving even a sole case for water stress or any other water stress-related issue where the markers were applied in wheat crops. Molecular work identified about 50 QTLs screened using DNA markers. However, among all these QTLs, only a single locus was found to be linked with better performance in less productive areas. These loci were resistant to soil with high boron, toxicity caused by aluminium, plant height (PH) and tolerance against the nematodes. According to Fleury *et al*. (2010), previous studies failed to map QTLs that could be used in plant breeding programmes for variety development.

Past failures of molecular and physiological breeding strategies imply that more work needs to be done in terms of identifying strategies to understand breeding for water stress resistance (Fleury *et al.*, 2010). Genetic strategies utilised nowadays could possibly lead in two directions: on the right direction or to those which were unsuccessfully tested or even further than that. Water stress resistance programmes should incorporate many disciplines with a common goal. A multidisciplinary approach should integrate aspects such as genome implementation, QTLs, plant physiology for water stress tolerance traits, microarrays and transgenic crops (Fleury *et al.*, 2010). For the success of research programmes, the strategy should also incorporate scientists from many disciplines such as plant physiologists, molecular biologists and plant breeders. According to Passioura (2007), the multidisciplinary approach was realised in the field by major companies in technology demonstration for water stress tolerance.

According to Cattivelli *et al*. (2008), a great effort has been made by primitive agriculturalists to improve grain yield and stability under water stress conditions. Detection of genetic determinants for plant physiological response to stress provides a great opportunity for progress of breeding programmes (Nezhadahmadi *et al*., 2013). Under water stress conditions, plant cells lose water and become dehydrated. Dehydration induces plant expression of water stress-related genes. According to Cattivelli *et al.* (2002), this provides a plant with an opportunity to escape or avoid

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stress-related problems and greater degree may be observed under transcriptional control.

Multiple water stress resistance-related genes from different plant species have been isolated over the past few decades. According to Kollipara *et al.* (2002), the complexity of water stress tolerance has been discovered and revealed by large transcriptome analyses. Kollipara *et al.* (2002) state that in ongoing research projects worldwide for crops growing under stress conditions, the complexity of aspects involved such as networking and communication among cells and/or within individual cells has been explored during plant response to water stress through use of molecular work. According to Xie *et al*. (2005), intensive research work has been done on model plants, involving screening and isolation of genes and transfer of these genes to new varieties. For efficient transfer of the genetic material, a technique called ‘genomic synteny’ is used. Molecular pathways offer a great opportunity to manipulate stress resistance (Nezhadahmadi *et al*., 2013). Furthermore, using this technique offers an opportunity to identify elements driving water stress response from different varieties.

Transgenic crops have been developed to generate, regulate and produce specific stress responses and physiological processes. Examples of transgenic crops are encoding genes such as *DREBs/CBFs* from cereal crops such as wheat, and rice crops (Dubouzet *et al*., 2003). Genetically modified crops have shown promise in breeding programmes following signs of enhanced stressed tolerance under water stress conditions (Wang *et al.*, 2003). Genetically modified crops’ downregulated genes that were highly associated with stress were found over induced. In addition, some stress components were detected such as protein prolines and increased amounts of soluble sugars (Wang *et al*., 2003). Over-expression of the *SNAC1* gene has been reported in transgenic rice under water stress conditions as a sign of stress resistance. Overexpression of this gene was associated with decreased water loss from the leaves, improved stomatal conductance (g_s) and decreased sensitiveness to abscisic acid (ABA) (Sivamani *et al*., 2000).

Nezhadahmadi *et al.* (2013) describe QTLs as important chromosomal locations where genes of quantitatively inherited traits influence phenotype. According to Ashraf *et al.* (2008), genetic material varies from one crop to the next; therefore, mapping of QTLs offers an opportunity to examine plants’ genetic variation and it enables one to

predict the degree of phenotypic effect and the pattern of the genetic activity. Several studies reported great efforts made to clone QTLs by 2005 by investigating target traits for water stress resistance (Salvi & Tuberosa, 2005; Tuberosa & Salvi, 2006; Cattivelli *et al*., 2008). Some studies reported mapping of QTLs responsible for water stress resistance in wheat and other cereals (Quarrie *et al*., 1994; Bernier *et al*., 2008).

ABA synthesis and increased concentration have been identified and described under water stress conditions (Quarrie *et al*., 1994). Chromosome location 5A has been reported to be responsible for transportation of genes associated with ABA concentration in wheat. Quarrie *et al*. (2005) mapped QTLs associated with dehydration tolerance in chromosomes locations 1A, 1B, 2A, 2B, 2D, 3D, 5A, 5B, 7A and 7B. Double haploid-induced wheat cultivars provide reliable QTLs for water stress tolerance traits. Recombinant inbred lines were utilised, crossing water stress tolerance and susceptible parental lines, thereby generating a potential base population required for QTL mapping and analysis. Improved yield under water stress conditions was thereby attained (Tuberosa *et al*., 2002). Target QTL analysis and mapping have been conducted for more than a decade; however, certain procedures should be followed when doing such analysis and mapping.

Firstly, genotypes should be evaluated from the very large population that is required for polymorphic markers (Nezhadahmadi *et al*., 2013). Secondly, it is very important to consider the importance of available genetic material. Lastly, it is generally significant to do statistically analysis to identify the QTLs and what influences the traits of interest. The QTL for water stress tolerance comes with disadvantages, including genetic and environmental interaction (Rana *et al*., 2011). Limitations may also include great genetic variation and mapping of incorrect populations. These drawbacks have restricted the approaches utilised for mapping QTLs associated with improved yield in water-deficit environments (Gupta *et al*., 1999).

2.9 Screening for water stress resistance

2.9.1 Traits associated with water stress resistance

2.9.1.1 Biomass

Plant biomass plays an important role in plant growth and development. A study by Wang *et al*. (2005) showed a reduction in total plant biomass as a result of water stress

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in spring wheat. Plant dry weight (DW) is influenced by a number of factors, including relative growth rate (RGR), photosynthesis active radiation (PAR) and photosynthesis (Koca & Erekul, 2016). Plant biomass increases from the seedling stage as the plant grows. Some research studies reported positive relationship between accumulation of plant DW and RGR (Ozturk *et al*., 2014). According to Shao *et al*. (2008), the number, size and longevity of the leaves of the entire plant can decline as a result of water stress. Water stress may significantly interfere with the development of wheat leaves and other plant organs (Singh *et al*., 1973). Khan *et al*. (2010) reported a decreased number of tillers (NT) as a result of water stress in wheat genotypes. The NT per plant plays a major role in yield contribution. Guo *et al*. (2018) showed that water stress significantly reduced total plant dry weight (TPDW) by reducing shoot length (SL), shoots dry weight (SDW) and roots dry weight (RDW).

2.9.1.2 Relative growth rate

Plasticity is the ability of plants to modify their growth behaviour in response (e.g metabolism) to water stress (Solomon & Labuschagne, 2009). Increased water use efficiency and reduced loss of water by a plant may increase grain yield (Whan *et al*., 1991). Rapid growth of a plant provides an opportunity to maximize amount of scarce available resources such as water. Plants with higher RGR and increased plasticity (morphological and/or physiological) have a greater chance to adapt in dry areas. This provides a better opportunity to screen and to make selections for water stress resistance. Simane *et al*. (1993) found that the relationship between RGR and morpho-physiological factors could play a significant role in utilising this trait in plant breeding programmes. Understanding the genetic background of the specific variety plays a significant role in variety improvement for a specific trait (Solomon & Labuschagne, 2009). Reduced RGR was observed in wheat genotypes under water stress conditions (Guo *et al*., 2018).

2.9.1.3 Root and shoot traits

Plant roots growing under water stress have the ability to grow deep into the soil thereby extracting available water from deeper soil (Hawes *et al.*, 2000). Of the three plant parts namely leaves, stems and roots, roots are the first to be affected by water stress. Root extension progresses under water stress whereas other plant parts are limited to growing up to a certain threshold (Franco *et al*., 2011). Thus, there is

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variation in growth and development of different plant organs under water-limited conditions. Such plant behaviour is empirical evidence of plant adaptation in a water-deficit environment. For optimal water uptake by a plant, the root-to-shoot ratio (R:S ratio) is increased during the desiccation period, and this relates to the abscisic acid content of the roots and shoots (Smirnoff, 1998). Surprisingly, Noctor & Foyer (1998) observed a decrease in root growth of wheat grown under moderate and severe water stress. Rao *et al*. (1993) reported a nonsignificant reduction in root growth under water stress. However, yield stability under water stress can be improved by paying more attention to root traits such as root biomass and root morphology (Becker *et al*., 2016).

Water stress affects not only R:S ratio but other factors such as RDW, roots fresh weight (RFW) and root length (RL) as well. Under water stress conditions, more assimilates are directed into roots for growth and development to ensure a deep root system for water uptake (Franco *et al*., 2011). The R:S ratio could be that roots grow faster than the shoots or that the shoots slow down or stop growing (Franco, 2011). Similar results were reported by Guo *et al*. (2018) with SDW and SL being reduced under water stress conditions. Rapid root growth may result in increased RDW, RFW and RL under water stress conditions. Reduced SDW, shoots fresh weight (SFW) and SL are expected under water stress conditions.

2.9.1.4 Chlorophyll content

Plant growth and yield are the end product of photosynthesis. The process of photosynthesis links several physiological and molecular processes occurring in the plant. According to Zlatev *et al*. (2017), biomass accumulation is the result of photosynthesis. Maxwell and Johnson (2000) state that Chl and photosynthesis activity are an important signal to indicate the stress levels and health status of a plant because of the changes in their usual concentration in response to stress. The Chl of the leaf determines the amount of light to be absorbed, thereby determining the end product (Murchie & Lawson, 2013). Biotic and abiotic stress interferes with the functioning of the most active enzyme, Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBisCO), in the photosynthesis process. Therefore, any form of stress may reduce Chl and/or RuBisCO functioning, and the damage can be observed from the chloroplast. Moderate water stress may reduce the stomatal aperture followed by reduced photosynthesis activity without severe damage of the chloroplast (Maxwell &

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Johnson, 2000). According to Grassi & Magnani (2005), extreme water stress may severely reduce the stomatal aperture up to 0.1 mol H_2O $m^{-2}s^{-1}$. Hence, the photochemical system will collapse, resulting in inhibition of carbon dioxide (CO_2) intake, reduced RuBisCO functioning and reduced photosystem II activity.

2.9.1.5 Leaf area

Research conducted by Rizza *et al*. (2004) reported a correlation between short plant stand, early maturity and reduced leaf area with water stress tolerance. Lonbani & Arzani (2011) observed a significant increase in flag leaf area length in relation to width, which did not change significantly in wheat genotypes grown under water stress conditions. Plant growth is reduced under water stress condition resulted by a reduction in leaf extension; this enables a balance between plant water status and water uptake by the roots (Maathuis *et al.*, 2003). Water stress was found to reduce leaf area, followed by a reduction in photosynthesis (Rucker *et al*., 1995). According to Shao *et al*. (2008), the number, size and longevity of the leaves of the entire plant can decline as a result of water stress. Water stress may significantly interfere with the development of wheat leaves and other plant organs (Singh *et al*., 1973).

2.9.1.6 Stomatal conductance

G_s was found to be the most reliable element to control water loss by the plant, and utilising this trait for screening could lead to success in variety development (Strauss & Agenbag, 2000). Measurements have been made more convenient with newly developed devices that determine leaf diffusion resistance. Several studies have been conducted for screening the g_s of leaves (Haworth *et al*., 2016; Khalilzadeh *et al*., 2016; Flexas & Medrano, 2002). Strauss & Agenbag (2000) recognised the rapid response of g_s to control leaf water potential in plants exposed to water stress in the field. However, this response was found to restrict plant CO_2 absorption followed by reduced biomass. Some research studies reported the production of ions (potassium) by the leaf to be responsible for closing and opening of the stomata (Chaves *et al.*, 2002; Davies & Zhang, 1991). Severe water stress results in stomatal closure and inhibits CO_2 intake, interferes with enzyme (RuBisCO) activity, inhibits production of ATP and limits photochemical activity (Flexas & Medrano, 2002).

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2.9.1.7 Leaf relative water content

Leaf relative water content (LRWC) can be defined as the quantity of water available in the leaf in relation to LDW. Higher LRWC indicates water stress resistance in cereal crops. According to Clavel *et al*. (2005) reduction of LRWC is a sign of sensitivity to water stress. It is very important for a plant to maintain the maximum water balance to allow growth and development (Strauss & Agenbag, 2000). Water use efficiency was found to increase under water stress conditions in winter wheat. Despite reported problems of yield decline (Xue *et al*., 2006). Water stress-tolerant genotypes were reported to have reduced water deficit-per-unit decrease compared to stress-sensitive genotypes' leaf water potential (Strauss & Agenbag, 2000). Research by Sullivan & Eastin (1974) suggests that water stress-susceptible and -resistant genotypes can be identified by using leaf water potential. Spring wheat and sorghum varieties showed increased water stress resistance in the population caused by increased leaf water potential in the specific varieties (Strauss & Agenbag, 2000).

2.9.1.8 Excised leaf water loss

Excised leaf water loss (ELWL) indicates the ability of the leaf to maintain the water balance between the continuous supply of water from the roots and loss of water through transpiration (Kaur *et al*., 2016). Genotypes that show reduced ELWL under water stress conditions can tolerate stress better. Such genotypes can easily recover from stress and produce secure yield (Lugojan & Ciulca, 2011). Cuticular transpiration rate can be used to distinguish between water stress-susceptible and -resistant varieties (McCaig & Romagosa, 1991). Low rate of water loss indicates cuticle resistance against stress, and such genotypes can be used for screening. Measuring ELWL is cost-effective and convenient and can be used for screening large populations (Kaur *et al*., 2016; Clarke & Townley-Smith, 1986). ELWL is a moderately heritable trait.

2.9.1.9 Photosynthesis active radiation and radiation use efficiency

The structure and size of the canopy significantly influence the light energy absorbed by the plant (Koca & Erekul, 2016). According to Dadashi *et al*. (2015), this trait significantly influences biomass accumulation and yield returns. Yield was reported to be highly dependent on the photosynthesis capacity of the leaves, light interceptance and leaf area index (LAI) (Duchemin *et al*., 2007). Increasing irrigation water was found

to proportionally increase LAI in wheat. PAR also increases with increased LAI. PAR was significantly decreased under severe water stress conditions followed by significant reduction of net photosynthesis in rice (Yang *et al*., 2014).

Accumulated plant biomass was reported to be directly associated with intercepted radiation (IR) (Monteith, 1977). According to Stöckle & Kemanian (2009), the production of DW per unit of radiation energy used can also be defined as radiation use efficiency (RUE). Hence, total plant biomass accumulated in grams per square metre may also be referred to as a product of the cumulative IR (MJ m^{-2}) and RUE (g MJ^{-1}) throughout the growing season.

Different phenotypic traits can be used for screening the material at different stages of growth, thereby developing new varieties or improving existing varieties.

2.9.2 Screening for water stress from early stages of growth

Water stress resistance of wheat from the early stages of growth can be considered as a selection criterion for varietal development. Utilising the appropriate screening methods from the early stages of growth could support variety development. This will allow selection of desirable lines for plant breeding programmes. Screening and selection of an actively growing young plant offer the chance to predict performance at a later stage. According to Noorka & Khaliq (2007), the yield of a plant can be determined through selection of vigorous seedlings at an early stage of growth. According to Alves and Setter (2000), water stress-resistant genotypes are characterised by several physiological parameters identified for characterisation of target plants. Water stress does not uniformly affect the early stages of growth, but the seedling stage is reported to be more sensitive to water stress and this varies with genotypes (Noorka, 2014). Therefore, the tillering and stem elongation stages are also sensitive to water stress. Environmental variability significantly influences production; therefore, under rain-fed conditions, production can be enhanced through development of potential genotypes. Early-stage characterisation is more convenient, less labour is required and it is cheaper. According to Rauf *et al*. (2008), at the seedling stage, genotypes show moderate to high variability behaviour throughout the environment. This variability is associated with additive genes, which offer a great advantage for screening at initial stages.

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2.9.3 Stages of growth and development of wheat

Many scales have been developed to describe the growth stages of cereal (grain) crops, such as those by Zadoks, Feekes and Romig. However, two common decimal scales are utilised worldwide, namely those of Zadoks *et al*. (1974) and Feekes (1941) as amended by Chiarappa (1971) and Large (1954) (Figure 2.5). In this study, Zadoks' decimal codes were used because they are commonly used in small-grain crops such as wheat due to their precision.

There are four principal stages of wheat development, namely tillering, stem extension, heading and ripening (Feekes, 1941) (Figure 2.6). Four principal stages are divided into ten substages of a scale of 00 to 99. Understanding these stages offers a great opportunity to carry out relevant management practices. This allows timeous application of relevant inputs such as fertilisers and/or data collection. Water stress has been considered as one the major environmental stresses. According to Abido & Zsombik (2018), approximately 25% of agricultural production worldwide is affected by water stress. Water stress is reported to significantly affect the growth and development of wheat from planting throughout the life cycle (Abido & Zsombik, 2018). However, the susceptibility of the plant to water stress largely depends on the stage of growth, the intensity and duration of the stress and the genotype.

2.9.3.1 Germination stage

The germination stage (Zadoks, 00 to 09) lasts from planting the seed in the soil until appearance of the first green leaf from the coleoptile. According to Zadoks *et al.* (1974), the planted kernels remain in the form of a dry seed in the soil. The imbibition process commences, and the kernel becomes swollen, followed by radicle protrusion from the seed. The coleoptile emerges from the seed, followed by the first green leaf just at the tip of the coleoptile. The process is called germination. Several studies conducted on the germination stage (from planting to emergence of green leaves) reported that minimum temperatures ranged from 2.4 °C to 4.6 °C (Almansouri *et al.*, 2001; Hampson & Simpson, 1990; Nyachiro *et al.*, 2002). Temperatures between 20.4 °C and 23.6 °C were reported to be optimum for seed germination, and maximum temperatures fall between 31.8 °C and 33.6 °C (Nyachiro *et al.*, 2002).

Increased water stress levels at the germination stage were found to reduce the germination percentage in wheat significantly (Guo *et al.*, 2013; Abido & Zsombik,

2018). At the germination stage, increased water stress levels were found to reduce enzyme activity, thereby affecting the process of metabolism (Guo *et al*., 2013). Reduced or no imbibition results in low water potential and reduced nutrient uptake. Eventually, changes in the seed hormones and modified activation of the enzyme occur (Abido & Zsombik, 2018).

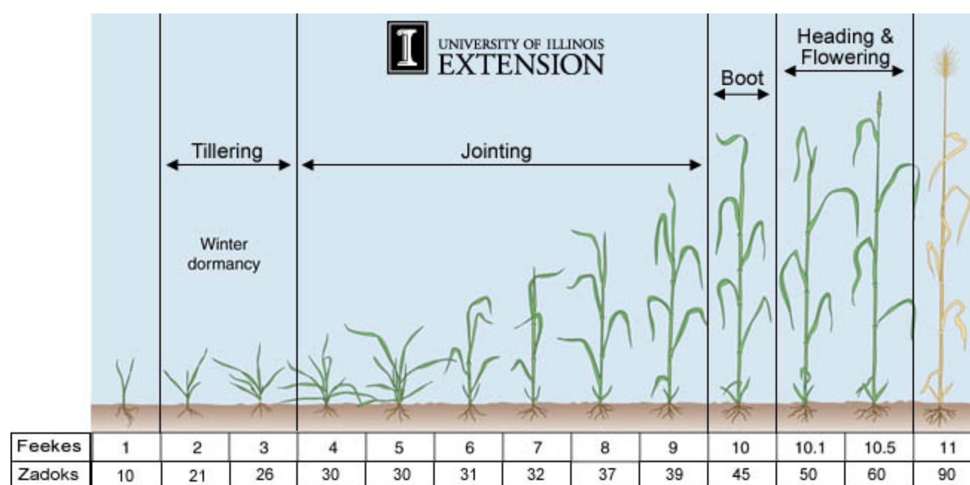

Figure 2.6: Diagram illustrating the wheat growth stages

Image acquired online: (https://www.westco.coop/pages/custom.php?id=33411)

Increased accumulation of organic osmolytes was reported in wheat seeds as a defence mechanism to cope with increased levels of water stress (Li *et al*., 2013). Prolonged periods of water stress interfere with mitochondrial and chloroplastic metabolism activity. This will result in production of reactive oxygen species as defence mechanism, and membrane stability may be destroyed (Li *et al*., 2013).

2.9.3.2 Seedling stage and leaf development

After germination and emergence of the first leaf, seeds continue to grow. The seedling stage lasts from the appearance of the first leaf until the emergence of nine or more leaves (Zadoks 10 to 19). Growth at this stage involves production of new leaves approximately every four to five days (Simmons *et al*., 1995). The seedlings frequently produce about eight to nine leaves in total. However, some genotypes may produce many leaves, especially those that mature at a later stage (Simmons *et al*., 1995). Flag leaf appearance is one of the most important periods of growth in wheat. Growth regulators and other chemical practices are synchronised with flag leaf appearance (Simmons *et al*., 1995). Water stress significantly affects the percentage

of germination and the early seedling growth of wheat (Li *et al*., 2013). Seedlings cannot tolerate water stress for long periods. Li *et al*. (2013) reported increased accumulation of proline content up to a threshold in seedlings under water stress. Thereafter, reactive oxygen species increased, followed by membrane damage content, indicating that seedlings cannot cope with prolonged water stress (Nezhadahmadi *et al*., 2013).

2.9.3.3 Tillering stage of development

The tillering stage begins with the emergence of one tiller from the main stem and ends when the plant has nine or more tillers (Zadoks, 20 to 29). The tillering stage is commonly associated with massive leaf development from the main stem, and this is a key to plant growth and development (Simmons *et al*., 1995). At this stage, the plant has an opportunity to accumulate a high NT in order to increase the plant population. Tillers are expected to be formed at the junction of the coleoptile and from the main stem at the lower leaves (Del Moral & Del Moral, 1995). The growth conditions and genotype usually determine the NT formed. Apparently, formation of tillers in the greenhouse and on the field, is not the same. More tillers are expected to form in the greenhouse compared to the field, supposedly three or more tillers from the main stem (Acevedo *et al*., 2002).

Among all the tillers formed, it is common that a few will produce seeds. Production of tillers also largely depends on plant proficiency; if the plant is not clustered with tillers or is overfertilised, more secondary tillers can be formed from primary tillers (Acevedo *et al*., 2002). Tillers that synchronise their appearance with the emergence of four to six leaves from the main tiller are expected to be highly competitive for development and to form grain. In contrast, tillers that emerge far beyond that period are not expected to perform well; abortion may result, thereby a loss of grain. Some tillers may persist longer, but this varies with genotypes to form tillers that are likely to abort seeds (Acevedo *et al*., 2002).

The stress level that a plant may encounter also plays a key role as abortion increases with increased water stress levels. Khan *et al*. (2010) reported decreased NT caused by water stress in wheat genotypes. NT per plant plays a major role in yield contribution. Water stress reduces RUE, followed by reduction in photosynthesis

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activity and production of carbohydrates. Therefore, tiller development is part of plant growth and development and is affected by water stress.

