THE RE-EVALUATION OF POLLEN MEDIATED GENE FLOW WITHIN SMALL GRAIN

by

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of

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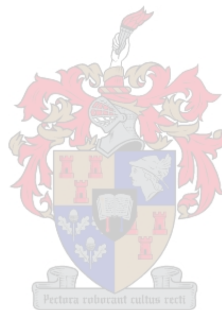

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March 2016

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ABSTRACT

South Africa is one of the few countries within Africa that have introduced genetically modified (GM) crops, and have been growing first generation GM crops since 1997 (Gouse *et al.* 2005). Within South Africa no emphasis has yet been set on the co-existence to establish management practices for the effective isolation of GM- and non-GM crops (Viljoen & Chetty 2011). There are currently no GM wheat or triticale varieties available for commercial production, but there are transgenic wheat and triticale varieties that are being successfully developed and field tested (Doshi *et al.* 2007; Kavanagh *et al.* 2012; Loureiro *et al.* 2012; Wree & Sauer 2015). The aim of this study was to determine the crossability as well as the potential of pollen mediated gene flow between wheat and triticale under dry-land conditions within the Western Cape.

A *Nelder* wheel field trial was conducted at Mariondahl experimental station, Stellenbosch, South Africa