2.9.3.4 Head differentiation, stem extension and head growth

The jointing stage begins when the plant has a pseudostem and ends when the ligule of a flag leaf is fully visible (Zadoks 30 to 39). Towards the end of the tillering stage is a jointing stage; tillers and the main stem begin to form the heads, and this is an important event for wheat development. However, heads that form at this point are microscopic and all other reproductive structures that will form floral parts and seeds have already been formed by this stage (Simmons *et al.*, 1995). At this stage, the internodes of the lower stem remain the same throughout the stage and plants usually consist of five leaves. Growth commences from the lower fourth internode that elongates when a plant has approximately nine leaves (Acevedo *et al.*, 2002). Internodes continue to elongate on the upper part of the plant. The peduncle elongates last. Moreover, the length of the peduncle accounts for a large part of the overall main SL. Growth management practices to control lodging risk by utilising growth regulators for good returns are scheduled at this stage. Other growth regulators are aimed at retarding the last three or four internodes, thereby retarding growth and influencing stem elongation. Consequently, plants with reduced height and firm shoots have reduced lodging risk (Simmons *et al.*, 1985).

Head growth lasts from when the leaf sheath begins to grow until when the first awns are visible (Zadoks 41 to 49). Stem elongation and rapid head growth occur simultaneously, and during this period individual florets get ready for pollination and fertilisation (Simmons *et al.*, 1985). Tillers have different phases of growth in a single plant. There is a variation in the emergence of tillers from the main stem in a single plant. Prior to heading, this variation is decreased from a few weeks to a few days. The flag leaf encloses the developing head just before it emerges, and this is called heading (Simmons *et al.*, 1985).

Water stress significantly affects wheat growth and development during the stem extension and the booting stages. Large numbers of tillers die in winter wheat exposed to severe water stress during the stem elongation and the booting stages (Nelson *et al.*, 1988), and these two stages were reported to be the most critical stages of growth of all (Zhang & Oweis, 1999). Water stress was found to reduce NT as a result of

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reduced photosynthesis products in the plant (Gholami & Poor Asadollahi, 2008). Gholami & Poor Asadollahi (2008) reported a reduced number of kernels per spike of wheat exposed under water stress conditions at joining stage.

2.9.3.5 Heading and flowering

The heading stage begins when the head appears from the leaf sheath and ends when it becomes completely visible (Zadoks, 50 to 59). This is caused by continuous growth of the stem and the peduncle, eventually exposing the head and become visible. The head is made up of two spikelets positioned opposite one another and held onto the peduncle by rachis (Simmons *et al*., 1985). A wheat spike consists of spikelets held onto the peduncle extension by rachis and alternating throughout the peduncle. Spikelets are made up of four segments, namely stigma, ovary, anthers and stamens. These four components give rise to a floret. Heading is followed by flowering after a few days. Flowering begins when a few anthers are visible around the middle of the head and ends when the head is fully surrounded by the anthers (Zadoks, 60 to 69). The temperature requirement for flowering is usually 11 °C to 13 °C (Simmons *et al.*, 1985). Flowering begins from central spikelets on the head and progresses upward and downward.

Wheat crops are primary self-pollinated; therefore, the pollen sacs shed pollen and remain attached to stigmatic branches. This is important to prevent outcrosses. Anthers can be noticed growing out from each floret, and they are usually yellow, grey or green (Simmons *et al*., 1995). The colour of the anther can be used to predict whether pollination was successful or not. Yellow or grey instead of green guarantees that pollination has occurred. Pollination often takes about four days per head. The stigmas become receptive for approximately 6–13 days. The sizes of the kernels are not uniform as from the pollination commences and maintain this variation until maturity (Simmons *et al*., 1985).

Water stress may result in yield loss due to loss of spikelets from the developing head. Severe water stress may result in abortion of the florets and pollen grains from the head prior to flowering (Ji *et al*., 2010). Anthesis or flowering was reported to be the second most critical stage of growth after stem elongation and booting (Zhang & Oweis, 1999). Water stress may result in severe yield loss if it occurs during the stem elongation and flowering stages in cereal crops. Anthesis is a critical stage of growth;

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the occurrence of stress at this stage reduces pollination and eventually causes yield loss (Ashraf & Azam, 1998). Sufficient water supply during the post-anthesis period enables increased photosynthesis rate and provides enough time to translocate carbohydrates to grains, thereby increasing grain sizes and ultimately grain yield (Zhang & Oweis, 1999). According to Ji *et al*. (2010), water stress was found to induce male sterility in wheat as a result of stress in the early reproductive stage and to cause reduced yield.

2.9.3.6 Grain development and ripening

Grain development begins at kernel water ripe and lasts until the hard dough stage (Zadoks, 70 to 89). Under normal growth conditions, the plant continues to grow and kernels are formed, which can take about three weeks (Acevedo *et al*., 2002). The endosperm cells at this period are fully developed, and the weight of a kernel does not change considerably (Ashraf & Azam, 1998). From the first week to the second week after pollination, an important event occurs; the kernel rapidly accumulates starch and protein, allowing continuous increase in DW (Ji *et al*., 2010). A constant supply of assimilate is required at this phase, which is possibly the determinant of final DW. The kernels are constantly in a soft dough stage at this period, and eventually the growth of the kernels decreases in about three weeks into grain filling (Acevedo *et al*., 2002).

The weight of a kernel approaches its maximum at physiological maturity. During the hard dough stage, the kernel continues to mature. If conditions become unfavourable during the growth of the kernel, accumulation of dry matter will be reduced, which will eventually reduce yield (Ji *et al*., 2010). Ripening begins when the kernel is hard up to the loss of secondary dormancy (Zadoks, 90 to 99).

Water stress interferes with accumulation of reserves, thereby reducing the number of kernels and their sizes and/or causing abortion of the grain (Ji *et al*., 2010). Water stress affects plant growth and development during all stages of growth; therefore, it is important to understand all stages of growth that may result in considerable yield loss (Acevedo *et al*., 2002). Understanding the complexity of water stress and plant developmental stages will not only aid in water stress management but will also enable the development of water stress-resistant varieties and lead to progress in plant breeding programmes and agronomic practices (Zhang & Oweis, 1999).

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2.10 Reticulated hydroponic system

RHS utilises subirrigated gravel culture as a growth media. Such a system enables the plant roots to anchor themselves in a coarse-textured growth medium, for example silica sand, form plastic or loose/gravel stones. Utilising gravel/coarse material as growth medium permits convenient sterilisation. This method may be costly in relation to others; however, it enables considerable manipulation of growth factors for good returns. The RHS advances screening of plants in the greenhouse or growth chamber, thereby enabling feasible planting and harvesting. At harvest, plants can be easily uprooted to observe root parameters.

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CHAPTER 3: MATERIAL AND METHODS

3.1 Introduction

The study was conducted at Stellenbosch University in Welgevallen experimental farm. The seed material used in this project was sourced from the SU-PBL and collaborators. MAS was utilised for molecular characterisation of wheat lines from the nursery using rust resistance markers. The following markers were used for screening the material in the nursery: *Lr34*, *Sr2*, *Sr31*, *Sr24*, *Lr37*, *Sr26* and *Lr19*. These markers were used for the female F1 segregating population and for donor lines phenotypically screened for water stress resistance.

Phenotypic screening of 60 male lines for water stress resistance utilising an RHS was carried out in the greenhouse. High-heritability Mendelian inherited and quantitative traits were identified and selected for screening the material. Selected genotypes were validated many times for certainty of the results. Selected genotypes were further screened using more identified and selected traits. Selected genotypes were cross-pollinated with females in the MS-MARS cycle nursery to introduce the traits of interest and/or novel germplasm into the SU-PBL breeding population. Correlation study was performed from the studied phenotypic traits and heritability estimates was explored from the literature.

Annual routine MS-MARS cycles practised in the SU-PBL provided sufficient male and F1 1:1 female segregating seed material from the 9th Wheat Rust Resistance Nursery pre-breeding programmes during 2014. Both males and females were planted as F1 base population in the greenhouse. The female segregating population were planted two weeks earlier in the greenhouse to synchronize flowering, followed by donors planted on separate benches. The female 1:1 segregating population was screened using rust resistance gene molecular markers from the MS-MARS cycle in the nursery.

Four benches of males and females were planted during MS-MARS Cycle 1. Leaf samples were taken (third leaf more than half visible, Zadoks 12) followed by molecular characterisation of wheat lines for rust resistance genes. Male-sterile female plant tillers were selected and crossed with donor tillers to produce seeds. An RHS was used to facilitate crosses designed for recurrent selection in the greenhouse. The

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male fertile F1 segregating population that remained was allowed to self-pollinate and produced seeds that were used during MS-MARS Cycle 2 as base population. Seeds produced during MS-MARS was used to make crosses with phenotypically screened genotypes for water stress resistance traits. Summary of the study flow is represented on Figure 3.1 below.

3.2 Screening the material

3.2.1 Molecular screening

The seed material used in this project was sourced from the SU-PBL and collaborators. MAS was utilised for molecular characterisation of wheat lines from the nursery using rust resistance markers. The SU-PBL has a standardised set of molecular markers routinely used for screening material in the wheat nurseries (Smit, 2013). A selected set of primers was used for the purpose of this study (Table 3.1).

Different markers were used during MS-MARS cycles 1 and 2 for both male and female lines. During MS-MARS Cycle 1, the following markers were used for both males and females: *Lr34*, *Sr2*, *Sr31*, *Sr24*, *Lr37*, *Sr26* and *Lr19* (Table 3.1). Only two markers were excluded from donor lines *Sr31* and *Sr26* in MS-MARS Cycle 1. During MS-MARS Cycle 2, only *Lr34* and *Sr2* were used for screening both male and female lines in the nursery. Wheat molecular characterisation began with DNA extraction from the laboratory.

3.2.1.1 DNA extraction

A shortened version of a protocol described by Doyle and Doyle (1990) was utilised to perform DNA extractions from wheat genotypes at seedling stage (third leaf more than half visible, Zadoks 12). Leaf material of approximately 0.1 g was sampled in the greenhouse and was put into 2 ml micro-centrifuge tubes. Three sterilised steel ball bearings were equally assigned to each tube, followed by addition of 500 µl of 2% (m/v) CTAB extraction buffer. The buffer solution was made up of 1.4 mM NaCl, 20 mM Na2EDTA (pH 8) and 100 mM Tris-HCl (pH 8). The leaf material contained in the centrifuge tubes was pulverised twice for two minutes at 30 Hz by placing the samples in the Qiagen®TissueLyser supplied by Qiagen (Pty) Ltd and distributed by Southern Cross Biotechnology, Claremont, RSA. This was followed by incubation of a green mixture of CTAB extraction buffer and pieces of plant material for 15 minutes in the

water bath at 60 ˚C. Thereafter, 500 µl of chloroform: isoamyl- alcohol (C: I: 24:1) were added to the solution and it was centrifuged for 10 minutes at 14 000 rpm. The next step was transferring the supernatant after centrifuging to new 1.5 ml sterilised micro-centrifuge tubes. Once more 400 µl of chloroform: isoamyl-alcohol were added to the supernatant and mixed by inversion, followed by centrifuging for five minutes at 14 000 rpm. After centrifuging once more, the supernatant was isolated and transferred to 1.5 ml new, clean and sterilised micro-centrifuge tubes. Five hundred microlitres of ice-cold ethanol and 50 µl of 7.5 M ammonium acetate (or 3 M sodium acetate [pH 5.0]) were added to the micro-centrifuge tubes. Genomic DNA precipitation was performed by several inverting micro-centrifuge tubes and followed by cold centrifuging for two minutes at 14 000 rpm. The supernatant was then removed.

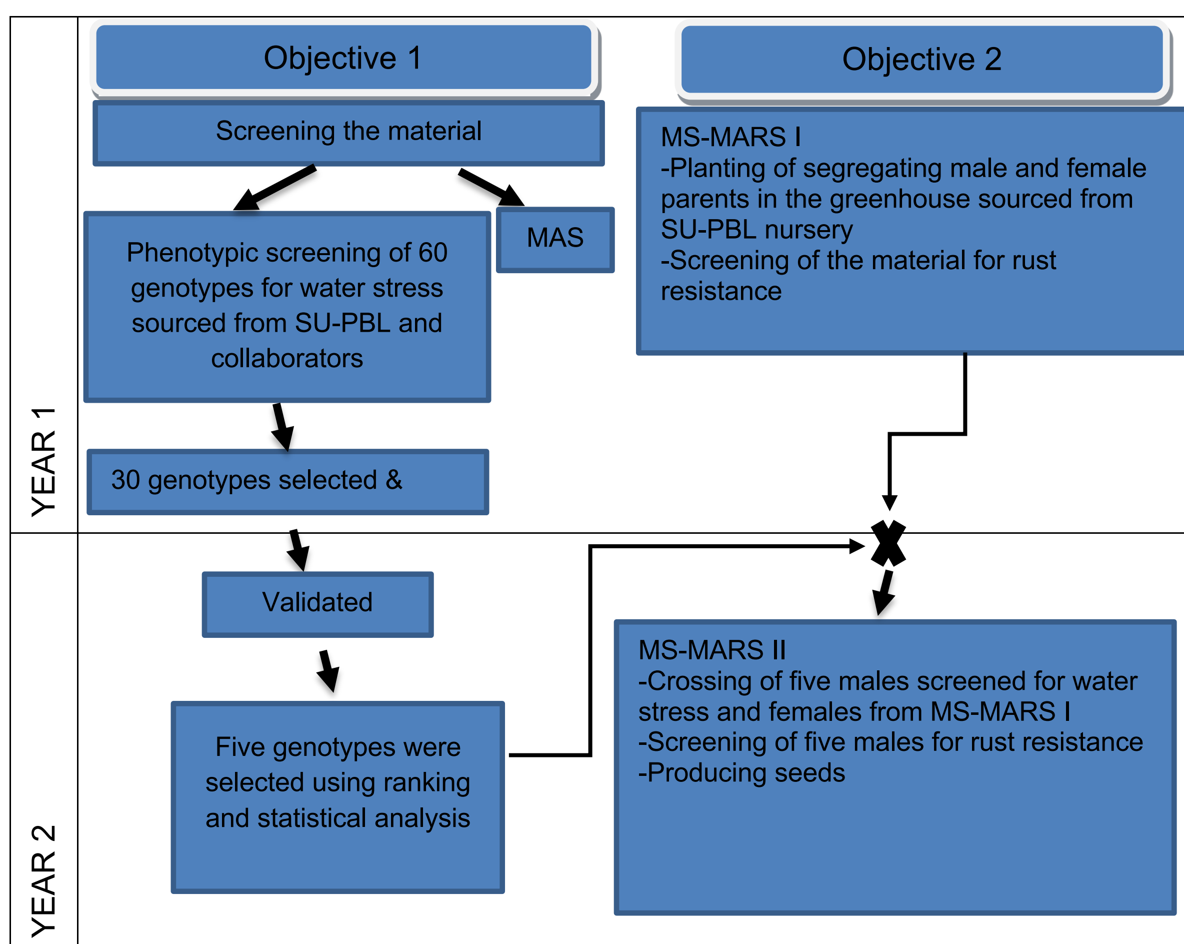

Figure 3.1: Representation of the study flow

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Table 3.1: Primers used for molecular screening of wheat lines

Trait	Marker	Primer	Primer sequences	Ta °C	Fragment size (bp)	References
Disease resistance genes	*Sr2*	csSr2 F csSr2 R	5'-CAAGGGTTGCTAGGATTGGAAAAC-3' 5'-AGATAACTCTTATGATCTTACATTTTTCTG-3'	60	53, 112, 172	Mago *et al.* (2011b)
	Sr24	SCS719-F SCS719-R	5'-TCGTCCAGATCAGAATGTG-3' 5'-CTCGTCGATTAGCAGTGAG-3'	55	719	Cherukuri *et al.* (2003)
	Sr26	Sr26#43 F Sr26#43 R	5'-AATCGTCCACATTGGCTTCT-3' 5'-CGCAACAAAATCATGCACTA-3'	60	207	Mago *et al.* (2005)
	Sr31	lag 95-F lag 95-R	5'-CTCTGTGGATAGTTACTTGATCGA-3' 5'-CCTAGAACATGCATGGCTGTTACA-3'	55	1030	Mago *et al.* (2005)
	Lr19	12C-F 12C-R	5'-CATCCTTGGGGACCTC-3' 5'-CCAGCTCGCATACATCCA-3'	60	119	Prins *et al.* (2001)
	Lr37	VENTRUIP LN2	5'-AGGGGCTACTGACCAAGGCT-3' 5'-TGCAGCTACAGCAGTATGTACACAAA-3'	65	259	Helguera *et al.* (2003)
	Lr34	L34DINT9-F L34PLUS-R	5'-TTGATGAAACCAGTTTTTTTTCTA-3' 5'-GCCATTTAACATAATCATGATGGA-3'	58	517	Lagudah *et al.* (2009)

Seventy percent ethanol was used to wash the DNA pellet twice. The micro-centrifuge tubes were then opened and placed horizontally on the table to allow air to flow into the tubes and dry the DNA pellet at room temperature. After allowing the pellets to dry out, they were resuspended in 30 µl deionised water and stored at -20 °C. The interesting step that followed was to determine the concentration of DNA in the tubes by utilising a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). DNase/RNase-free water was used to dilute the DNA to a required concentration ranging from 90 ng/µl to 120 ng/µl.

All primers used in this study were manufactured by Integrated DNA Technologies (and distributed by Whitehead Scientific Inc., Stikland, RSA), and PCR reagents were

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manufactured by KapaBiosystems (distributed by Lasec SA (Pty) Ltd, Cape Town, RSA). PCR machine number 2 (GeneAmp® PCR System 2720 Thermal cycler) supplied by Applied Biosystems® Life Technologies, Johannesburg, RSA and/or a TECHNE TC-5000 (distributed by Lasec, Cape Town, RSA) was used to perform all the PCR analysis reactions.

The reactions were performed based on the genes of interest; therefore, specific primer sequences and molecular markers were used (Table 3.1). Most of the PCR reactions performed for characterisation of wheat lines were multiplex reactions. However, the exception was CAPS for stem rust (*Sr2*) reactions. The multiplex reaction for molecular marker *Lr34/Yr18* was tested on the female population, consisting of wheat lines postulated to carry leaf and stripe rust resistance genes. The *Lr34* codominant *CSLV*+ and negative marker *CSLV*- were added to aid identification of *Lr34* genes in a multiplex reaction. The *Lr34* reactions were performed using PCR tubes with final concentration of 17.6 µl. The reaction mix included 4.6 µ1 dH2O, 10 µl 2X KAPA 2GTM Fast Multiplex PCR Mix, 0.6 µ1 Dinta forward primer, 0.6 µ1 Lr34 reverse primer, 0.25 µl of each *csLV34* forward and reverse primer, and 1.3 ng of gDNA.

Micro-centrifuge tubes were placed into the PCR reaction for analysis under the following cycling conditions: The starting point was the denaturation steps for five minutes at 94 ºC. This was followed by 35 cycles at the same temperature of 94 ºC for one minute. Annealing step during which temperature went to 57 ºC for one minute. Then one minute at 72 ºC and finally 72 °C for seven minutes. The PCR machine automatically stored the samples at 4 ºC when the cycle was completed. Samples were removed from the PCR machine and electrophoresed in 1%, 1.5%, 2% and/or 3% agarose gel. For visualisation, 4, 6, and 7 microlitres of ethidium bromide were added to 80 ml, 160 ml and 200 ml agarose gel respectively and mixed well. Gels were visualised under ultraviolet (UV) light by using the Uvitec gel imaging system.

Multiplex reaction was performed for six markers, namely *Sr31*, *Lr34*, *Lr24/Sr24*, *Lr37/Sr38*, *Sr26* and *Lr19*. These markers were used to test the female population in the greenhouse (Springfield, 2014). The PCR tubes' final concentration contained for multiplex reaction differed from the others; it was 21.7 µl. The PCR tubes contained the following reagents: two times 12.5 µl KAPA2GTM Fast Multiplex PCR Mix, 1.0 µl

of each Iag95 of both forward and reverse primers, 0.5 µl Dint-forward and 0.5 µl Lr34Plus-reverse primers, 0.5 µl of 719 both forward and reverse primers, 0.5 µl Vent and 0.5 µl of Ln2, 0.5 µl of each Sr26#43 both forward and reverse primers, before the last 1.0 µl of each 12C both forward and reverse primers, and lastly the genomic DNA of approximately 1.2 ng/µl. Tubes were put into the PCR machine under the following cycling conditions: Initially, the denaturation step for three minutes at 94 °C.

Subsequently, 30 cycles at the same temperature of 94 °C for 30 seconds. Thereafter, the temperature slightly differed for annealing at 57 °C for 30 seconds. Then one minute at 72 °C and finally 72 °C for 10 minutes. The PCR machine automatically stored the samples at 4 °C when the cycle was completed. Samples were removed from the PCR machine and electrophoresed in 1%, 1.5%, 2% and 3% agarose gel. For visualisation, 4, 6, and 7 microlitres of ethidium bromide were added to 80 ml, 160 ml and 200 ml agarose gel, respectively and were mixed well. Gels were visualised under UV light by using the Uvitec gel imaging system.

*Sr*2 CAPS molecular markers were utilised to characterise the female F1 segregating base population at seedling stage (third leaf more than half visible, Zadoks 12). Wessels & Botes (2014) utilised standardised molecular markers in the SU-PBL to map desirable genes in the nurseries. The most recent CAPS was used to perform PCR analysis for *Sr2* (Table 3.2).

Table 3.2: The conditions and reaction volumes for *Sr2* marker characterisation

Reaction reagents	**Reaction volume (µM)**	**Temperature (°C)**	**Time (min)**
dH_2O	3	95	2
2X Green	7.5	95	0.5
Cssr2F	0.45	60	0.40
Cssr2R	0.45	72	0.50
gDNA	1.5	72	5
Total	12.9	**40 cycles**	

The markers were *Cssr2F* and *Cssr2R*. Both markers were tested on both the male and female populations in the nursery. The mixture concentration in the tubes for PCR reaction is shown in Table 3.2. The PCR machines automatically stored the samples at 4 ºC when the cycle was completed. Samples were removed from the PCR machine

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and electrophoresed in 1% agarose gel. For visualisation 4, 6, and 7 microliters of ethidium bromide were added to 80 ml, 160 ml and 200 ml agarose gel respectively and were mixed well. The Uvitec gel imaging system that uses ultraviolet light was utilised to visualise the gel. Samples that carried a band were then selected, and the researcher proceeded to the next step. Thereafter, the enzyme digestion step followed. This step involved the addition of 2.5 µl of PagI enzyme (Thermo Scientific) to selected tubes of genomic DNA that carried a band, followed by incubation under 37 ºC for an hour. Prior to 2.5% agarose gel electrophoresis, about four µl of loading dye was added to the original genomic DNA samples throughout, mixed and aliquoted. This was followed by visualising under UV light with the use of the Uvitec gel imaging system. The presence of the *Sr2* gene was identified by a band pattern of 53 bp, 112 bp and 172 bp, and the absence of the gene showed a band size of 225 bp and 112 pb.

3.2.1.2 Preparation of agarose gel electrophoresis

For gel electrophoresis, 1%, 1.5% and 1.8% of SeaKem® LE agarose, supplied by Lasec, (Pty) Ltd, Cape Town, SA, was used. Agarose was weighted on an Advuture™ Ohaus supplied by United Scientific (Pty) Ltd, Cape Town, SA. Agarose powder was transferred to a 200 ml volumetric flask, and 80 ml, 160 ml or 200 ml of TBE solution was added and mixed well. One TBE buffer was diluted from 5X TBE stock solution, 0.5 M Tris (hydroxymetyl) aminomethane, 0.5 m Boric acid and 0.5 M Ethylenediamine tetra acetic acid disodium salt dehydrates that were autoclaved for 30 minutes.

The buffer solution was heated in a microwave until all the suspensions were fully dissolved or cleared. The agarose gel solutions were allowed to cool down at room temperature before adding ethidium bromide supplied by Sigma-Aldrich (Pty) Ltd, Cape Town, SA. For visualisation 4, 6 and 7 microlitres of ethidium bromide were added to 80 ml, 160 ml and 200 ml of agarose gel, respectively and were mixed well. The gel was casted slowly into plastic boxes/trays to prevent formation of bubbles. This step was followed by putting 25 or 50 channel combs (respective to gel sizes) into the gel trays. The gels were allowed to cool followed by gentle removal of the combs. The gels were immersed in the electric field with 1X TBE followed by loading of DNA samples into the sample. For the *Sr2* PCR product, 4 µl of

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cresolsulfonephthalein (cresol red) were added to each PCR tube and loaded onto the agarose gels. The gels were run at 100/120 V for 45 minutes and visualised under UV light, using the Uvitec gel imaging system (distributed by Whitehead Scientific Inc., Stikland, RSA).

While phenotypically screening the genotypes for water stress resistance using target traits and at the same time, MS-MARS was conducted in the greenhouse for MS-MARS validation. Selected water stress resistance genotypes were cross-pollinated using the MS-MARS recurrent scheme to screen and validate the presence of rust resistance markers.

3.2.2 MS-MARS cycle

The female segregating population used for crosses was sourced from the ninth Wheat Rust Resistance Nursery pre-breeding programmes during 2014. This is an ongoing annual MS-MARS project for rust resistance molecular screening. The F1 base population utilised in this study consisted of a large number of rust resistance genes (Table 3.1) established in 1999 Botes (2001). The plant material grown in the greenhouse consisted of 1:1 male sterile and fertile segregating populations. The male population already consisted of several biotic and abiotic resistance genes and previously was proved to deliver improved yield under field conditions.

Seeds of equal size were visually selected prior to sowing. Seeds can be visually assessed whether they were cross-pollinated or self-pollinated. Self-pollinated seeds are thick and swollen whereas cross-pollinated seeds are tiny and shrunken.

The MS-MARS breeding technique was utilised as part of Objective 2 of this study, namely phenotypic validation of several wheat rust resistance genes (Springfield, 2014). Among the cross-pollinated wheat lines, the degree of cross-pollination was also evaluated. The male and female' wheat lines were planted in the SU-PBL nursery. The nursery contained eight benches; four were used for donors and four for the F1 1:1 female segregating population. The benches for donor plants were planted a week prior to the benches for female plants. The female lines consisted of a 1:1 ratio of male sterile female and male fertile female segregating population (Marais & Botes, 2000). From the female segregating population, desirable tillers were selected. To make crosses, selected tillers had to be male sterile females (Figure 3.2A & 3.3A). At the

flowering stage, donor lines and male sterile females ready for MS-MARS were carefully selected. Tillers of selected plants were cut and immediately suspended in a 20 L bucket half-filled with a standard nutrient solution. Thereafter, female tillers were transferred to galvanised steel trays with a dimension of 600 mm x 450 mm x 160 mm.

During cross-pollination and rearing, diseases such as fungus might have infected the tillers; therefore, black antifungal paint was used to paint the trays on the inside thereby preventing infection. All leaves from selected female tillers were detached except the flag leaves, which were kept intact. Tweezers were used to open the florets for visual observation. and laboratory scissors were used to cut them open to facilitate crossings (Figure 3.2B). Prior to transferring tillers to trays, they were cut into the same height. The maximum capacity of each tray was 230 tillers.

Figure 3.2: MS-MARS cycle. Cross-pollinations were carried out in the greenhouse as using male sterile female tillers as shown in Figure 3.2A. Male sterile female glumes were cut open to facilitate pollination (Figure 3.2B). The donor lines were orientated above the female lines to facilitate cross-pollination. Figure 3.2D shows cross-pollinated female tillers in seed development stage.
Source: Springfield (2014).

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Tillers were suspended in a standard nutrient solution that fully filled the steel trays. Prior to pollen shedding, male tillers were cut from the main plants and all leaves were removed. Buckets were used to collect the tillers that were suspended in a standard nutrient solution. From the female tillers, flag leaves were kept intact whereas from the male tillers, all leaves were removed. This was followed by proper arrangement of the tillers in two narrow galvanised steel trays positioned parallel to each other and held up by four-legged pod stands (Figure 3.2C).

The female tillers were positioned below the donors, with the donors arranged as if forming a shelter above the females. This has easy the dropping of pollens from the dour to the females. The donors' galvanised steel trays were narrow, enabling sequential positioning of the wheat tillers. Similar to the female tillers, the donor tillers were also suspended in a nutrient solution. The donors were kept at the pollinating position for about a week (although pollen shedding takes approximately three days) for maximum pollination (Figure 3.2C), and thereafter they were discarded. Female tillers were reared by trimming their base constantly thereby preventing formation/development of fungal infection and changing the nutrient solution after two weeks, thereby promoting seed development (Figure 3.2D). When seeds were fully developed and ripened, tillers were transferred into brown paper bags and oven dried for a week at 21 °C. Male fertile plants were allowed to self-pollinate and were harvested at the end of the MS-MARS cycle and used for single-seed descent in the field. Selection of male sterile tillers involved visual assessment and selection throughout the MS-MARS cycles.

3.2.2.1 Visual assessment and selection

Visual assessment for tiller selection included the colour of the spike. When male plants were ready to shed pollen, the colour was dark green and if the pollen was visible, it was yellowish. Male plants with white pollen were not selected (off-types). Female plants were yellowish in colour. Plants were also visually observed through carefully opening their glumes using tweezers to observe the presence of pollen grains. If the male reproductive system was poorly developed and no pollen grains had been formed, the plants were considered sterile and if the reproductive system was fully developed with pollen grains, the plants were considered fertile (Figure 3.3A).

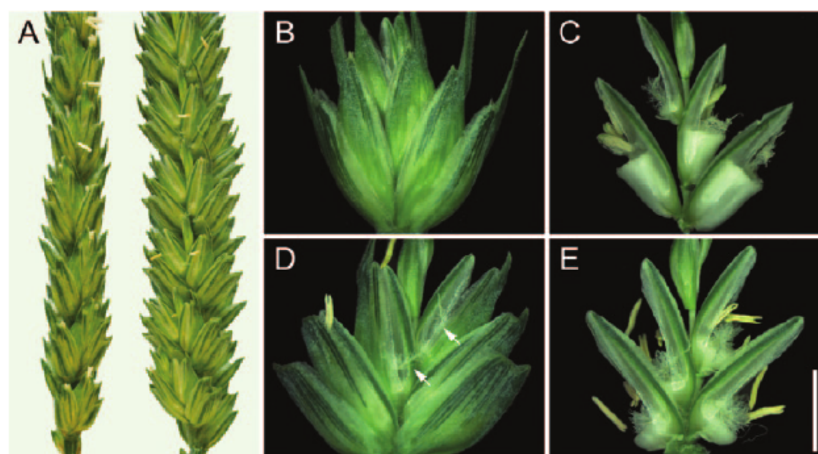

Figure 3.3: Visual assessment of spikes and florets. Figure 3.3A shows two heads of wheat, male fertile on the left and male sterile on the right. Figure 3.3B shows an individual spikelet removed from the spike. Figure 3.3C shows the fertile spikelet with cut-open glumes and lemmas from the spikelet in B. Figure 3.3D shows a male sterile spikelet, and the white arrows in the picture show that stigmas were pushed out of the flower. Figure 3.3E shows a male sterile spikelet pushed out glumes and lemmas in D.
Source: Whitford *et al*. (2013).

When the ovaries are not fertilised, they swell in a sideways direction, thereby opening the flowers. Soon after the MS-MARS cycles, phenotypic screening of genotypes for water stress commenced. More traits were added and used for validation of material in the growth chamber.

3.2.3 Phenotypic screening

Phenotypic screening of 60 genotypes was done in the growth chamber using water stress resistance-related traits and an RHS. High-heritability Mendelian-inherited and quantitative traits were identified and selected for screening the material. Thirty genotypes were selected from 60 genotypes and further validated for water stress resistance genes. Five genotypes selected from the 30 were further validated for water stress resistance genes using more traits of interest. Five water stress resistant genotypes were molecularly characterised using SU-PBL rust resistance markers used for screening the material in the nursery (Table 3.1). Thereafter, five genotypes screened for water stress resistance were crossed with female lines from the nursery

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to introduce the traits of interest and/or novel germplasm into the SU-PBL breeding population.

3.2.3.1 Phenotypic traits and measurements

Measurements were taken from the tillering stage (shoot and one tiller, Zadoks, 21) to stem elongation (fourth node detectable, Zadoks, 34). Measurements were taken at different times and for different durations (Table 3.3). Some were taken at the onset of stress throughout the stress period and some at harvest. The following measurements were taken daily from the onset of stress throughout the stress period: LAI, LAE, NL and NT. Measurements taken at harvest included RFW, SFW, LFW and TPFW. DW measurements included RDW, SDW, LDW and TPDW. Measurements taken by graduated ruler included RL and SL. Water-related measurements were taken at specific stages of plant growth, such as LRWC and ELWL. These measurements were used for screening 60 genotypes; thereafter, additional measurements were included for further screening of the top five selected genotypes. These measurements included R:S ratio, CCI, RGR, g_s and PAR. Before applying water stress, DW measurements were taken for all plants (grown in duplicates) in a polyvinyl chloride (PVC) pipe to calculate change in DW. Measurements included IRDW, ISDW, ILDW and total plant initial dry weight (TPIDW).

3.2.3.2 Plant biomass measurements

The number of leaves and tillers were counted from the onset of water stress at tillering stage (shoot and one tiller, Zadoks 21) up to stem elongation (fourth node detectable, Zadoks 34). Destructive measurements for plant biomass were taken at the end of the stem elongation stage (fourth node detectable, Zadoks, 34). Plants were carefully uprooted and separated into three parts: roots, stems and leaves. RL and SL measurements were taken using a graduated ruler. The plants were then put in brown envelopes and quickly taken into the laboratory to determine the RFW, SFW and LFW measurements using the Ohaus Adventurer^{TM} scale supplied by United Scientific (Pty) Ltd. This step was followed by oven drying up to constant DW at 50 °C, and then RDW, SDW and LDW measurements were determined. TPFW and TPDW measurements were determined by adding FW and DW components, respectively. R:S ratio was calculated as:- R:S ratio $= \frac{SDW + LDW}{RDW}$; calculations were carried out from all genotypes.

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3.2.3.3 Excised leaf water loss

ELWL measurements were taken during the intense water stress period. Leaf material was sampled, preferably young, fully expanded leaves (leaf sheath) (Pask *et al.*, 2012). Samples were taken from all replicates at the tillering stage (main shoot and seven tillers, Zadoks 27) and placed into small envelopes. Samples were rushed into the laboratory to determine FW by weighing the samples on the Ohaus Adventurer™ scale. Samples were then incubated for six hours at 28 °C. This was followed by reweighing the samples to determine weight after six hours. Lastly, oven drying of the samples at 60 °C for 24 hours was done, and DW was determined. The percentage of ELWL was calculated as proposed by (Clarke, 1987):

$$\% \text{ of ELWL} = \frac{FRESH\ WEIGHT - WEIGHT\ AFTER\ 6\ HOURS}{DRY\ WEIGHT} \times 100$$

3.2.3.4 Leaf relative water content

LRWC measurements were taken at the stem elongation stage (second node detectable, Zadoks 27). Flag leaf samples were randomly taken from all the genotypes. The leaves were cut on both ends. Any dry or dead leaf part was removed (Pask *et al.*, 2012). The samples were quickly taken into the laboratory. FW measurements were taken by weighing the samples on the Ohaus Adventurer™ scale. Ten-ml tubes were then labelled according to the representative leaf samples, and 1 ml of distilled water was added to each tube. The rack of tubes was placed in a 4 °C refrigerator for 24 hours to reach full turgor. Leaf samples were removed from the distilled water and carefully dried using blotter paper (Pask *et al.*, 2012). Turgid weight was determined from leaf samples by weighing them again. Thereafter, samples were put in small brown envelopes and oven dried at 60 °C up to constant DW. Samples were removed from the oven, DW measurements were determined by weighing the samples on the balance scale and the data was recorded. The following formula was used to calculate LRWC (Smart & Bingham, 1974; Turner, 1981): -

$$\% \text{ of LRWC} = \frac{FRESH\ WEIGHT - DRY\ WEIGHT}{TURGIT\ WEIGHT - DRY\ WEIGHT} \times 100$$

Table 3.3: Summary of studied phenotypic trait measurement details

Traits	Time	Stress period	Duration	Non-destructive/ destructive	Method	Unit	H^2 %	Quantitative/ Mendelian	Marker/QTL	Plant organ	References	Page no.
LAE	12 am	Cont.	5 sec	Non	Ruler	mm	67	Quantitative	-	Leaf	Mohsin *et al.* (2009)	29
NT	12 am	Cont.	5 sec	Non	Count	n/a	55	Quantitative	1D, 2D, 6A	Plant	Ullah *et al.* (2007)	26
NL	12 am	Cont.	5 sec	Non	Count	n/a		Quantitative	-	Plant	Mbave *et al.* (2013)	26
RGR	12 am	Cont.	5 sec	Non	Protocol	mm	77	Quantitative	1D, 4D, 7D	Plant	Hunt et al. (2003)	27
LRWC	12 am	End	4 days	Des	Protocol	%	64	Quantitative	2A	Leaf	Pask *et al.* (2012)	30
ELWL	12 am	End	24 hours	Des	Protocol	%	94	Quantitative	1A, 1B, 2B	Leaf	Clarke (1987)	30
G_s	12 am	Cont.	30 sec	Non	Porometer	$m^{-2}s^{-2}$	99	Mendelian	1A, 1B, 2B,	Leaf	Becker *et al.* (2016)	29
SFW	12 am	End	1 day	Des	Scale	g	46	Quantitative	1A, 1B, 2B,	Shoot	Hussein and Zaki (2013)	27
SDW	12 am	End	4 days	Des	Scale	g	76	Quantitative	-	Shoot	Ahmad *et al.* (2014)	27
RDW	12 am	End	4 days	Des	Scale	g	9	Quantitative	Xgwm296.1	Roots	Becker *et al.* (2016)	27
RFW	12 am	End	1 day	Des	Scale	g	87	Quantitative	-	Roots	Naeem *et al.* (2015)	27
RL	12 am	End	1 day	Des	Ruler	mm	62	Quantitative	Xksuh9d	Roots	Khan *et al.* (2010)	27
SL	12 am	End	1 day	Des	Ruler	mm	31	Quantitative	Xcdo456a	Shoot	Khan *et al.* (2010)	27
R:S ratio	12 am	End	4 days	Des	Calculations	n/a	44	Quantitative	-	Plant	Ahmad *et al.* (2014)	27

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Traits	**Time**	**Stress period**	**Duration**	**Non-destructive/ destructive**	**Method**	**Unit**	**H^2 %**	**Quantitative/ Mendelian**	**Marker/QTL**	**Plant organ**	**References**	**Page no.**
CCI	12 am	Cont.	2-3 sec	Non	CCM-200	n/a	9	Mendelian	Xgwm63	Leaf	Panio *et al*. (2013)	28
PAR	12 am	Cont.	30 sec	Non	Ceptometer	%		Mendelian	4B, 5B	Plant	Sandaña *et al*. (2012)	30
RUE	12 am	Cont.	30 sec	Non	Ceptometer	MJ-1		Mendelian	-	Leaf	Sandana *et al*. (2012)	30
TPDW	12 am	End	1 day	Des	Scale	g	96	Quantitative	-	Plant	Tatar *et al*. (2016)	26
TPFW	12 am	End	1 day	Des	Scale	g		Quantitative		Plant	Hussein and Zaki (2013)	26

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3.2.3.5 Relative growth rate

RGR is one of the most important traits used to measure water stress effect. Measurements were taken from the tillering stage (shoot and one tiller, (Zadoks 21) up to the end of the stem elongation stage (fourth node detectable, Zadoks 34). Since five genotypes were investigated, replicated three times per RHS, and five seeds were planted per PVC pipe, there was enough material for RGR sampling. To examine RGR, each plant from all replicates was harvested on each PVC pipe at the tillering stage, allowing one plant to grow per PVC pipe. Plants were uprooted and separated into three parts, namely roots, stems and leaves, and put into labelled brown envelopes. Plant material was incubated at 50 °C to a constant DW, and then the final DW was determined. The remaining plants were allowed to grow and were harvested at the end of the stem elongation stage. Similarly, the harvested plants were separated into roots, stems and leaves and put into labelled brown envelopes. Again, samples were incubated at 50 °C to a constant DW, and then the final DW was determined. Total DW of all varieties under investigation was calculated using logarithmic transformation (Hunt *et al*., 2003). The following formula was used to calculate RGR:

RGR ($g\ g^{-1}\ d^{-1}$) = $\left[\frac{(\ln W2 - \ln W1)}{(t2 - t1)}\right]$

Where ln represents logarithmic transformation, W2 final DW, W1 initial DW, t2 final time/days of taking measurements and t1 initial time/days of taking measurements.

3.2.3.6 Photosynthetic active radiation and leaf area index

PAR and LAI measurements were taken every day from 11:00 to 14:00 (Pask *et al*., 2012). Measurements were taken from the onset of water stress at the tillering stage up to stem elongation (fourth node detectable, Zadoks, 34) (Pask *et al*., 2012). Measurements were taken using a hand-held Decagon AccuPAR LP-80 ceptometer supplied by Campbell Scientific Africa (Pty) Ltd. The ceptometer measures light wavelengths within 400 to 700 nm (PAR) and utilises waveband as determinant of LAI. A bubble spirit level was used to ensure that the ceptometer was level when taking measurements from 0.5 m to 1.0 m above the leaf canopy. Measurements were taken diagonally from plant canopies on each Addis Rough tote (ART) which is a black bucket carried plants (Figure 3.5A below). Three measurements were taken for above-canopy PAR, with the ceptometer being carefully aligned horizontally above the leaf canopies. Measurements took about 30 seconds. Similarly, for below-canopy PAR,

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the probe was held in the same position below the canopy. The measurement for canopy reflectance was taken similar to above-canopy PAR, except that the ceptometer was held inverted (Pask *et al*., 2012). The percentage of light interception was calculated using the following formula: -

$$\% \text{ of LI} = \left[\frac{(A-B)-C}{(A-B)}\right] \times 100$$

Where A = above canopy, B = reflected and C = below canopy. The ceptometer can simultaneously calculate the above- and below-canopy PAR; hence, when the ceptometer is placed below the canopy to take readings, light interceptance (F) can be calculated as follows: -

$$F = 1 - \frac{TRANSMITTED}{INCIDENT}$$

Light extinction coefficiency (K) can be defined as the capacity of a substance to absorb light at a given wavelength per unit of mass density. The extinction coefficient was calculated using the formula: -

$$K = \frac{-LN\,(1-F)}{LAI}$$

Where F is the interceptance and LAI is the leaf area index (Farahani *et al*., 2009).

Because PAR was sampled in a quadrat, it was possible to calculate RUE. Moreover, PAR sampling was done prior to destructive measurement of plants. This provided efficiency and accuracy to calculate the canopy coefficient. RUE was therefore calculated for each RHS by investigating a change in biomass over interceptance throughout the water stress period. The following equation was used to calculate RUE (g MJ^{-1}) (Pask *et al*., 2012): -

$$RUE\ (g\ MJ^{-1}) = \left[\frac{MJt2-MJt1}{DWt2-DWt1}\right]$$

Where MJ is PAR accumulative interceptance (MJ m^{-2}) from the initial and final readings, and DW is the initial and final DW time in days.

3.2.3.7 Chlorophyll content index

Leaf CCI was determined using CCM-200 plus a Chl meter manufactured by Opti-Sciences, Inc. and distributed by Campbell Scientific Africa (Pty) Ltd. Relative CCI was determined at the stem elongation stage (second node detectable, Zadoks 32) (Pask *et al*., 2012). A young, fully expanded leaf was used to take measurements from all genotypes (Elshafei *et al*., 2013). The CCI meter sampled 71 mm^2 to calculate CCI utilises receptors and calibrated light-emitting diode (LED). CCI is the transmission percentage ratio of the waveband through a leaf at 931 and 653 nanometres (Mishra *et al*., 2015). The estimated time to take measurements is 2-3 seconds per leaf tissue, and the device weights 162 grams. The CCI meter was calibrated by taking a blank reading prior to taking measurements. Measurements were taken from all the genotypes, and the arithmetic mean was calculated per genotype.

3.2.3.8 Stomatal conductance

G_s measurements were taken using a hand-held SC-1 Leaf Porometer, manufactured by Decagon Devices, Inc. and distributed by Campbell Scientific Africa (Pty) Ltd. G_s measurements were taken from the mid-tillering stage (main shoot and five tillers, Zadoks 25) and (main shoot and seven tillers, Zadoks 27) (Pask *et al*., 2012). Porometer measuring units are mmol m^{-2} s^{-1}, m^2s mol^{-1} and sm^{-1} sample chamber aperture measures 6.35 mm^2. Measurements were taken from 11:00 to 14:00 from all genotypes. Measurements were taken from selected young, fully expanded leaves (because of full exposure to light), preferably the leaf sheath, constantly across all genotypes. Measurements were taken on the upper (adaxial) leaf sheath surface. Each leaf measurement took approximately 30 seconds in auto-mode. Measurements were taken from well-watered and water-stressed plants. Anticipated g_s ceptometer readings were from 300 to 700 mmol m^{-2} s^{-2} for well-watered plants and from 80 to 300 mmol m^{-2} s^{-2} for water-stressed plants (Pask *et al*., 2012). The g_s meter is a sophisticated, time-consuming tool that requires time and patience when working with large populations.

3.2.3.9 Leaf area

Leaf area (LAE) measurements were taken every day from the onset of water stress from the tillering stage (shoot and one tiller, Zadoks, 21) up to stem elongation (fourth node detectable, Zadoks, 34). Measurements such as leaf length and maximum width

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were taken on young, fully expanded leaves using a graduated ruler and rounded off to the nearest millimetre. Leaf length measurements were taken by placing the ruler from the bottom of the leaf blade towards the tip. Maximum width was measured by placing the ruler five times across the leaf blade. Leaf area in mm^2 was calculated as follows: -

$$A = L \times W \times K$$

Where L is the leaf length, W is the leaf maximum width and K is a multiplying factor or constant for wheat (0.65) (Sestak *et al.*, 1971).

3.2.3.10 Association of the traits and their heritability estimates

Correlation study was performed for all the phenotypic traits under investigation thereby enhancing indirect selection for several traits of interest. Correlation co-efficiencies of both water stressed and well-watered genotypes were calculated and compared to previous studies from the literature. Moreover, in order to consider heredity of the target traits, heritability estimates of the phenotypic traits were explored from the literature. Considerable additive genes and reduced environmental effect can provide a great deal of selection (Fellahi *et al*., 2013).

3.2.3.11 Planting

Planting began on the 30th of August 2015, but the experiment was terminated because of poor physical appearance of the plants before inducing water stress. Plants were unhealthy and yellowish. The next planting began on the 6th of September 2015, and again the experiment was terminated due to high temperatures (Figure 3.4) inside the greenhouse in mid-October 2015, caused by failure of the cooling system so that the plants wilted and showed signs of yellowing.

The next planting commenced on the 15th of October 2015, but due to high temperatures plants showed signs of heat stress e.g. wilting (Time and date, 2017) (Figure 3.4). Wheat crop requires the minimum and maximum temperature of 18 °C to 22 °C respectively, throughout a growing season. Temperatures exceeding 22 °C may result in heat stress thereby affecting the plant growth and development. Heat stress may result in pollen sterility and/or ear abortion thereby affecting the yield returns. Therefore, RHSs were moved into the growth chamber under temperatures of 18 °C

to 22 °C. Inside the growth chamber, plants showed unidentified stress before water stress was applied. Later it was discovered that the air supply had been switched off accidentally in the growth chamber. This triggered suffocation of the plants, and they could not recover; the experiment again had to be terminated. New planting began on the 9th of November 2015. Water stress was applied on the 3rd of December 2015, data was collected and the experiment was terminated on the 16th of December 2015.

At the beginning of the year 2015, screening for water stress resistance continued, and planting of the second 30 genotypes out of 60 commenced on the 21st of January 2016. The experiment was terminated on the 23rd of February 2016, and data was collected. The results obtained from sets one and two were evaluated by planting all 60 genotypes at once. The next planting, which was to evaluate plants, started on the 25th of February 2016, and data was collected. The experiment was terminated on the 29th of March 2016. For every round of planting, a new RHS had to be reconstructed. Evaluation of 60 genotypes was followed by selection of the five best genotypes, which were than planted in replicates.

The five best selected genotypes were planted on the 9th of May 2016. Unfortunately, the experiment was terminated in two weeks' time because some of the seeds had been treated with Poncho (seed chemical) while others had not been treated. The treatment caused variation in growth. On the 19th of May 2016, the next planting began using untreated seeds only. Reconstruction of three RHSs commenced, and seeds were planted in replicates. The RHSs carried five genotypes, each randomly assigned to three replicates per genotype. After obtaining unsatisfying results, planting again commenced on the 9th of August 2016.

Two weeks after planting, inexplicable yellowing of the plants resulted in termination of the experiment. This could have been caused by a continuous day-and-night supply of LED light in the growth chamber. This was an artificial incident whereby LED light was switched from auto to manual (provided continuous light throughout the night for three weeks) and/or insufficient electronic conductivity of a nutrient solution used. The experiment was repeated. Planting began on the 22nd of August 2016, data was collected and the experiment was terminated on the 14th of November 2016. Each RHS consisted of 15 PVC pipes; five seeds were planted per PVC pipe, and ultimately 75 seeds were planted per RHS. All seeds were fully germinated at Zadoks' Stage 10.

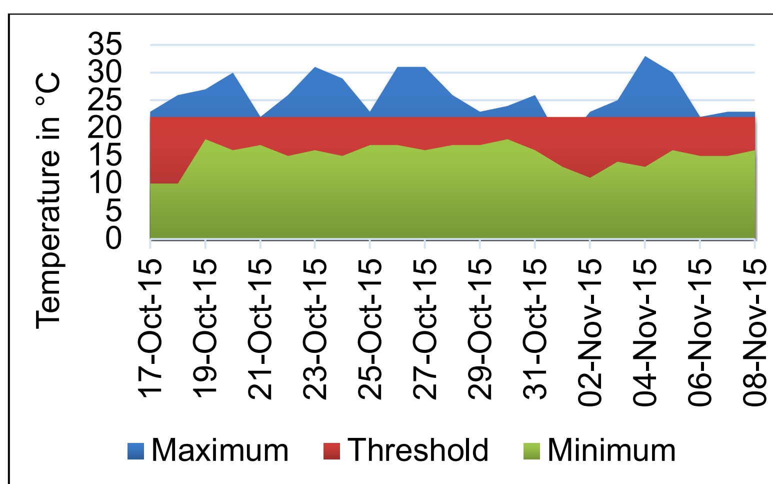

Figure 3.4: Temperatures recorded during planting

Source: Time and date.com (2017).

Plants of equal length were selected and thinned down to one plant per PVC pipe. This allowed plants of equal height to continue to grow. Thinning of plants was done at Zadoks, 13 stages after plants establishment (for all conducted experiments).

3.2.3.12 Growth conditions

Experiments were conducted in a controlled environment at SU's Welgevallen Experimental Farm. Both greenhouses and the growth chamber were used for planting. The growth chamber's minimum and maximum temperatures were 18 °C to 22 °C. The greenhouse temperatures ranged from 10 °C to 25 ºC. The greenhouse temperatures were not stable since they increased with increasing daytime temperatures. LED light was used in the growth chamber at pulse width modulation (PWM) dimmer 13 (maximum) set up as a long day length. The external fresh air supplier was always switched on to ensure a constant fresh air supply for the plants.

3.2.3.13 Irrigation

A standard nutrient solution was used for irrigation with electrical conductivity of 2.5 dS/m. The nutrient composition of the irrigation solution was Microflex GA chelated trace elements supplied by Yara, distributed by Farmisco (Pty) Ltd. Irrigation was carried out by hand using a watering can from sowing to emergence. After germination (Zadoks, 10), the irrigation system was set to irrigate automatically. The ARTs on the floor were fully filled with the standard nutrient solution. Timers were set to irrigate four times a day. Silica sand was used as growth medium. Due to its rapid-drying

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properties, the irrigating time interval was set at 07:00, 11:00, 15:00 and 18:00 daily. Irrigation in the morning gave plants water to begin the day, followed by irrigation 11:00 to wet the soil due to the rapid-drying properties of silica sand. Irrigation again took place at 15:00 during the hottest time of the day and later in the day at 18:00 to provide plant water balance by compensating water lost through evaporation during the day.

The sub-irrigated gravel culture RHS allowed plants to be supported by their own roots. Submersible pumps were used to pump the nutrient solution from the ARTs on the floor into the ARTs with plants on the benches. The time required to fill up the ARTs was calibrated. Each ART was set up to be irrigated based on the allocated time. When the ARTs on top of the benches were filled up, the submersible pump stopped. The nutrient solution immediately drained back into the reservoir or the ARTs placed on the floor. Two Emjay filters prohibited foreign material from entering the ARTs on the floor during drainage. A standard nutrient solution was used for irrigation in the nurseries. Watering cans were used for irrigation from planting to emergence, followed by a drip irrigation system until harvesting. The electric conductivity of the standard nutrient solution was 2.5, and it was increased to 4.0 at the grain filling stage.

3.2.3.14 Application of water stress

Water stress was induced by withholding nutrient solution supply. Control genotypes received optimum nutrient solution supply throughout the growing period. The flow of the nutrient solution was suspended by cutting off the power supply at the source. The nutrient solution was withheld for 14 days, from the tillering stage (shoot and one tiller, (Zadoks, 21) to stem elongation (fourth node detectable, Zadoks, 34). After 14 days of water stress, the experiment was terminated and the plants were harvested, followed by data collection.

3.2.3.15 Selection of the genotypes

Simple statistical analysis using Microsoft excel (2013) was performed to select the genotypes for the traits studied. Mean values were used to distinguish between the upper and lower limit. Mean values of traits that fell below the lower limit were excluded. Mean values of traits that fell above the upper limit were used to rank the genotypes. Genotypes that ranked the highest and integrated more traits were selected. The selected genotypes were advanced to the next screening.

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3.2.4 Reticulated hydroponic system

An RHS was used for planting. Seeds were planted in blue PVC pipes (Figure 3.5A), followed by installing the irrigation system (Figure 3.5B). A submersible pump was installed to ensure that the nutrient solution was pumped into the ART above and PVC ball valve to prevent the nutrient solution from returning to the ART (Figure 3.5C) and lastly, the ART with nutrient solution (Figure 3.5D).

Figure 3.5: Reticulated hydroponic system used. A-ART with plants, B-Complete set of RHS with irrigation system installed, C-Submersible pump for pumping up nutrient solution, D-ART fully-filled with nutrient solution.

Timers were used to calibrate irrigation time. Irrigation system is described as follows. A: complete RHS with wheat plants. B: Fully installed RHS together with irrigation system. C: Shows PVC ball valve and submersible pump setting. D: position of lower ART elevated with nutrient solution for irrigation to support the pump.

3.2.5 Experimental design

The experiment was set up as a completely randomised two-way factorial design. Two factors were investigated: water levels and variety. Water treatment consisted of withholding nutrient solution and allowing continuous flow of nutrient solution. The

experiment comprised 60 wheat genotypes reduced to five, namely 15HYLD-22, 15HYLD-26, 15HYLD-29, 15HYLD-30 and 15HYLD-18, randomly assigned to three RHSs and replicated three times on each system.

3.2.6 Data collection and statistical analysis

The data collected was analysed using Agrobase Generation II® Agromix software (2008-2011). The means were separated by least significant difference at 5%.

3.2.7 Weather data

The historical weather data for Welgevallen Experimental Farm was obtained from the Department of Agriculture in the Western Cape.

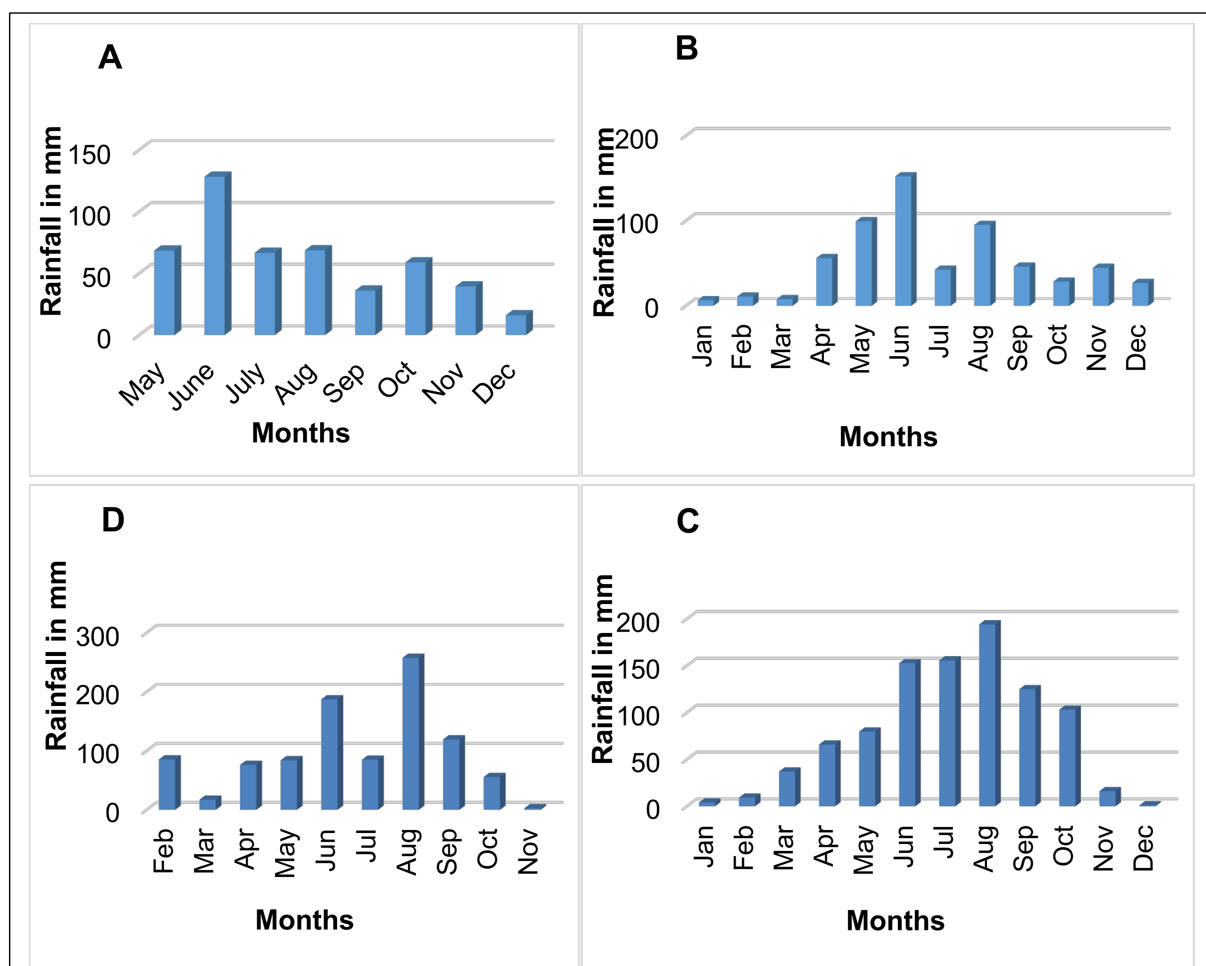

Figure 3.6: Historical weather data from Welgevallen Experimental Farm. The figure shows monthly average rainfall pattern from year 2010 to 2013. A- March to December, B and C- January to December and D-February to November.

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Investigating water stress resistance at the initial stages of plant growth is justified by empirical evidence of the effects of minimal water supply during the early stages of plant growth. The present study entailed screening of wheat genotypes. Temperatures of about 10 °C to 20 °C permitted optimum germination of this crop (Buriro *et al.*, 2011). Consequently, planting in April and May at the latest provided favourable conditions for germination and emergence.

However, favourable temperatures for this cool-season crop coincide with minimal water supply at the initial stages of growth, specifically in the Western Cape. Less rainfall is received during April and May compared to June (Figure 3.6). Furthermore, the most recent drought conditions affected the whole of SA, particularly the Western Cape.The annual rainfall pattern for the Welgevallen Experimental Farm fluctuated over the past decades.

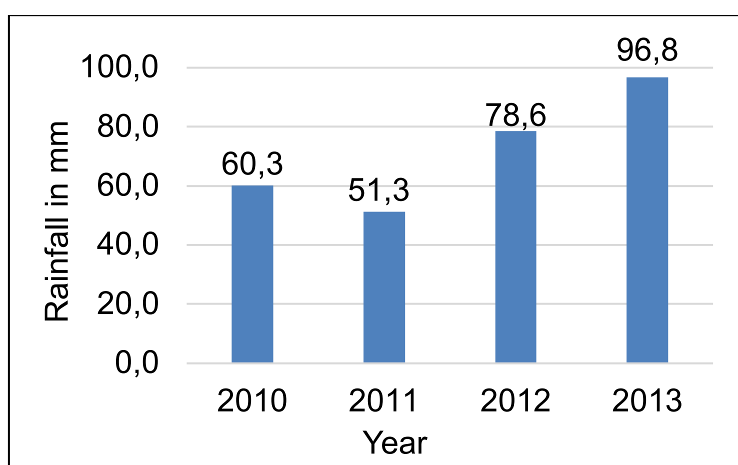

Figure 3.7: Average annual rainfall pattern from year 2010 to 2013. Recorded annual rainfall distribution in Welgevallen experimental farm at Stellenbosch University from year 2010 to 2013.

Average annual rainfall pattern indicated in (Figure 3.7) clearly shows a change in rainfall from one year to the next. The rainfall decreased from 2010 to 2011 by 9 mm and increased sharply from 2011 to 2013 (Figure 3.7). It is unfortunate that the data for 2014 to 2018 is not available for Welgevallen Experimental Farm from the weather station for more detailed assessment of the historical rainfall pattern of the farm.

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CHAPTER 4: RESULTS AND DISCUSSION

4.1 Wheat molecular characterisation

4.1.1 Molecular marker screening and validation

Molecular diversity assessment of wheat lines linked to rust resistance genes such as leaf rust (*Lr34*), stem rust (*Sr2*) and number of markers in a multiplex reaction was carried out. Corresponding identified markers for rust resistance genes were subjected to PCR multiplex analysis, thereby optimising and validating these markers.

4.1.1.1 Lr34 *markers*

Molecular markers specific for *Lr34* included *L34DINT9-F* and *LR34PLUS-R,* followed by a codominant marker and a negative marker (*CSLV*) to assist in identifying *Lr34*. The PCR reactions were performed for the male and F1 1:1 female segregating populations.

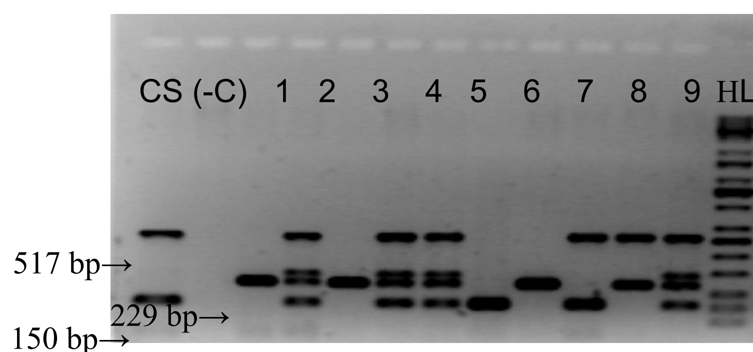

Figure 4.1: Gel electrophoresis UV image for optimisation and validation of *Lr34* resistance and codominant marker

The gel electrophoresis shown in Figure 4.1 is described as follows: HL – hyper ladder II; gel lanes 1 to 10 genotypes were screened for the presence of *Lr34* markers. Chinese spring (CS) was used as positive control, and negative control (-C) was deionised water (d.H2O). Band amplification of 517 bp indicated the presence of *Lr34* genes, followed by 229 bp, which is a negative marker for absence of *LR34* and by 150 bp, which is a codominant marker assisting in identifying the presence of the *Lr34* marker. These results were observed during screening for the *Lr34* marker for rust resistance genes. Chinese spring 517 pb was partially visible but present, and 150 bp

was clearly visible and no contamination was observed in negative control. Markers were successful in distinguishing between *Lr34* resistance and susceptible genotypes.

4.1.1.2 Sr2 *markers*

Molecular screening for the *Sr2* CAPS marker used were *csSr2F* and *csSr2R.* The PCR reactions were performed for male and F1 1:1 female segregating populations (figure 4.2 and 4.3).

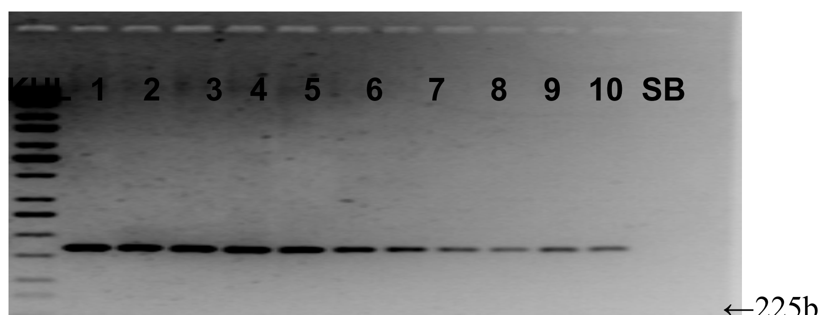

Figure 4.2: Gel electrophoresis UV image for optimisation and validation of *Sr2*

Two markers (*csSr2F* and *csSr2R*) were postulated to carry stem rust resistance genes. The KUL-Kappa ladder was used for *Sr2* optimisation and validation. The genotypes screened indicated in lanes 1 to10 showed a band size of 225 bp. Steenbras (SB) was the positive control, and the negative control (-C) was deionised water (d.H2O). The UV imaging system showed the presence of a band size of 225 bp, indicating the possibility of *Sr2* resistance genes being present. Genotypes indicating the bands were selected, and then the enzyme digestion step followed. Addition of 2.5 µl of PagI enzyme to the selected tubes and incubation at 35 ºC for an hour followed. The samples were re-run for gel electrophoresis, and UV imaging system-

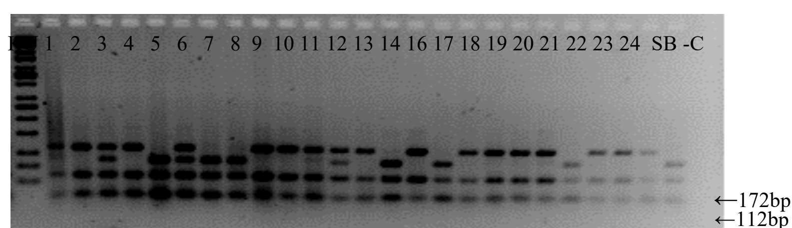

Figure 4.3: Gel electrophoresis UV image for optimisation and validation of *Sr2*

showed (Figure 4.3). After UV image optimisation and validation of *Sr2* markers, the PagI enzyme digestion step followed to confirm the presence of the marker. The KUL-

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Kappa ladder was used for band sizes. Genotypes studied ranged from 1 to 24, followed by Steenbras (SB) for positive control and negative control (-C) through deionised water. The presence of the *Sr2* markers was successfully identified with band sizes of 112 bp and 172 pb. Negative genotypes for *Sr2* showed the presence of band sizes of 112 bp and 225 bp.

4.1.2 Marker-assisted selection screening

The F1 1:1 segregating population and donor lines were tested for the presence of *Lr34* (*L34DINT9-F* and *LR34PLUS-R*) markers with the help of *Cslv* markers in a multiplex reaction, and the *Sr2* CAPS (*csSr2F* and *csSr2R*) were also tested for both male and female populations in an anon-multiplex reaction. Another multiplex reaction was also performed for several panel of markers, including *Sr31* (*Iag95-F* and *Iag95-R*), *Lr34* (*L34DINT9-F* and *LR34PLUS-R*), *Lr24/Sr24* (*719-F and 719-R*), *Lr37/Sr38/Yr17* (*Vent* and *Ln2*), *Sr26* (*Sr26#43-F* and *Sr26#43-R*) and *Lr19* (*12C-F* and *12C-R*) to test both the male and F11:1 female segregating populations. Only two sets of markers were not included in the donor lines PCR reaction: *Sr26* (*Sr26#43-F* and *Sr26#43-R*) and *Sr31* (*Iag95-F* and *Iag95-R*).

Allele frequencies were calculated using the Hardy-Weinberg equation. All the markers screened were present in the population, although to a different extent. The highest allele frequency in a population for MS-MARS Cycle 1 was observed from the *Lr/Sr24* (96,9%) marker, and the lowest was *Lr19* (1%). Allele frequencies of *Lr34* (61%), *Sr31* (44%), *Lr24* (96%), *Lr37* (19%), *Sr26* (3%), *Lr19* (1%) and *Sr2* (38%) (Figure 4.4) were recorded for MS-MARS Cycle 1 for the F1 1:1 female segregating population. For MS-MARS Cycle 2 screening, the F1 1:1 female segregating population consisted of two molecular markers: *Lr34* and *Sr2* CAPS. There was an increase in allele frequency in the second MS-MARS cycle for both markers. *Lr*34 for MS-MARS Cycle 2 was 67%, and *Sr*2 was 57%. There was a 5% increase in *Lr34* allele frequency from 2015 (MS-MARS Cycle1) to 2017 (MS-MARS Cycle 2). There was an 18% increase in *Sr2* allele frequency from 2015 to 2017 (MS-MARS cycles 1 and 2, respectively) (Figure 4.5).

An increase in allele frequency was also observed by Springfield (2014) in the F1 1:1 female segregating population in MS-MARS cycles 1 and 2. Interestingly, an increase in allele frequency equivalent to that in the current study was observed by Springfield

(2014). *Lr34* was observed to increase by 8% and *Sr2* by 11% in MS-MARS cycles 1 and 2. Springfield (2014) estimated a significant increase in *Sr2* allele frequency in the F1 1:1 female segregating population, which was observed in the current study.

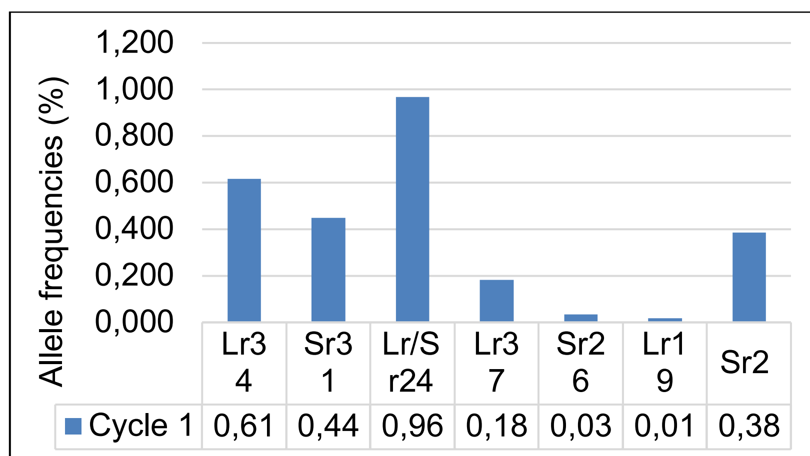

Figure 4.4: MS-MARS Cycle 1 allele frequency for rust resistance markers from female population

There is thus evidence of a continuous increase in allele frequency in every MS-MARS cycle. Low genetic frequencies in MS-MARS Cycle 1, specifically *Lr19*, *Sr26* and *Lr37*, are expected to increase in the following series of MS-MARS cycles. Evidence is emerging of marker *Sr26* in the population, which was reported absent in the female F1 segregating population (Springfield, 2014). Recurrent selection requires at least four cycles to improve low desirable allele frequencies in a population from 5% to 70% (Marais & Botes, 2009). Springfield (2014) concluded that absence of *Sr26* and *Sr45* markers occurred because it had been introduced recently into a population.

Furthermore, due to random seed selection to produce 2% of the plants from a 100% produced seed, absence of the desired marker can only be 2% true. The markers *Sr2*, *Lr24*, *Lr34*, *Lr37* and *Lr19* were used in MS-MARS Cycle 1 for screening the males for rust resistance genes. Very low allele frequencies were observed for donor lines. The minimum and maximum allele frequency observed was *Sr2* (9%) and *Lr24* (41%), respectively. Other allele frequencies observed were *Lr34* (18%) and *Lr37* (30%) (Figure 4.6). Marker *Lr19* was not visible in the male population. The frequency of *Lr34* was two times lower (43%) than the allele frequency reported by Springfield (2014). The frequencies of *Sr2* and *Sr24* were half the allele frequencies reported by Springfield (2014) of 19% and 46%, respectively.

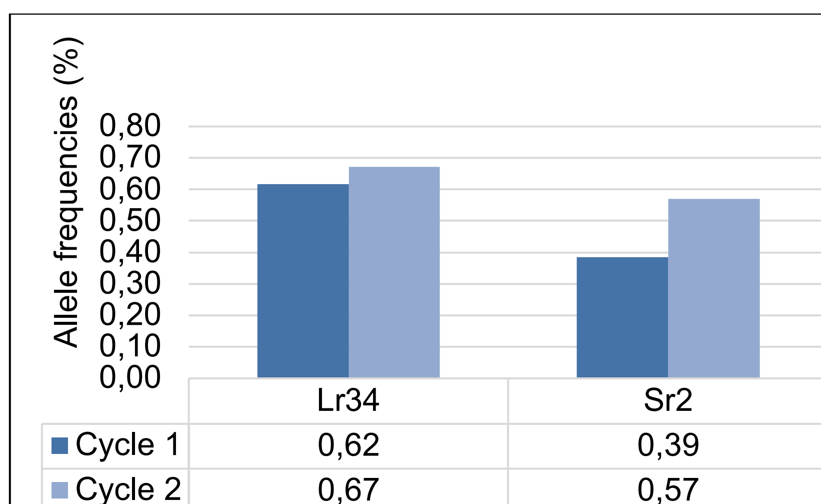

Figure 4.5: MS-MARS cycles 1 and 2 female population allele frequency comparison

The allele frequencies of marker *Lr37* was 64% lower than reported by Smit (2013). The significant differences in the allele frequencies were the result of the genotypes used. Some of the genotypes were externally sourced and combined with wheat nursery genotypes from the SU-PBL. As mentioned, allele frequencies are expected to increase with recurrent selection cycles, and the absence of the *Lr19* marker does not necessarily mean that it is not present in the population.

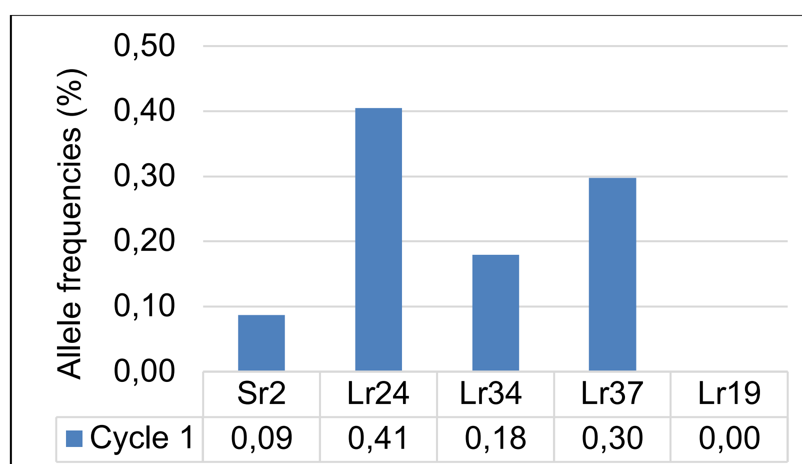

Figure 4.6: MS-MARS Cycle 1 allele frequencies for rust resistance markers from 60 male genotypes

MS-MARS Cycle 2 screening of rust resistance genes from donors involved screening of five selected genotypes used for cross-pollination in the nurseries. Two molecular markers of interest were utilised for screening: *Lr34* and *Sr2*. The allele frequencies

observed for *Lr34* and *Sr2* were 27% and 33%, respectively (Figure 4.7). An increase in allele frequency from 2015 (MS-MARS Cycle I) to 2017 (MS-MARS Cycle 2) was again observed from the donor lines. *Sr2* was increased by 24% and *Lr34* by 9%. Again, a significant increase in *Sr2* allele frequency was observed, similar to the female lines.

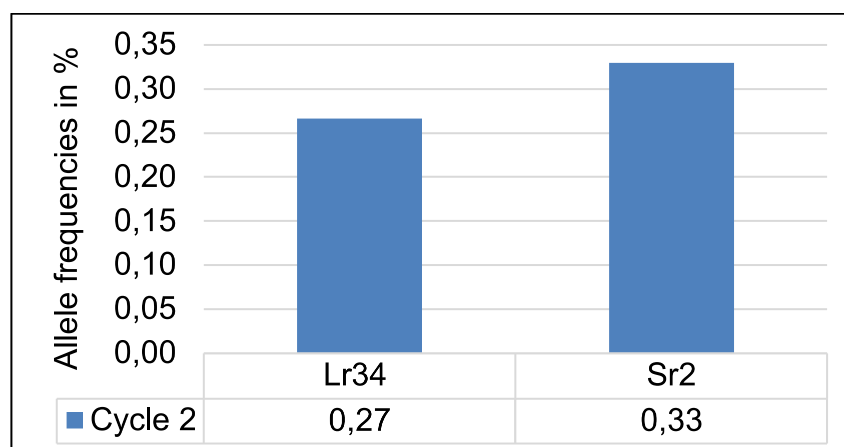

Figure 4.7: MS-MARS Cycle 2 allele frequencies for rust resistance markers from 60 male genotypes

This data further supports the findings and conclusions of Springfield (2014) about an expected significant increase in *Sr2* allele frequency. According to Springfield (2014), the effectiveness of recurrent selection can be observed through a significant increase of allele frequency in the population. Higher allele frequencies in the population can play a significant role in variety development for disease resistance in MS-MARS breeding programmes. Furthermore, pyramiding these genes with higher allele frequencies can provide broad-spectrum resistance. After molecular characterisation of wheat lines using MAS in the greenhouse, plants were allowed to grow. This was followed by selection of male sterile and donor lines to make cross-pollinations in the growth chamber using the MS-MARS cycles.

4.2 MS-MARS breeding technique validation

4.2.1 MS-MARS Cycle 1

A total of 1 437 male fertile plants and a total of 1 451 male sterile plants were cut, leading to 303 085 possible combinations (Table 4.1). At harvest, 8 616 seeds were obtained, weighing about 295 g.

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Table 4.1: MS-MARS Cycle 1 year 2015

Week	Male fertile	Male sterile	Possible combination	Harvest	Sterile plants	Harvested seeds	Grain mass (g)
1	59	66	3 894	1	229	1 394	48
2	172	179	30 788				
3	192	231	44 352	2	444	2 704	93
4	261	266	69 426				
5	222	263	58 386	3	452	2 752	94
6	235	183	43 005				
7	283	211	50 218	4	290	1 766	60
8	58	52	3 016				
Total	**1 437**	**1 451**	**303 085**		**1 415**	**8 616**	**295**

MS-MARS Cycle 1 was statistically analysed using the chi-square test to determine male sterility genetic inheritance for the F1 1:1 segregating population. A good probability to fit a 1:1 ratio was observed from all the tables (Table 4.2), indicating nonadditive genetic epistasis. Therefore, it could be concluded that only a single dominant gene controlled male sterility in the heterozygous state in MS-MARS Cycle 1. The second MS-MARS cycle was also conducted in Greenhouse 3. Greenhouse 3 contained four benches; benches 1 and 2 contained donor lines, and benches 3 and 4 contained the F1 1:1 female segregating population. Benches 1 and 2 accommodated 64 and 110 donor lines, respectively. Benches 3 and 4 accommodated 110 plants of the F1 1:1 female segregating population. Four seeds were planted in each pot. Molecular characterisation took place prior to cutting tillers.

Table 4.2: MS-MARS Cycle 1 probability to fit 1:1 ratio

Table number	Sterile	Fertile	X^2	Probability to fit 1:1 ratio
1	213	207	0,085	0,77
2	219	213	0,085	0,77
3	228	209	0,83	1,2
4	242	215	1,59	0,5
Total	**902**	**844**	**1,93**	**0,16**

4.2.2 MS-MARS Cycle 2

Cuttings took three consecutive weeks. A total of 254 and 165 cuttings were made from the donor lines and male sterile females, respectively. This gave about 7 117 possible combinations for MS-MARS Cycle two (Table 4.3). The total number of harvested seeds from MS-MARS Cycle 2 was 759 and weighed 4,300 g. The harvested seeds were very shrivelled and shrunken. This indicated success of the cross-pollinations. A similar observation was reported by Springfield (2014). However, due to higher temperatures during the anthesis and grain-filling stages from December 2016 to January 2017 (Figure 4.8), MS-MARS Cycle 2 did not do well (Time and date, 2018). Maximum temperatures were above 23 °C throughout the reproductive stage. Most of the tillers did not produce seeds. Heat stress resulted in less seed production. Khatun *et al*. (2016) reported a shortened grain-filling stage as a result of failure of starch conversion caused by higher temperatures.

Environmental stress such as heat stress was found to affect fertility and development of floral organs (Smith & Zhao, 2016). In addition, the failure of the LED lighting in the growing rooms for three weeks may have resulted in poor seed formation and development during cross-pollination. This resulted in a massive decline in the number of selected tillers for cross-pollination in MS-MARS Cycle 1 compared to MS-MARS Cycle 2. No seeds were obtained from the last crosses made, and this could be the reason for not obtaining any seeds. Hence, probability to fit 1:1 ratio was not calculated for MS-MARS Cycle 2.

Table 4.3: MS-MARS Cycle 2 year 2017

Week	Male fertile	Male sterile	Possible combination	Harvest	Sterile plants sourced	Harvested seeds	Grain mass (g)
1	27	17	459	1	37	131	0,67
2	64	20	1 280			144	0,86
3	51	28	1 428	2	47	161	0,93
4	56	43	2 408			200	1,25
5	30	15	450	3	20	123	0,59
6	26	42	1 092			0	0
Total	**254**	**165**	**7 117**		**104**	**759**	**4,300**

Seed material produced during MS-MARS was used for cross-pollination with selected phenotypically screened genotypes from the greenhouse for water stress resistance.

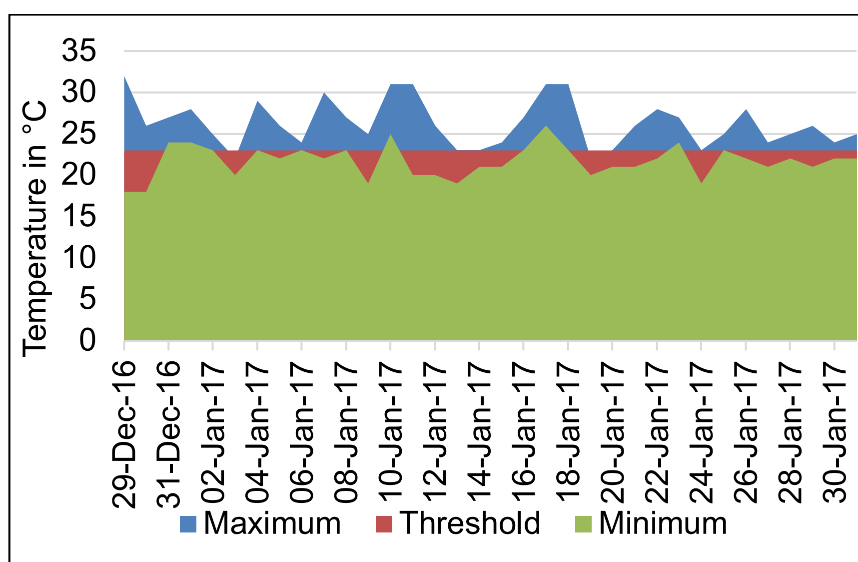


Figure 4.8: Temperatures recorded during the reproductive stage

Source: Time and date.com (2018).

4.3 Phenotypic traits

The five best performing genotypes were selected based on their ranking (R) among the 60 genotypes studied (Table 4.4). After statistical analysis, the top five genotypes were considered. Selection was based on the genotypes that integrated more traits, and these were then selected. Prior to inducing water stress, the plants' DW components were sampled and analysed to closely examine anticipated changes in DW. As anticipated, statistical analysis exhibited no significant differences ($p > 0,05$) in ILDW, ISDW, IRDW and TPIDW (Table 4.5).

Table 4.4: Selected genotypes based on their ranking

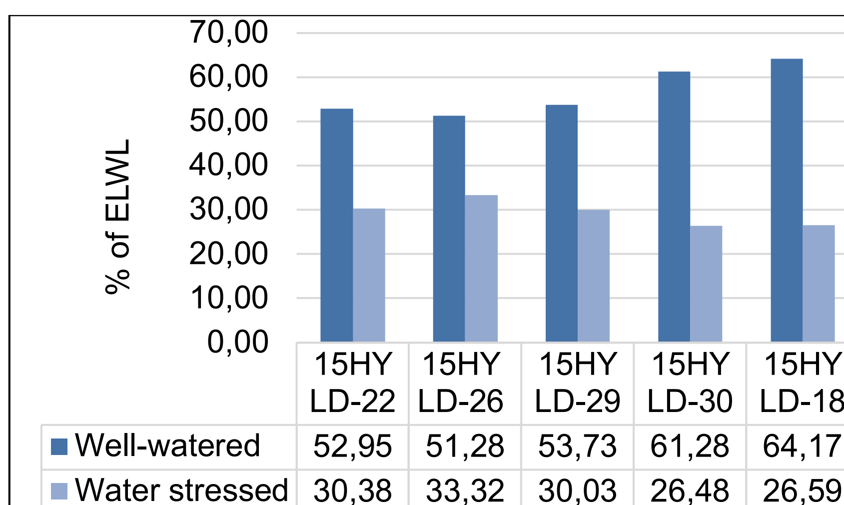
Genotypes	NT	R	NL	R	RL	R	FW	R	DW	R	LRWC	R
15HYLD-18	10	3	9	13	24	31	8,9	3	1,9	3	78,7	10
15HYLD-22	9	6	10	12	24	32	9.5	2	1.9	4	80	6
15HYLD-26	8	10	5	41	32	1	5	14	1,1	21	78	13
15HYLD-29	11	1	7	25	27	8	8,8	4	2	2	77,3	19
15HYLD-30	2	40	5	43	24	35	1,1	58	0,41	49	44,3	56

This implies that at the beginning of water stress, there were no significant differences amongst the plants since they were grown under the same environmental conditions with a continuous supply of nutrient solution.

The statistical analysis and mean comparison for target traits are indicated in tables 4.6, 4.7, 4.8 and 4.9. The tables show the means of the genotypes and the reduction percentages between water-stressed and well-watered conditions. Important values such as covariance (CV), R-square and grand mean were recorded.

4.3.1 Excised leaf water loss

Water stress treatment significantly ($p \leq 0.0000$) influenced ELWL. The differences among the varieties did not reach the level of statistical significant. Treatments combined and the environment (water stress and genotypes) did not cause any significant differences in ELWL. The percentage of decrease was very high (-48.28%) for ELWL (Table 4.6). The percentages calculated for ELWL ranged from 26.48% to 64.17% (Figure 4.9). The highest value observed was in the control genotype 15HYLD-18 (64.17%) and the lowest in 15HYLD-26 (51.28%).



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	52,95	51,28	53,73	61,28	64,17
Water stressed	30,38	33,32	30,03	26,48	26,59

Figure 4.9: ELWL of five wheat genotypes grown under water-stressed and well-watered conditions

Water-stressed genotypes showed a maximum loss of water content of 33.32% in 15HYLD-26 and minimal loss of water of 26.48% in 15HYLD-30 (Figure 4.9). Lugojan and Ciulca (2011) obtained results that ranged from 54.16% to 84.09% while screening winter wheat inbred lines and hybrid varieties for ELWL. A slightly different range of results (2.05% to 41.33%) was recorded by Dabiry *et al*. (2015) while screening wheat genotypes under water-stressed and well-watered conditions. The results of the current study overlap with and fall between the ranges of these two studies. A higher percentage of water loss was recorded for the controls 15HYLD-18

and 15HYLD-30, indicating a reduced rate of stomatal closure compared to their corresponding water-stressed genotypes (Kaur *et al*., 2016). This resulted in increased water loss through transpiration and evaporation. These two genotypes showed better water stress resistance under water stress conditions.

The rate of ELWL observed from the genotypes under different water treatments can be explained by the rate of stomatal closure and cuticle ability to lose water under stress conditions. Similar results observed by Kaur *et al*. (2016) showed severe water loss from excised leaves under water and heat stress treatments. Water loss caused by drought was recorded as the highest amongst all the stresses after stress revival (Kaur *et al*., 2016). According to Lugojan & Ciulca (2011), cuticle transpiration rate is one of the important traits that can be utilised for characterisation of wheat lines.

Table 4.5: ANOVA and mean comparison of DW measurements prior to instigation of water stress

Treatment	**Trait**			
	ILDW	ISDW	IRDW	TPIDW
Genotypes				
15HYLD-22	0,18	0,07	0,05	0,30
15HYLD-26	0,16	0,06	0,05	0,27
15HYLD-29	0.17	0,06	0,05	0,28
15HYLD-30	0,17	0,06	0,05	0,27
15HYLD-18	0,19	0,07	0,05	0,31
Block	ns*	ns*	ns*	ns*
Variety	ns*	ns*	ns*	ns*
CV (%)	16,37	29,78	13,97	13,98
Grand mean	0,176	0,062	0,049	0,287
R-square	0,2101	0,0752	0,2241	0,2174

ns – nonsignificant at 5% probability. Means of the same letters show no significant difference from each other.

Cuticle transpiration rate can be estimated through differences among the varieties for water stress resistance breeding. ELWL is an important trait to screen for water stress resistance, and it is linked to the ability of the plant to supply water to the leaves and the rate of transpiration (Kaur *et al*., 2016). The ability of the plant to supply water to

the leaves at a minimal transpiration rate enables the plant to sustain the water balance in the leaves, thereby producing considerable yield under water stress conditions. Dabiry *et al*. (2015) reported a negative correlation coefficient for ELWL and grain yield under well-watered and water-stressed conditions of r = -0.526 and r = -0.723, respectively.

Table 4.6: ANOVA and mean comparison of treatment effects

Treatment	Trait						
	ELWL %	LRWC %	CCI	g_s, $mmlm^{-2}s^{1}$	RGR gg^{-1} d^{-1}	LEA m^2	NT
Water levels							
Well-watered	56,68	85,13	13,95	491,35	0,12	12,33	9,33
Water stressed	29,36	74,60	10,49	431,47	0,10	10,46	9,07
% of decrease	-48,20	-12,37	-24,80	-12,19	-16,67	-15,17	-6,87
Genotypes							
15HYLD-22	41,66	78,94	10,00	440,77	0,11	11,57	10,50
15HYLD-26	42,30	76,99	9,10	458,06	0,10	10,12	8,67
15HYLD-29	41,88	78,15	11,37	485,78	0,12	12,25	9,33
15HYLD-30	43,88	85,63	11,97	474,18	0,10	11,76	8,83
15HYLD-18	45,38	79,62	18,68	448,28	0,12	11,28	8,67
Genotypes	ns*	ns*	**	ns*	**	**	**
Water levels	**	**	**	**	**	**	ns*
Interaction	ns*	ns*	**	ns*	**	**	**
CV (%)	15,74	7,86	11,00	12,79	8,47	11,39	9,90
Grand mean	43,02	79,86	12,223	461,41	0,109	10,95	9,200
R-square	0,880	0,7031	0,9358	0,5302	0,827	0,753	0,7695

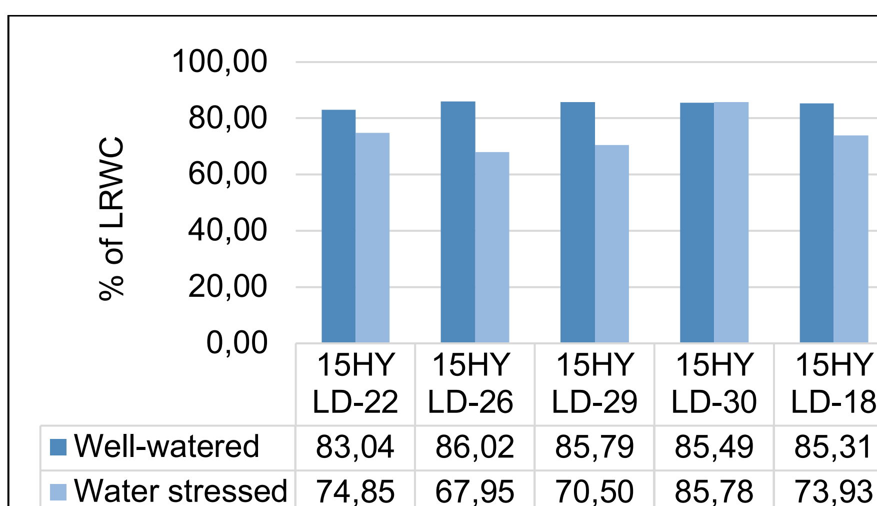
Significance level at 5% probability. ns* – nonsignificant and ** – significant.

However, a positive correlation coefficient (PCC) was also reported between ELWL and LRWC under both well-watered (r = 0.311) and water-stressed (r = 0.395) conditions. Higher percentages of broad-sense heritability (H^2) were recorded and ranged from 89.36% to 96.70% for ELWL in wheat (Chandra & Islam, 2003). Naeem *et al*. (2015) reported a higher H^2 estimate of 94.4% in wheat under water stress

conditions. Since ELWL is associated with g_s, QTLs have been mapped for g_s in chromosome locations 1A, 1B, 2B, and 7A (Shahinnia *et al*., 2016). The different responses from the genotypes under investigation for ELWL provide sufficient choice of selection of water stress-resistant plants. This is crucial for variety improvement in plant breeding programmes.

4.3.2 Leaf relative water content

ANOVA showed significant differences ($p \leq 0.0002$) for LRWC. The interaction of the treatments (water stress and the genotypes) and the environment did not have any significant effect on LRWC. The percentage of decrease for LRWC was -12.37%, which was relatively lower compared to other traits. The highest value for LRWC observed was from the control genotype 15HYLD-26 (86.03%), and the minimum value of 67.95% was for the water-stressed genotype (Figure 4.10). Hence, the values for LRWC ranged from 86.03% to 67.95% for the studied genotypes. Similar results were recorded in wheat genotypes by Munjal & Dhanda (2016), ranging from 69.20% to 91.60% in morpho-physiological traits assessment under both well-watered and drought conditions.



	15HYLD-22	15HYLD-26	15HYLD-29	15HYLD-30	15HYLD-18
Well-watered	83,04	86,02	85,79	85,49	85,31
Water stressed	74,85	67,95	70,50	85,78	73,93

Figure 4.10: LRWC of five wheat genotypes grown under water-stressed and well-watered conditions

Genotype 15HYLD-30 showed higher LRWC of 85.78% under water stress conditions. Water-stressed genotypes showed decreased LRWC compared to control genotypes, except 15HYLD-30. Similar findings were reported by Khatoon *et al.* (2016) in wheat under water stress conditions. Big differences in LRWC between water stress-

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susceptible and -resistant genotypes of wheat were observed by Kraus *et al*. (1995). Varieties that maintain the optimum water balance provide a significant advantage of yield stability and/or improved yield (Lugojan & Ciulca, 2011). Genotype 15HYLD-30 tolerates water stress conditions better compared to all the studied genotypes. According to Khatoon *et al.* (2016), plants under optimum water conditions remain turgid and has 98% LRWC. Most plant species have about 60% to 70% LRWC for wilting and about 40% for extreme dehydration.

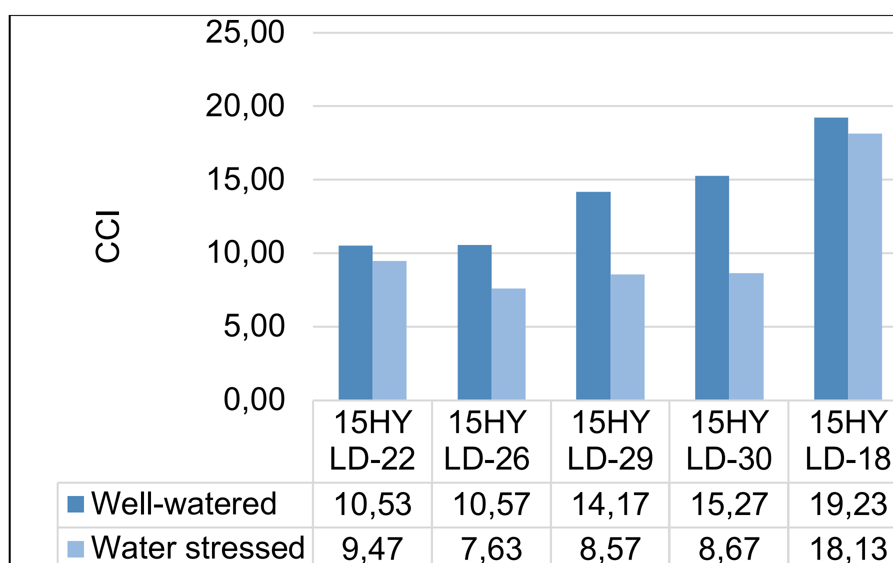
LRWC plays a significant role in indicating plant water status in relation to other aspects (Jongdee *et al*., 2002). LRWC was found to be closely linked to the balance between transpiration rate and plant available water signal (Khatoon *et al*., 2016). This offers an opportunity for a plant to recover from various abiotic stresses, thereby positively influencing yield returns. When LRWC is used for screening plants that are resistant to water stress, genotype 15HYLD-30 could be a good option. Munjal & Dhanda (2016) observed PCC (r = 0.48) between LRWC and grain yield under water-stressed conditions. Dabiry *et al*. (2015) reported PCC between LRWC and grain yield under both well-watered and water-stressed conditions of r = 0.628 and r = 0.403 respectively.

LRWC H^2 estimates were recorded to be 94.5% and 97.55% under well-watered and water-stressed conditions, respectively (Jatoi *et al*., 2012). Thus, plant breeding programmes can utilise LRWC for screening genotypes under water-limited conditions. Malik *et al*. (2015) utilised composite interval mapping to identify the QTLs associated with LRWC, cell membrane stability and photosynthesis. The study successfully mapped QTLs in chromosome location 2A, one each for LRWC and photosynthesis and two for cell membrane stability. This information may be useful in MAS for variety development under water stress conditions. LRWC is considered as an inexpensive and convenient trait to measure for screening purposes in water stress environments (Boutraa *et al*., 2010); therefore, it can be used as a selection criterion for screening.

4.3.3 Chlorophyll content index

ANOVA for CCI showed highly significant differences for water stress treatment ($p \leq 0.0000$), variety ($p \leq 0.0000$) and treatment combination ($p \leq 0.0049$). The percentage of decrease was -24.80%, relatively higher compared to most of the traits.

Lunagaria *et al*. (2015) conducted a study to evaluate the relationship between CCM-200 and SPAD chlorophyll meters. Richardson *et al*. (2002) found a linear relationship and a highly significant regression model between the two. Consequently, both devices' values can be interconverted to each other and into chlorophyll concentration in µg cm^{-2}. Hence, CCM-200 is a relevant tool that can be used. The average maximum value for CCM-200 was 19.23 and the average minimum was 7.63 from genotypes 15HYLD-18 and 15HYLD-26, respectively (Figure 4.11). Minimum reduction was observed from genotypes 15HYLD-22 and SST1755. The results obtained fall within the range of results recorded by Richardson *et al*. (2002) of 2 to 24 using a CCI-200 meter. Khalilzadeh *et al*. (2016) recorded the range of total Chl of 5 to 8 in wheat genotypes grown under water-limited conditions.



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	10,53	10,57	14,17	15,27	19,23
Water stressed	9,47	7,63	8,57	8,67	18,13

Figure 4.11: CCI of five wheat genotypes grown under water-stressed and well-watered conditions

Water stress significantly reduced the CCI of all the genotypes grown under water stress conditions. Reduced Chl was observed by Khalilzadeh *et al*. (2016) under water-limited conditions in wheat. According to Wang *et al*. (2009), any form of stress may result in damaged chloroplast and reduces Chl content and according to Khalilzadeh *et al*. (2016) reduced Chl content damages pigment photo-oxidation and, subsequently, reduced photosynthetic rate. According to Del Pozo *et al*. (2016), chlorophyll degradation caused by increased water stress eventually leads to increased leaf senescence, caused by reduced LAE and photosynthesis activity.

According to Jyothsna & Murthy (2016), leaf senescence is associated with Chl degradation; hence, potential delayed senescence was observed from the genotype 15HYLD-18 throughout the water stress experiments. Based on the empirical evidence, it can be concluded that genotype 15HYLD-18 has a stay-green character. Interestingly, genotype 15HYLD-18 showed exceptionally higher and surprising values of CCI for control and water-stressed genotypes of 19.28 and 18.13, respectively. This genotype indicates better water stress tolerance.

Talebi (2011) reported higher yield gained from genotypes with higher Chl under both irrigated and water stress environments. The Chl reduction was found to be much quicker in the water stress-susceptible than in the water stress resistant variety (Naeem *et al*., 2015). Therefore, it can be concluded that genotypes 15HYLD-26 and 15HYLD-29 are moderately susceptible to water stress. Chl was found to be associated with yield gain under stress and is regarded as one of the target traits for wheat crop improvement under water-limited conditions.

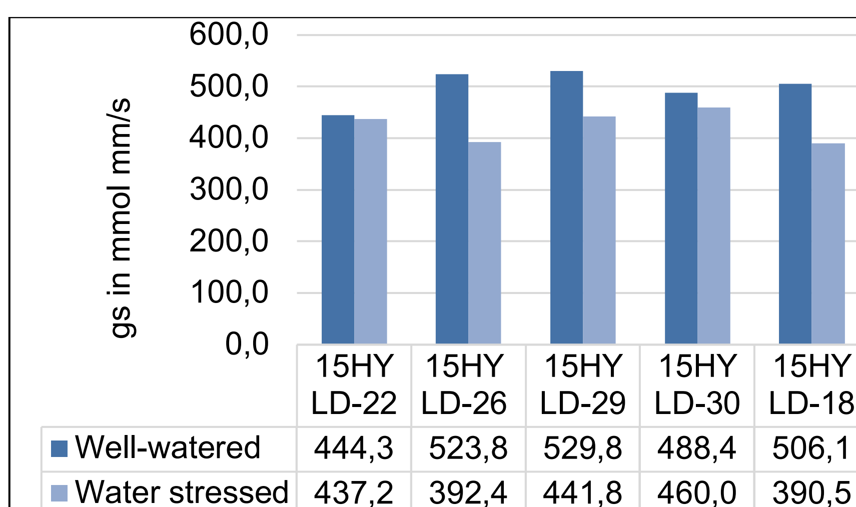
The Chl trait serves as a signal of water stress resistance; therefore, it should be considered as ae valuable tool for wheat screening for water stress. Chlorophyll concentration can be used to distinguish between susceptible and resistant wheat varieties under water stress conditions: the higher the concentration, the higher the resistance and vice versa.

PCC was reported by Fellahi *et al*. (2013) between Chl and grain yield (r = 0.601), harvest index (r = 0.561), thousand-kernel weight (r = 0.468), spikes per plant (r = 0.883), spike length (r = 0.301) and PH (r = 0.331). Al-Tahir (2014) reported PCC between Chl and LAE (r = 0.739), FW (r = 0.299) and grain yield (r = 0.567). Weak PCC (r = 0.223) was also recorded for specific CCI (Fellahi *et al*., 2013). H^2 of 60.44% was recorded for Chl in bread wheat. Lambrides *et al*. (2004) also recorded higher narrow-sense heritability and H^2 estimates for Chl of 0.82 and 0.80, respectively. According to Talebi (2011), higher Chl has a PCC with high yield under water stress conditions; therefore, more yield gains are expected from genotypes with higher CCI. Panio *et al*. (2013) mapped QTLs associated with Chl in chromosome locations 1B, 3B (2 QTL) and 6B. Chl QTLs were identified utilising different SRAP primers in

chromosome locations 1D, 2A, 2B, 3A and 7A under water stress conditions (Elshafei *et al*., 2013).

4.3.4 Stomatal conductance

ANOVA for g_s revealed significant differences ($p \leq 0.0124$) for water stress. No significant differences were recorded for the genotypes and treatment combination. The decrease percentage was recorded to be -12.19%. The average minimum and maximum g_s values observed ranged from 390.5 mmol m^{-2} s^{-1} for genotype 15HYLD-18 to 529.8 mmol m^{-2} s^{-1} for genotype 15HYLD-26, respectively (see Figure 4.12 below). Similar average minimum values of 416.4 mmol m^{-2} s^{-1} and maximum of 511.2 mmol m^{-2} s^{-1} were recorded by Haworth *et al*. (2016) in wheat genotypes. Water stress resulted in reduction of g_s in the genotype studied; however, minimal reduction was observed in genotype 15HYLD-22, followed by 15HYLD-30.



	15HYLD-22	15HYLD-26	15HYLD-29	15HYLD-30	15HYLD-18
Well-watered	444,3	523,8	529,8	488,4	506,1
Water stressed	437,2	392,4	441,8	460,0	390,5

Figure 4.12: G_s of five wheat genotypes grown under water-stressed and well-watered conditions

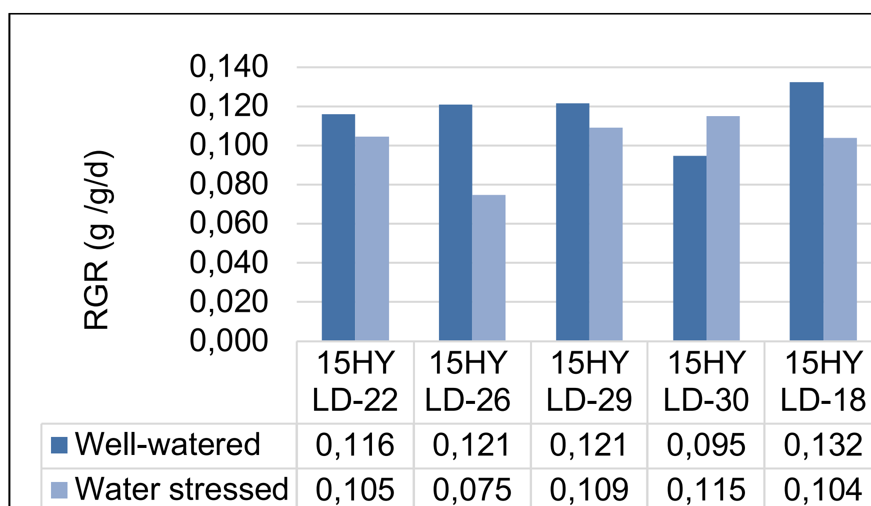
Under water stress conditions, the activity of the enzyme RuBisCO is reduced. According to Flexas & Medrano (2002), reduction of g_s lower than 100 mmol m^{-2} s^{-1} causes reduction of photosynthesis activity followed by reduced yield. The control genotype 15HYLD-29 showed the best g_s (529.8 mmol m^{-2} s^{-1}) of all the genotypes. Genotype 15HYLD-30 showed higher g_s of 460 mmol m^{-2} s^{-1} under water-stressed conditions. Extreme water deficit significantly reduces g_s (Khalilzadeh *et al*., 2016); hence, genotypes with higher g_s tolerate water stress better. Genotypes with increased reduction of g_s under severe water stress conditions may experience stomatal closure

followed by decreased CO_2 in the leaf, reduced ATP production caused by reduced RuBisCO activity, modified metabolism and reduced photosynthetic activity (Flexas & Medrano, 2002).

Persistence of water stress may lead to photochemical activity inhibition (photo-inhibition) and may result in loss of yield. G_s can be utilised as a tool in plant breeding for screening plants under water-limited conditions. QTLs for g_s have been identified in both field and greenhouse conditions in chromosome locations 1A, 1B, 2B and 7A (Shahinnia *et al*., 2016). Nonetheless, a few studies reported a negative correlation between g_s and yield (Shahinnia *et al*., 2016; Jatoi *et al*., 2012). A positive correlation of r = 0.79 was reported by Khakwani *et al*. (2012) for wheat genotypes under water stress conditions. H^2 estimates for g_s were reported by Jatoi *et al.* (2012) for both well-watered and water-stressed conditions to be 99.6% and 99.3% respectively at the anthesis stage in spring wheat.

4.3.5 Relative growth rate

ANOVA for RGR showed highly significant differences for water stress treatment ($p \leq 0.010$), variety ($p \leq 0.0002$) and treatment combination ($p \leq 0.0001$).



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	0,116	0,121	0,121	0,095	0,132
Water stressed	0,105	0,075	0,109	0,115	0,104

Figure 4.13: RGR of five wheat genotypes grown under water-stressed and well-watered conditions

The recorded percentage of decrease for RGR was -16.67%. An average RGR maximum value of 0.151 g g^{-1} d^{-1} was observed in the control genotype 15HYLD-18

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and an average minimum value of 0.075 g g^{-1} d^{-1} in the water-stressed genotype 15HYLD-26 (Figure 4.13).

Similar RGR results were obtained by Kahrizi *et al*. (2010), ranging from 0.058 g g^{-1} d^{-1} to 0.162 g g^{-1} d^{-1} in durum wheat. Water stress resulted in reduced RGR in four genotypes. Genotype 15HYLD-30 had a higher RGR in water stress treatment compared to the control genotypes. Similar results were observed by Saeidi *et al*. (2015) whereby water-stressed genotypes showed a higher value of harvest index compared to the corresponding control genotypes in wheat. This can be explained by additive genes. Upregulation of water stress resistance genes or genetic overexpression may have resulted in exponential growth of the genotype. Genetic differences in the genotypes studied may have resulted in significant differences in RGR. Genotype 15HYLD-26 showed moderate susceptibility to water due to higher reduction in RGR. A study by Abid *et al*. (2016) reported significant decrease in RGR caused by drought stress in various stages of plant growth.

The negative impact of drought stress was reported to be even higher in the jointing and booting stages compared to the anthesis stage. Water stress interferes with the physiological processes of wheat in the vegetative growth stage, thereby affecting plant establishment, net photosynthesis and eventually yield (Abid *et al*., 2016). According to Abid *et al*. (2016) continuous respiration inhibits net photosynthesis followed by reduced assimilates. Strong PCC between RGR and yield of $r = 0.91$ in wheat was reported by Karimi & Siddique (1991). The QTL for RGR was mapped in chromosome locations 1D, 4D and 7D, together with LAE and biomass (Ter Steege *et al*., 2005).

4.3.6 Photosynthetic active radiation

PAR statistical analysis showed nonsignificant differences ($p \geq 0.05$) between the RHSs under investigation. The environment also showed no significant differences between the RHSs studied (Table 4.7). Average light interceptance values of 72.8% and 73.3% were recorded for both the well-irrigated and water-stressed RHSs, respectively (Table 4.8). The water-stressed RHS showed a higher extinction coefficient (9.26%) than the control RHS (9.1%).

Light interceptance was approximately 20% less than that recorded by Pask *et al.* (2012) in the field (97% to 99%). Seemingly, the differences were caused by different environmental conditions. The LAI for the control RHS was 0.18 and 0.16 (Table 4.8). There was no big difference from the results for LAI recorded by Lunagaria & Shekh (2006) for minimum and maximum seasonal mean values of 0.77 to 0.86, respectively. This can also be explained by differences in the environments, growth system used (RHS), plant spacing, the genotypes and the stage of plant growth.

Table 4.7: ANOVA and mean comparison of extinction coefficiency and light interceptance parameters

	Traits	
Treatment	Interceptance	Extinction coefficient
RHS1 (control)	48.75	0.81
RHS2 (water stressed)	48.02	0.81
RHS3 (water stressed)	47.27	0.80
RHS	ns*	ns*
CV (%)	8.74	3.71
Grand mean	48.016	0.07
R-square	0.523	0.464

Significance level at 5% probability. ns* – nonsignificant and ** – significant.

LAI was found to be highly associated with increased grain yield in wheat and maize crops in dry areas (Yin *et al.*, 2016). Variation in plant LAI significantly influences plant populations' micro-environment and spatial distribution, thereby influencing photochemical distribution and photosynthetic efficiency (Giunta *et al.*, 2008). Optimum LAI of the plant indicates increased yield gains, associated with organ development and stability, and facilitates the source to sink strength relationship in the plant (Yin *et al.*, 2016).

The maximum average light interceptance value of 73% was observed from the water-stressed RHS throughout the stress period (Table 4.8). Soleymani & Shahrajabian (2012) reported that a maximum thousand-grain weight was obtained from the studied genotypes, consisted of higher values of extinction coefficient, light interceptance and LAI. RUE values ranged from 0.07 g MJ^{-1} to 0.08 g MJ^{-1} for both the water-stressed

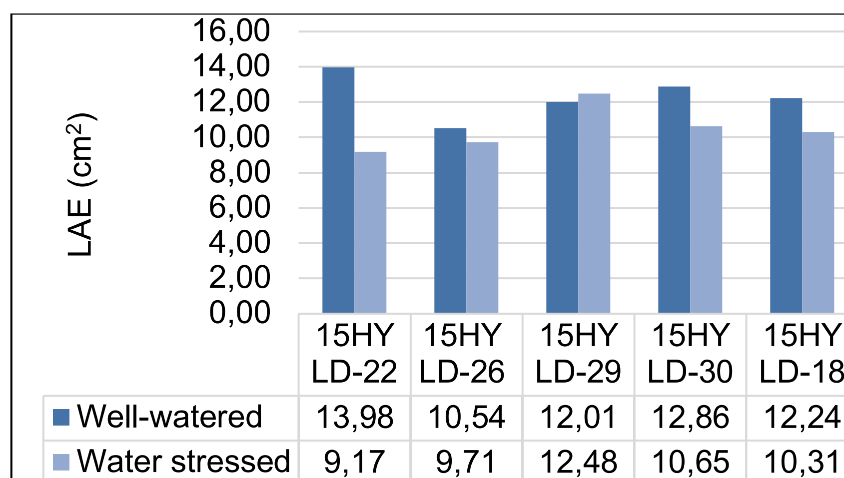
and the control RHS respectively. However, the RUE values fell below those reported in the literature. Sandaña *et al*. (2012) reported a minimum of 1.10 g MJ^{-1} and a maximum of 1.65 g MJ^{-1} RUE in both wheat and pea crops. Such differences are believed to be caused simply by differences in data collection time intervals, genotypes and the environment. The data for the current study was collected during a 14-day period whereas in other experiments in the literature, it was collected throughout the growing season up until harvest. Efficient use of the energy from the sun by the plant provides a great opportunity for realised yield (Soleymani & Shahrajabian, 2012). Therefore, PAR can be used as a selection criterion for screening plants in plant breeding programmes. QTLs for PAR were mapped at chromosome locations 4B, 5B and 6B under rain-fed conditions (Diab *et al*., 2008).

Table 4.8: Means of PAR measurements from the control and water-stressed RHSs

RHS	Mean	Extinction coefficient	LAI	RUE (g MJ^{-1})
Control	0.728	9.1	0.181	0.08
Water stressed	0.733	9.26	0.168	0.07

4.3.7 Specific leaf area

ANOVA for LAE showed highly significant differences for water stress treatment ($p \leq 0.0387$), variety ($p \leq 0.0002$) and treatment combination ($p \leq 0.0081$). The percentage of decrease was recorded to be -15.17%.



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	13,98	10,54	12,01	12,86	12,24
Water stressed	9,17	9,71	12,48	10,65	10,31

Figure 4.14: LAE of five wheat genotypes grown under water-stressed and well-watered conditions

An average maximum value of about 13.98 cm^2 was recorded from genotype 15HYLD-22 and an average minimum of about 9.71 cm^2 from genotype 15HYLD-26 (Figure 4.14). Among the water-stressed genotypes, 15HYLD-29 showed higher LAE size of 12.48 cm^2 and a minimum value of 9.17 cm^2 was recorded from genotype 15HYLD-22. Similar results for LAE were recorded by Guendouz (2016) and Yang *et al*. (2016), with minimum and maximum values ranging from 11.46 cm^2 to 19.37 cm^2 and 8.80 cm^2 to 27.09 cm^2 respectively under irrigated and non-irrigated conditions in wheat. Hence, the different between water stress and well-watered genotypes was -15.17% for LAE. Average LAE size was reduced during water stress in all water-stressed genotypes except 15HYLD-29. Water stress was found to reduce cell turgor, cell division and eventually cell expansion (Guendouz, 2016). Genotype 15HYLD-29 showed a higher LAE value of 12.48 cm^2 compared to the control genotypes under water stress conditions.

This indicates the influence of additive genes expressed under water stress conditions. This trait is very important for variety improvement. Chaudhary *et al*. (2012) concluded that LAE was one of the most important traits for plant growth analysis and yield estimation. Photosynthesis is the primary activity of the leaf; hence, LAE size serves to indicate photosynthesis capacity and potential grain yield. Reduced distance between the spikes and flag leaf (peduncle length) offers the opportunity for a flag leaf

to be the primary source of photosynthate to the grain and continuous supply through its delayed senescent properties.

Tiryakioglu (2015) observed PCC (r = 0.54) between spike grain yield and flag leaf area under water stress conditions. Al-Tahir *et al*. (2014) reported PCC between LAE and FW (r = 0.548), Chl (r = 0.739) and grain yield (r = 0.806). Genotype 15HYLD-29 showed higher LAE size; therefore, there is potential for increased yield under water-stressed conditions in relation to other genotypes. Higher H^2 estimates for LAE of 86.57% were reported by Ahmed *et al*. (2004) and 0.89% by Khan & Hassan (2017). Markers associated with flag leaf senescence in wheat were identified by Barakat *et al*. (2015) and included SSR (*Xgwm328*), ISSR (Pr8, AD5, AD2 and AD3) and RAPD (Pr9), indicating water stress resistance genes.

4.4 Agronomic traits

4.4.1 Root length

ANOVA results for RL showed no significant differences for water stress treatment. The variety and treatment combination did not show any statistical differences in the experiment. The percentage of decrease was recorded as the lowest, namely -6.87%. The average maximum and minimum RL values recorded were 23.67 cm and 23.50 cm from the control genotypes 15HYLD-29 and 15HYLD-18, respectively.

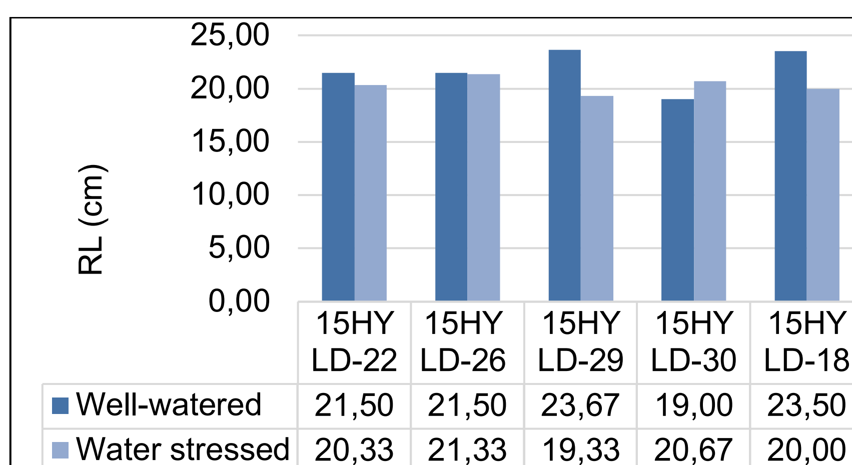


Figure 4.15: RL of five wheat genotypes grown under water-stressed and well-watered conditions

Minimum values of 9.33 cm and 20.00 cm were also recorded from the same genotypes under water stress conditions (Figure 4.15). Similar results were recorded

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by Ahmad *et al*. (2014), with minimum and maximum mean values of 9.35 cm to 19.98 cm and 3.94 cm to 17.866 cm for well-watered and water-stressed conditions, respectively. The slightly higher values of the current study may be due to data collection during the stem elongation stage whereas Ahmad *et al*. (2014) collected data during the seedling stage. Water stress resulted in reduction of RL except in genotype 15HYLD-30, but no significant differences recorded from statistical results, therefore this could be caused by nature or chance. Nonetheless, reduction of RL under water stress conditions was also observed by Ahmad *et al*. (2014) and Becker *et al*. (2016). Genotypes with increased RL under water-stressed conditions were found to tolerate water stress better and to have increased water and nutrient uptake from deep soil (Ahmad *et al*., 2014).

An H^2 estimate of 80.28% was recorded from wheat genotypes under water stress conditions (Ahmad *et al*., 2014). Becker *et al*. (2016) observed a positive association between RL and g_s under water stress conditions in wheat. A strong PCC was recorded by Ahmad *et al*. (2014) for RL and SL ($r = 0.874\%$), SFW ($r = 0.882\%$) and SDW ($r = 0.702\%$) under water-stressed conditions. A moderate H^2 estimate of 62.660% was recorded for RL by Khan *et al*. (2010). QTL associated with root hair length was mapped in chromosome locations 1A, 2A, 6A and 2BL. PCC was reported between root hair length and yield (Horn *et al*., 2016). QTL (*QRl.ccsu-2B.1*) for RL was mapped at chromosome location 2B (Bharti *et al*., 2014). RL may be used as a selection criterion under water-limited conditions for variety development.

4.4.2 Roots dry weight

ANOVA results for RDW showed significant differences ($p \geq 0.0000$) for water treatment and no significant differences for the variety and treatment combination. The percentage of decrease for RDW recorded was -30.12%. The average minimum and maximum values of RDW for genotype 15HYLD-26 were 0.98 g and 0.55 g for both control and water-stressed conditions (Figure 4.16).

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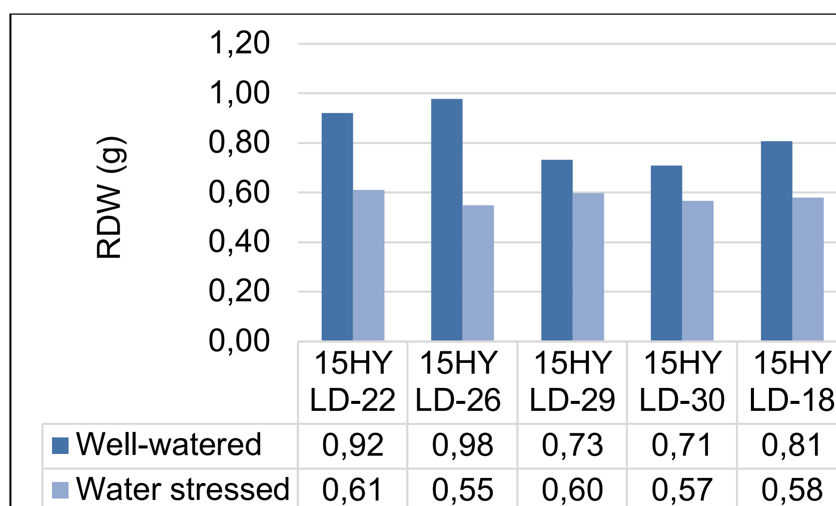


Figure 4.16: RDW of five wheat genotypes grown under water-stressed and well-watered conditions

The current study results are higher than those recorded by Mujtaba *et al*. (2016) at the seedling stage, ranging from 0.004 g to 0.01 g under osmotic stress. Similar results were recorded by Tatar *et al*. (2016) that ranged from 0.7 g to 1.2 g for both water-stressed and well-watered conditions. Water stress resulted in reduction of RDW in all genotypes.

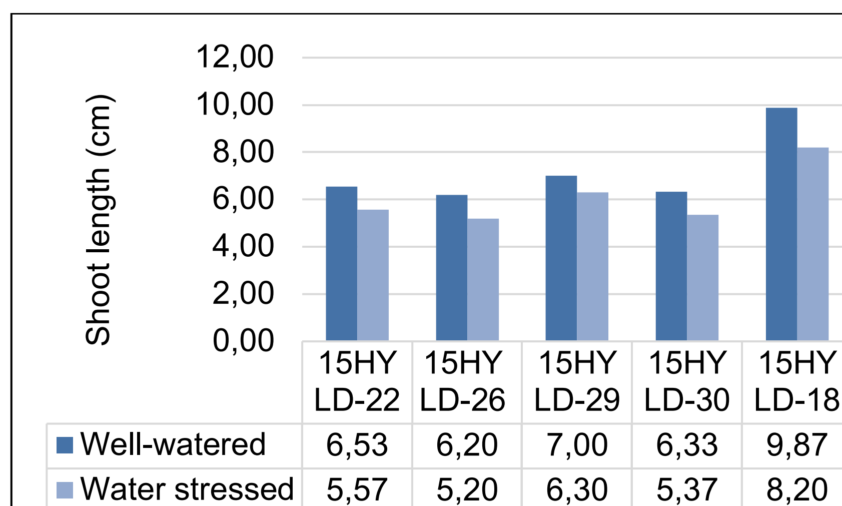
Minimum RDW reduction was recorded for BHYLD14-08 and 15HYLD-29 under water stress conditions. These genotypes may be useful for root traits development in plant breeding programmes. Greater reduction was observed from genotypes 15HYLD-22 and 15HYLD-26, showing signs of moderate susceptibility to water stress. A number of studies reported reduction in RDW in wheat under water stress conditions (Tatar *et al*., 2016; Ahmad *et al*., 2014; Sivamani *et al*., 2000). Narayanan *et al.* (2014) found increased RDW directly proportional to the increase of SDW and NT. Therefore, activities of the root system increase with increased root surface area. Hence, the more the plant root is in contact with the soil particles, the greater the water and nutrient uptake is. Roots may accumulate more dry matter under water stress compared to well-watered genotypes. Reduction in water and nutrient supply to the roots triggers a signal to the roots, leading to production of root hormones, thereby avoiding water stress (Dhanda *et al*., 2004). Narayanan *et al*. (2014) reported a positive but weak association (r = 0.41) between RDW and SDW in wheat. A strong

PCC of r = 0.86, r = 0.90 and r = 0.90 were recorded in bread wheat for RDW and RN, RL and root volume, respectively (Bharti *et al*., 2014).

Naeem *et al*. (2015) recorded lower H^2 estimates for RDW of 27% and 25.3% for water-stressed and well-watered conditions, respectively. Bharti *et al*. (2014) successfully mapped six QTLs for root traits in distinct chromosome locations. Among the six QTLs, four were for root volume (*QRv.ccsu* in chromosome locations 1A, 1B, 2D and 4A) and the remaining two for RDW (*QRDw.ccsu-2A.2*) and RL (*QRl.ccsu-2B1*). Such information may be useful for variety development for yield improvement under water stress conditions. Root structure is very important in plant performance, especially genotypes grown under conditions of limited moisture and nutrient supply.

4.4.3 Shoot length

ANOVA for SL showed highly significant differences for varieties ($p \leq 0.0000$), water stress treatment ($p \leq 0.0020$) and treatment combination ($p \leq 0.0000$). The reduction percentage was recorded to be -16.83%. The average minimum and maximum SL values recorded were 6.20 cm to 9.87 cm for control and 5.20 cm to 8.20 cm for water-stressed genotypes, respectively (Figure 4.17).



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	6,53	6,20	7,00	6,33	9,87
Water stressed	5,57	5,20	6,30	5,37	8,20

Figure 4.17: SL of five wheat genotypes grown under water-stressed and well-watered conditions

Maqbool *et al.* (2015) recorded an average minimum and maximum range of 0.87 cm to 55.02 cm for PH in wheat at the tillering stage. Water stress and variety treatments resulted in reduction of SL in all the genotypes studied. However, genotype 15HYLD-

18's higher SL value of 9.87 cm indicated better water stress tolerance. According to Saleem (2003), tolerant genotypes show increased PH under water stress conditions. SL measurements at the tillering and stem elongation stages can be used to predict PH at maturity and, subsequently, yield.

The recorded results are similar to those reported in the literature (for example Ahmad *et al*., 2014; Mujtaba *et al*., 2016; Khan & Naqvi, 2011). Despite differences in stages of growth, Ahmad *et al*. (2014) and Mujtaba *et al*. (2016) reported substantial reduction of SL because of water stress treatment in wheat genotypes. More reduction was observed by Ahmad *et al*. (2014) from the stem than the roots. A linear trend in SL reduction was observed from all the genotypes. According to El-Kholy & Gaballah (2005), water stress conditions may result in protoplasm dehydration and reduction of relative turgidity, thereby suppressing cell division and expansion though turgor loss.

Positive associations were observed for SL and RL, FW, DW and g_s of r = 0.841, r = 0.749, r = 0.640 and r = 0.410, respectively, under water stress conditions (Khan *et al*., 2010). PCC was recorded by Ahmad *et al*. (2014) for SL and RL (r = 0.670), SFW (r = 0.911) and SDW (r = 0.831). A high H^2 estimate was reported for SL of about 98.27% (Khan *et al*., 2010) and 83.41% (Ahmad *et al*., 2014). Ellis et al. (2002) reported availability of molecular markers for screening PH in wheat named dwarfing genes, namely *Rht-B1* and *Rht-D1* These markers are grouped into three categories: dwarfing (*Rht-B1c*), semi-dwarfing (*Rht-B1b*) and tall plants (*Rht-B1a*) (Kocheva *et al.*, 2014). Kocheva *et al.* (2014) reported better performance of semi-dwarfing (*Rht-B1b*) genotypes compared to dwarfing (*Rht-B1c*) genotypes under water stress conditions. Breeding for required optimum PH confer indispensable productivity, stability and safety of yield on varieties grown under a wide range of environmental conditions (Bognár *et al*., 2007).

4.4.4 Number of tillers

ANOVA NT showed significant differences for variety ($p \leq 0.0122$) and no significant differences for water stress treatment. However, treatment combination showed significant differences ($p \leq 0.0002$) for NT. Reduction percentage of about -6.87% was recorded for NT. The highest value for tiller development was recorded from the control genotype 15HYLD-29 (11.00) and the lowest value from 15HYLD-30 (7.67) (Figure 4.18). The highest and lowest values observed for water-stressed genotypes

were from 15HYLD-29 and 15HYLD-18 (7.67). Maqbool *et al.* (2015) obtained similar results with minimum and maximum average values ranging from 8.51 to 9.37 for total NT.

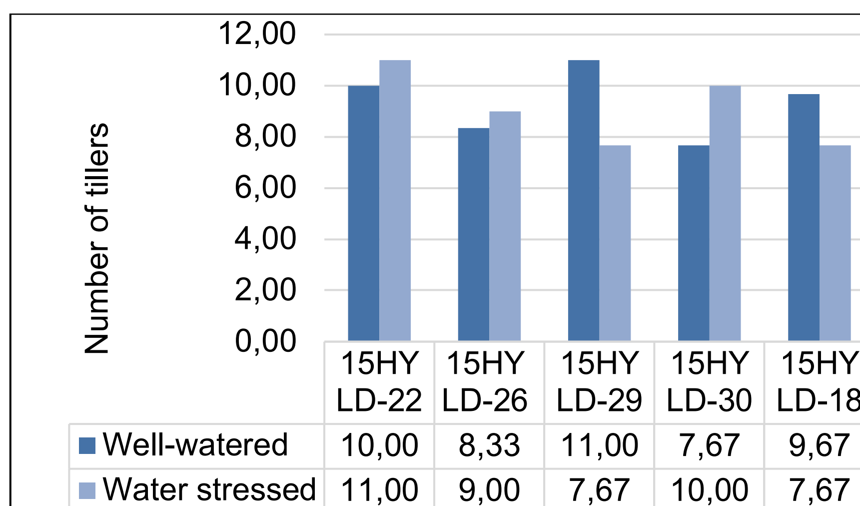


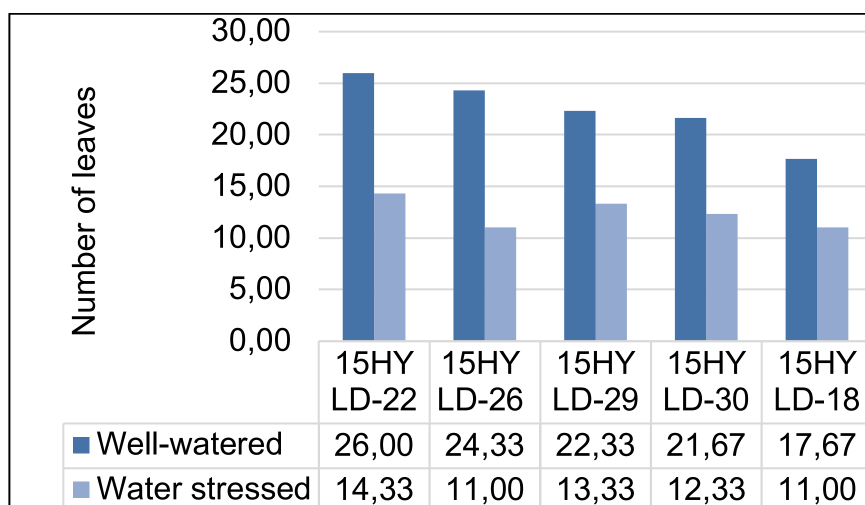
Figure 4.18: NT of five wheat genotypes grown under water-stressed and well-watered conditions

Water stress resulted in a reduction in NT in genotype 15HYLD-29 only. Increased NT was observed from four other genotypes under water stress conditions. Maqbool *et al.* (2015) reported reduction in NT under water stress conditions. Most genotypes under water stress conditions showed increased NT, indicating tolerance to water stress (Figure 4.18). Similar findings were reported by Khan and Naqvi (2011).

Genotype 15HYLD-22 showed a higher rate of tiller development and had the highest NT under water stress conditions, indicating better tolerance. This behaviour is associated with the additive genetic effect expressed under water stress conditions. Not all the tillers produced grain yield; however, the greater the NT under water stress conditions, the greater the proportion of fertile to sterile tillers and the better the chances of greater final yield. Weak PCC of NT of r = 0,281 for well-watered environment and r = 34,5 for water-stressed environments was reported by Naeem *et al.* (2015). QTLs associated with NT were mapped for pre-winter NT (*QMtw5D*), maximum NT in spring (*QMts5D* and *QMts6D*) and NT at harvest (*QEth6A*), followed by H^2 estimate of 0.94, 0.81 and 0.91%, respectively (Li *et al.*, 2010). NT can be used as a water stress selection criterion in plant breeding programmes in water stress environments and to predict better yield.

4.4.5 Number of leaves

ANOVA for NL showed significant differences for variety ($p \leq 0.0053$) and water stress treatment ($p \leq 0.0000$). No significant differences were observed for treatment combination. The value of reduction among all the genotypes was recorded as -42.85%.



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	26,00	24,33	22,33	21,67	17,67
Water stressed	14,33	11,00	13,33	12,33	11,00

Figure 4.19: NL of five wheat genotypes grown under water-stressed and well-watered conditions

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Table 4.9: ANOVA and mean comparison of treatment effects from studied traits

Treatment	Trait												
	RL (cm)	SL (cm)	RFW (g)	SFW (g)	LFW (g)	TPFW (g)	RDW (g)	SDW (g)	LDW (g)	ABGB DW(g)	TPDW (g)	R:S ratio	NL
Water levels													
Well-watered	21.83	7.19	2.49	4.88	8.83	15.55	0.83	0.35	0.65	1.06	1.23	0.93	22.40
Water stressed	20.33	6.13	1.85	2.80	4.82	10.11	0.58	0.25	0.63	0.91	1.16	0.61	12.40
% of decrease	-6.87	-16.3	-25.70	-42.62	-45.41	-34.98	-30.12	-28.57	-3.08	-14.15	-5.69	-34.4	-42.85
Genotypes													
15HYLD-22	20.92	6.05	2.43	3.77	6.60	12.79	0.77	0.26	0.72	1.10	1.23	0.72	20.17
15HYLD-26	21.42	5.70	1.63	3.09	5.55	10.26	0.77	0.36	0.48	0.86	0.98	0.80	17.67
15HYLD-29	21.50	6.65	2.41	4.30	7.95	14.66	0.70	0.25	0.71	0.62	1.25	0.80	17.83
15HYLD-30	19.83	5.85	1.87	3.38	6.06	11.31	0.67	0.24	0.57	1.03	1.07	0.75	17.00
15HYLD-18	21.75	9.03	2.53	4.65	7.96	15.14	0.64	0.39	0.73	1.30	1.46	0.72	14.33
Genotypes	ns*	**	**	**	**	**	ns*	**	**	**	**	ns*	**
Water levels	ns*	**	**	**	**	**	**	**	ns*	**	**	**	**
Interaction	ns*	ns*	**	**	ns*	ns*	ns*	**	**	**	**	ns*	ns*
CV (%)	11.36	8.63	10.25	12.03	9.33	7.76	17.01	8.07	10.14	10.72	6.12	16.81	12.77
Grand mean	21.08	6.657	2.174	3.837	6.822	12.83	0.707	0.552	0.639	0.983	1.197	0.771	17.40
R-square value	0.448	0.904	0.894	0.926	0.954	0.949	0.717	0.756	0.870	0.929	0.938	0.763	0.910

Significance level at 5% probability. ns* – nonsignificant and ** – significant.

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The average minimum and maximum values recorded for control genotypes were 26.00 and 24.33 for 15HYLD-22 and 15HYLD-26, respectively. For the 15HYLD-26 and 15HYLD-18 genotypes, water stress average minimum values of 11.00 were recorded (Figure 4.19). Similar findings were reported by Mbave (2013), ranging from 11.75 to 25.00. Leaf development for water-stressed and nonstressed genotypes indicated plant development. Genotype 15HYLD-18 showed lower reduction of NL under water stress conditions, indicating better tolerance. As much as genotypes 15HYLD-22 and 15HYLD-26 had higher values than the controls, higher reduction was observed from the two genotypes, showing moderate susceptibility to water stress.

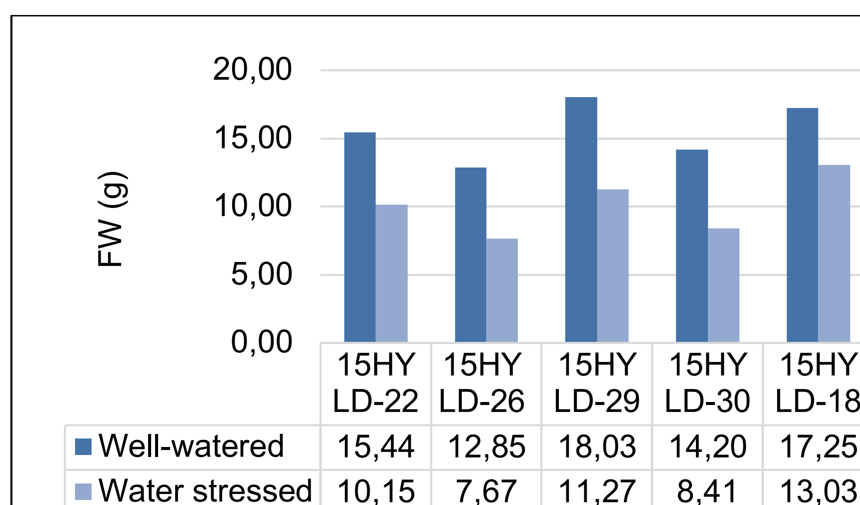
The primary function of leaves is photosynthesis; therefore, higher NL will result in higher interception and increased photosynthetic activity and yield (Mbave, 2013). Few research studies reported failure of plants to recover from the stress when water stress was experienced at the vegetative stage (Mbave., 2013; Vurayai *et al*., 2011). Plants that can recover from water stress conditions and produce yield are ideal for plant breeding programmes. According to Anjum *et al*. (2011), source-sink strength relationship is reduced under severe water stress conditions, resulting in reduced NL and LAE. Slafer *et al*. (1994) state that more knowledge is required of the genetic and environmental control for rate of leaf development and final NL to back up the yield improvement results from many regions. According to Stapper and Fischer (1990), several research works reported significant changes in rate of leaf development once a specific NL have appeared. A positive correlation between NL and grain yield was recorded for biological yield in the booting stage (r = 0.38) and the anthesis stage (r = 0.67) (Gupta *et al*., 2001). Very limited or no information is available on NL; therefore, as it is one of the most important agronomic traits, more research needs to be done, especially molecular work. The NL plays a significant role in photosynthesis rate and capacity; therefore, it should be taken into consideration.

4.4.6 Total plant fresh weight

ANOVA for TPFW measurements showed significant differences among the genotypes for RFW ($p \leq 0.0000$), SFW ($p \leq 0.0001$), LFW ($p \leq 0.0000$) and TPFW ($p \leq 0.0000$). ANOVA showed significant differences for water stress for RFW ($p \leq 0.0000$), SFW ($p \leq 0.0000$), LFW ($p \leq 0.0000$) and TPFW ($p \leq 0.0000$). Treatment combination showed significant differences for RFW ($p \leq 0.0500$) and SFW

($p \leq 0.0026$). No significant differences were observed for treatment combination in LFW and TPFW. Higher values were recorded for percentage of decrease in RFW, SFW, LFW and TPFW, namely -25.70%, -42.62%, -45.41% and -34.98%, respectively. The highest average value of TPFW for control genotypes was observed for genotype 15HYLD-29 (18.03 g), and the lowest value was observed for genotype 15HYLD-26 (12.85 g) (Figure 4.20). Similarly, maximum and minimum average TPFW values for water-stressed varieties were recorded for genotypes 15HYLD-18 (13.03 g) and 15HYLD-26 (7.67 g).

Water stress resulted in a significant reduction in FW. Less reduction was observed for genotype 15HYLD-18. Such genotypes can be used in varietal development in wheat under water stress conditions. Genetic variability resulted in a difference in growth response of the five wheat varieties. Hussein & Zaki (2013) reported a decline in TPFW under conditions of delayed watering and withholding of water. Growth depends on several factors, such as genetic factors and growing conditions. Several parameters are associated with growth inhibition under water stress, including physiological, morphological, biochemical and molecular. According to Smirnoff (1998), water stress may result in oxidative stress, which may denature enzymes and protein functions and inhibit growth



	15HYLD-22	15HYLD-26	15HYLD-29	15HYLD-30	15HYLD-18
Well-watered	15,44	12,85	18,03	14,20	17,25
Water stressed	10,15	7,67	11,27	8,41	13,03

Figure 4.20: TPFW of five wheat genotypes grown under water-stressed and well-watered conditions

A positive correlation was reported between TPFW and SL (r = 0.749), RL (r = 0.633), DW (r = 0.781%) and g_s (r = 0.551) under water stress conditions (Khan *et al*., 2010).

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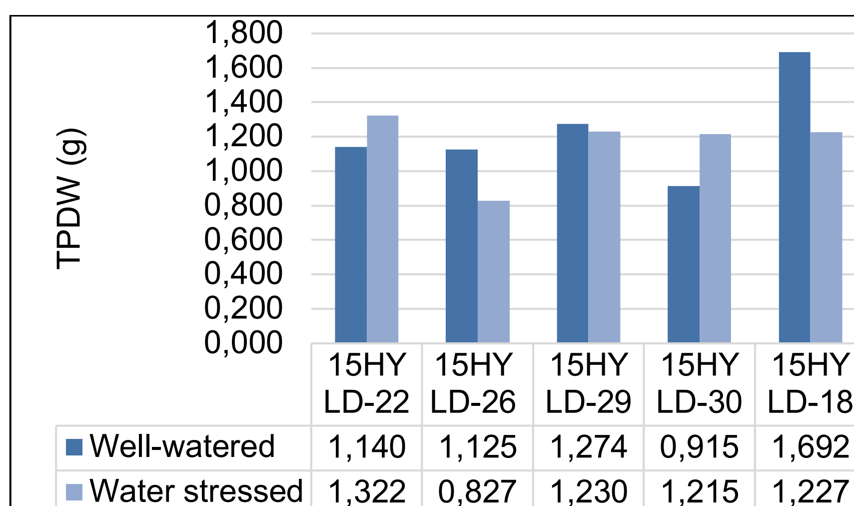
Al-Tahir (2014) recorded a positive association between TPFW and flag leaf area (r = 0.548), Chl (r = 0.299) and grain yield (r = 0.695). A high H^2 estimate of 94.59% was recorded for TPFW (Khan *et al*., 2010). H^2 estimates for RFW and SFW of 87.5% and 46.1%, respectively, were also recorded by Naeem *et al*. (2015). TPFW is one of the most important traits associated with yield as is evident from the correlation and heritability values; therefore, it can be used as selection criterion under water-stressed conditions.

4.4.7 Total plant dry weight

ANOVA showed significant differences among the varieties for SDW ($p \leq 0.0000$), LDW ($p \leq 0.0000$), aboveground biomass (ABGB) ($p \leq 0.0000$) and TPDW ($p \leq 0.0000$). Water stress treatment showed significant differences for SDW ($p \geq 0.0000$), ABGB ($p \geq 0.0008$) and TPDW ($p \geq 0.0278$). Water stress did not have any significant differences for LDW. Treatment combination showed significant differences for SDW ($p \geq 0.0000$), LDW ($p \leq 0.0001$), ABGB ($p \geq 0.0008$) and TPDW ($p \leq 0.0000$). The percentages of decrease for SDW, LDW, ABGB and TPDW were -28.57, -3.08, -14.15 and -5.69, respectively.



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	1,140	1,125	1,274	0,915	1,692
Water stressed	1,322	0,827	1,230	1,215	1,227

Figure 4.21: TPDW of five wheat genotypes grown under water-stressed and well-watered conditions

Higher TPDW in the control genotypes was observed from genotype 15HYLD-18 (1.692 g), and lower TPDW was observed from genotype 15HYLD-30 (0.915 g). Average maximum and minimum TPDW values from water-stressed genotypes were recorded from genotypes 15HYLD-22 (1.322 g) and 15HYLD-29 (1.230 g) (Figure

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4.21). Similar average values for total plant biomass were recorded by Saeidi *et al*. (2015) and Tatar *et al*. (2016) under water-stressed conditions. Reduction was observed from genotypes 15HYLD-26, 15HYLD-29 and 15HYLD-18. Genetic variation in genotypes and water stress resulted in reduction in total plant biomass. Several studies reported reduction in TPDW under water stress conditions (Saeidi *et al*., 2015; Ahmad *et al*., 2014; Tatar *et al*., 2016). Moderate and severe water stress conditions significantly reduced the number of spikes and the grain in wheat (Tatar *et al*., 2016). Moreover, the stem elongation phase coincides with spike formation and is regarded as a critical stage of growth, which resulted in loss of yield.

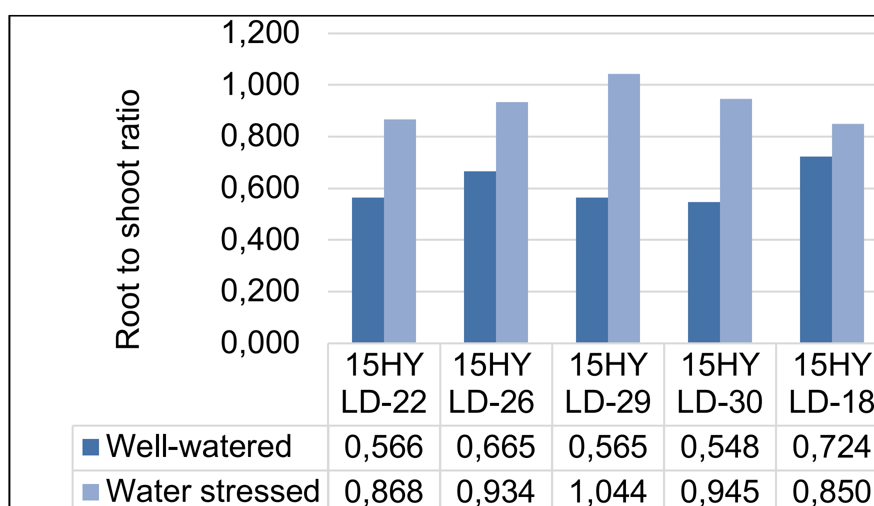
Water stress interferes with assimilates production; thus, assimilates are partitioned into different plant organs such as roots and assimilates directed to the grain are minimised. According to Gupta *et al*. (2001), the stem plays a significant role in the supply of reserve nutrients to the grain under severe water scarcity to compensate for reduced food synthesis. Noorka *et al.* (2013) found higher fructane reserves from the stems of water stress-resistant varieties to sustain developing grain under water-limited conditions. Therefore, plants that can accumulate more dry matter under water-limited conditions can be used to predict improved yield from the vegetative growth stage. Genotypes 15HYLD-22 and 15HYLD-30 showed increased dry matter under water stress conditions, indicating tolerance to water stress. The effect of additive genes can once again be observed from these genotypes. PCC was recorded for DW and SL (r = 0.64), RL (r = 0.54), FW (r = 0.781) and g_s (r = 0.42) under water stress conditions (Khan *et al*., 2010). Kahrizi *et al*. (2010) reported PCC for LDW and peduncle length (r = 0.51), NT (r = 0.20) and PH (r = 0.62). A positive association was also reported between SDW and LDW (r = 0.64), peduncle length (r = 0.74), PH (r = 0.65), NT (r = 0.34) and yield (r = 0.1) (Kahrizi *et al*., 2010). Lastly, Gupta *et al*. (2001) reported a positive association between SDW and yield for biological yield at booting stage (r = 0.62) and the anthesis stage (r = 0.57).

Moderate H^2 estimates for LDW and SDW were reported to be 58.08% and 69.00%, respectively (Kahrizi *et al*., 2010). High H^2 estimates of 96.72% were recorded for TPDW in wheat genotypes (Khan *et al*., 2010). Sivamani *et al*. (2000) introduced the *HV1* gene from barley induced by ABA synthesis; transgenic plants resulted in significantly increased DW, SDW and water use efficiency. Multiple methods can be

employed for variety improvement under water-limited conditions. Improved DW accumulation at the vegetative stage under water stress conditions guarantees carbohydrate supply to the developing grain at the grain filling stage. Therefore, DW can be used as a selection criterion under water stress conditions for varietal characterisation.

4.4.8 Root-to-shoot ratio

ANOVA for R:S ratio showed significant differences ($p \leq 0.0000$) for water stress treatment and no significant differences for the variety and the treatment combination. The reduction percentage recorded was -42.85%. Average maximum and minimum values of about 0.665 and 0.724 for controls were recorded from genotypes 15HYLD-26 and 15HYLD-18, respectively. A significantly higher R:S ratio was recorded from water stress genotype 15HYLD-29 (1.044), and the lowest was observed from genotype 15HYLD-18 (0.850) (Figure 4.22). The current study's R:S ratio results range from 0.548 to 1.044. Ahmad *et al*. (2014) recorded related results ranging from 0.334 to 0.597 and 0.339 to 0.602 for control and water-stressed genotypes, respectively. Increased R:S ratio was observed from all the genotypes.



	15HY LD-22	15HY LD-26	15HY LD-29	15HY LD-30	15HY LD-18
Well-watered	0,566	0,665	0,565	0,548	0,724
Water stressed	0,868	0,934	1,044	0,945	0,850

Figure 4.22: R:S ratio of five wheat genotypes grown under water-stressed and well-watered conditions

Similar results were observed by Ahmad *et al*. (2014) and Boutraa *et al*. (2010) whereby water-stressed genotypes increased the R:S ratio. Dhanda *et al*. (2004) reported that increased R:S ratio was attributed to greater inhibition of stem extension compared to roots due to water stress. Genotypes 15HYLD-26 and 15HYLD-29

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showed significant increase in R:S ratio, indicating that shoots were significantly affected by water stress. Genotype 15HYLD-18 showed a lesser degree of being affected by water stress. Water stress interferes with soluble sugar accumulation in the stem and entire plant, enzyme activity for soluble sugar degradation and carbohydrate partitioning (Xu *et al.*, 2015). Under water stress, the plant invests more nutrients in deep root growth into the soil for water and nutrient extraction to avoid stress (Dhanda *et al*., 2004). R:S ratio measurements provide an opportunity to estimate dry matter partitioning into different plant organs and indicates the effect on roots and shoots (Xu *et al.*, 2015).

Strong and moderate PCC was recorded for R:S ratio and soluble sugar proportion in roots (r = 0.88) and leaves (r = 0.45). Moderate PCC was also recorded between proportion of starch in the roots and R:S ratio (r = 0.60). Moderate H^2 was observed for R:S ratio of 0.44% (Khan *et al.*, 2001). The current study explored heritability estimates from the literature. According to Fellahi *et al*. (2013), a quantitative trait such as heritability is one of the most important to consider heredity and surrounding conditions to determine the trait genetic expression. Less environmental effect interference and considerable additive effect may provide an effective opportunity for selection.

4.5 Variety rankings and percentage of decrease

The percentage of decrease was calculated for all varieties, and it varied across all the traits studied. Minimum reduction was observed for NL, LDW, TPDW, RL, NT, RDW and SL, indicating better water stress resistance. The higher the percentage of decrease, the lower the chances of using the trait for screening. Reduction was also calculated for genotypes studied under water stress conditions (Table 4.10 below). Subsequently, ranking of the genotypes based on minimum reduction under water stress conditions for all the traits studied was done (Table 4.11). The sum of the rankings was calculated for all genotypes (Table 4.12). Ranking was based on the lowest to the highest values. Genotype 15HYLD-30 performed exceptionally well, followed by genotype 15HYLD-22. Genotype 15HYLD-29 was third and performed better than genotypes 15HYLD-18 and 15HYLD-26 (Table 4.12).

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4.6 Correlation study

Exploring information regarding the traits association from five wheat genotypes at the vegetative stage of growth could enhance the opportunity of indirect selection for several characteristics (Ahmad *et al*., 2014). Hence, valuable information could be presented to the breeder. To supplement the information discussed in the literature, correlation coefficients of the traits were explored. Negative and positive (weak, moderate and strong) correlation coefficients were observed from all the traits studied (Table 4.13), under both water-stressed and well-watered environments. However, the study focused on the strong positive correlation of the traits. The following correlations were observed under water-stressed conditions: A strong positive association between FW components included LFW and RFW (r = 0,884), followed by TPFW with FW components such as RFW (r = 0,848), SFW (0,922) and LFW (r = 0,920). A strong positive association was also recorded between SFW and SL (r = 0,832), CCI and SL (r = 0,835) and SFW (0,890). Lastly, TPDW with RGR (r = 0,879) and LDW (r = 0,872). A strong positive association was recorded under well-watered conditions namely TPFW showed a strong positive association with SFW (0,872), LFW (r = 0,920), TPDW with SL (r = 0,877) and LDW (r = 0,841). The correlation information provides a way forward in varietal development under water stress conditions.

Table 4.10: The reduction differences calculated from each trait studied

No.	Genotype	ELWL	LRWC	CCI	G_S	RGR	LAE	RL	RDW	SL	TN	NL	FW	DW	RSR
1	15HYLD-22	22,57	8,19	1,07	7,13	0,01	4,81	1,17	0,31	0,97	-1,00	11,67	5,28	-0,18	-0,30
2	15HYLD-26	17,95	18,07	2,93	131,3	0,05	0,83	0,17	0,43	1,00	-0,67	13,33	5,18	0,30	-0,27
3	15HYLD-29	23,70	15,29	5,60	88,03	0,01	-0,47	4,33	0,13	0,70	3,33	9,00	6,76	0,04	-0,48
4	15HYLD-30	34,81	-0,29	6,60	28,45	-0,02	2,22	-1,67	0,14	0,97	-2,33	9,33	5,79	-0,30	-0,40
5	15HYLD-18	37,58	11,38	1,10	115,5	0,03	1,93	3,50	0,23	1,67	2,00	6,67	4,21	0,46	-0,13

Table 4.11: Ranking of the genotypes based on minimum to maximum reduction

No.	Genotype	ELWL	LRWC	CCI	G_S	RGR	LAE	RL	RDW	SL	NT	NL	TPFW	TPDW	RSR	Total
1	15HYLD-22	2	2	1	1	2	5	3	4	3	2	4	3	2	3	37
2	15HYLD-26	1	5	3	5	5	2	2	5	4	3	5	2	4	4	50
3	15HYLD-29	3	4	4	3	3	1	5	1	1	5	2	5	3	1	41
4	15HYLD-30	4	1	5	2	1	4	1	2	2	1	3	4	1	2	33
5	15HYLD-18	5	3	2	4	4	3	4	3	5	4	1	1	5	5	49

Table 4.12: Final genotype ranking

No.	Genotype	Total	Rank
1	15HYLD-22	37	2
2	15HYLD-26	50	5
3	15HYLD-29	41	3
4	15HYLD-30	33	1
5	15HYLD-18	49	4

Table 4.13: Correlation coefficiencies of all traits studied

TMT	Well-watered genotypes																				
	Traits	**NL**	**NT**	**RL**	**SL**	**RFW**	**SFW**	**LFW**	**TPFW**	**RDW**	**SDW**	**LDW**	**TPDW**	**LRWC**	**ELWL**	**CCI**	**AB.G**	**R:S R**	**RGR**	**LAE**	**SC**
Water stressed genotypes	**NL**		0,130	-0,280	-0,659	-0,265	0,044	-0,390	-0,251	0,523	-0,246	-0,305	-0,470	0,240	-0,622	-0,629	-0,405	0,298	-0,280	0,355	-0,297
	NT	0,316		0,666	0,318	0,339	0,648	0,660	0,753	0,009	-0,184	0,757	0,501	0,519	-0,153	-0,008	-0,160	-0,191	0,504	-0,553	-0,079
	RL	-0,270	0,189		0,473	0,262	0,281	0,439	0,487	-0,104	0,273	0,739	0,705	0,557	0,102	0,173	0,056	-0,308	0,683	-0,205	0,343
	SL	-0,307	-0,648	-0,236		0,428	0,428	0,667	0,566	-0,192	0,413	0,681	0,877	0,179	0,505	0,698	0,574	0,082	0,574	0,016	-0,049
	RFW	0,198	-0,361	-0,263	0,609		0,458	0,525	0,561	-0,300	-0,287	0,479	0,389	-0,137	0,244	0,400	0,446	0,125	0,125	0,245	-0,177
	SFW	-0,246	-0,460	-0,263	0,832	0,686		0,751	0,872	-0,446	-0,420	0,447	0,224	0,164	0,244	0,249	-0,277	0,171	0,171	0,119	-0,175
	LFW	-0,014	-0,464	-0,263	0,686	0,884	0,751		0,968	-0,395	-0,079	0,714	0,681	0,045	0,315	0,584	0,104	-0,220	0,486	0,002	-0,039
	TPFW	-0,048	-0,400	-0,015	0,773	0,848	0,922	0,920		-0,411	-0,213	0,724	0,599	0,154	0,277	0,490	-0,002	-0,002	0,432	0,092	-0,081
	RDW	-0,058	0,044	0,399	0,048	0,261	0,160	0,380	0,302		0,366	0,011	0,027	0,361	-0,706	-0,364	0,094	0,123	0,188	0,207	0,148
	SDW	0,136	0,044	0,076	0,428	0,651	0,642	0,540	0,716	0,351		0,060	0,494	0,307	0,028	0,158	0,334	0,094	0,482	-0,464	0,264
	LDW	0,372	0,310	0,138	0,063	0,275	0,166	0,314	0,362	0,247	0,412		0,841	0,484	0,109	0,385	0,332	0,334	0,762	0,128	0,208
	TPDW	0,361	0,216	0,085	0,220	0,549	0,366	0,497	0,577	0,274	0,702	0,879		0,365	0,251	0,574	0,574	0,332	0,790	-0,072	0,193
	LRWC	0,249	-0,403	-0,180	0,089	0,237	0,083	0,265	0,230	-0,171	0,289	0,005	0,102		-0,287	-0,256	-0,026	0,554	0,513	0,163	0,119
	ELWL	0,346	-0,079	-0,382	-0,304	-0,143	-0,394	-0,372	-0,406	-0,583	-0,358	-0,388	-0,374	0,245		0,583	0,395	-0,026	0,057	-0,239	-0,248
	CCI	-0,299	-0,387	-0,084	0,835	0,483	0,890	0,529	0,767	-0,002	0,596	0,087	0,303	0,059	-0,223		0,476	0,395	0,106	-0,001	0,128
	AB.G	-0,036	0,562	0,229	-0,152	-0,416	-0,230	-0,417	-0,275	0,068	0,201	0,180	0,181	-0,253	-0,314	0,028		0,476	0,238	0,257	-0,152
	R:S Ratio	-0,152	0,031	0,178	0,028	-0,165	-0,166	0,004	-0,045	-0,031	0,009	0,075	-0,016	0,427	-0,012	-0,093	0,164		-0,112	0,414	-0,552
	RGR	0,302	0,157	0,021	0,117	0,368	0,215	0,329	0,388	0,005	0,493	0,635	0,831	0,119	-0,368	0,178	0,244	-0,085		-0,372	0,266
	LAE	0,154	-0,553	-0,235	0,145	0,443	0,145	0,456	0,277	0,080	-0,083	-0,025	0,118	0,227	0,004	-0,055	-0,610	-0,460	0,311		-0,398
	SC	0,239	0,425	-0,149	-0,361	-0,130	-0,312	-0,024	-0,171	0,082	-0,217	0,515	0,308	-0,261	-0,250	-0,366	0,158	-0,271	0,332	0,166	

The green-highlighted cells in Table 4.13 show strong positive correlations among the traits, and the grey-highlighted cells show moderate to partially strong positive correlations. The un-highlighted cells show weak, moderate and strong negative correlations and weak positive correlations.

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CHAPTER 5: CONCLUSION

Molecular marker work successfully identified rust-susceptible and rust-resistant wheat genotypes from the germplasm. The following markers were successfully identified from both the male and female populations: *Lr34/Yr18/Pm38*, *Sr31*, *Lr24/Sr24*, *Lr37/Sr38/Yr17*, *Sr26* and *Sr2.* Molecular marker work showed increased allele frequencies in both the male and female populations. The male population showed a low level of rust resistance genes compared to the female population. Some rust resistance genes such as *Lr19* could not be identified in the male population; however, that does not mean that they are not present in the population. Due to the large number of seeds produced during the MS-MARS cycles, less than 2% of the seeds were selected for planting. That left a large number of unselected seeds with rust resistance genes. Molecular markers can be used to successfully select the rust resistance genotypes prior to field evaluation.

During MS-MARS Cycle 1, crossing parents were effectively selected from the segregating population, which resulted in higher seed production of 295 gram. MS-MARS Cycle 1 confirmed the effectiveness of the technique, through high seed throughput and increased allele frequencies from one MS-MARS cycle to the next. However, MS-MARS Cycle 2 was not effective since it was carried out during the warm season of the year, which resulted in reduced seed production. Higher temperatures may have resulted in sterility of the plants. It is recommended that MS-MARS projects be conducted during spring and winter since wheat requires lower temperatures of 18 °°C to 23 °C. Phenotypic selection and molecular marker collaboration can result in successful plant breeding programmes, thereby improving wheat genotypes for water stress and disease resistance.

Traits associated with water stress resistance were thoroughly investigated using an RHS. The system was associated with some difficulties in growing healthy plants in the growth chamber. Therefore, thorough investigation of the effectiveness of the RHS should be done, involving optimum amount of nutrient solution to be used and optimum LED lighting in the growth chamber. The system was used to screen the plants for water stress resistance traits in the growth chamber. The growth chamber is associated with sophisticated equipment such as a light meter that should be kept on automatic and air supply that should be kept on at all times for healthy plant growth.

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Physiological and agronomic water characterisation is labour intensive and requires patience and dedication. Especially the use of sophisticated tools such as the leaf porometer may be time-consuming, which may lead to taking wrong measurements.

Water stress was found to affect wheat growth and development significantly. Induction of water stress at the tillering and stem elongation stages can result in considerable yield lost. Water stress resistance-related traits were successfully isolated from 60 water stress-susceptible genotypes. Genetic variation of five selected genotypes was examined. The genotypes showed variation in water stress resistance across the traits of interest. The genotypes under investigation were successfully ranked based on their performance under water stress conditions for all traits studied. The ranking was as follows: 15HYLD-30, 15HYLD-22, 15HYLD-29, 15HYLD-18 and 15HYLD-26. However, the ranking does not imply that genotypes at the bottom are susceptible but the ranking was based on the best performance.

Traits used for wheat genotype characterisation showed significant correlation with yield prediction parameters. A strong positive correlation was observed between dry and fresh weight and their components under water stress conditions compared to well-watered conditions. A correlation between RGR, SL and SFW and a correlation between TPDW and CCI under water stress conditions were observed. A strong heritability estimate was well researched from the literature for all the traits studied. Therefore, these traits can be used to predict yield from the vegetative growth stage and can be used as selection criteria for water stress resistance screening in wheat genotypes.

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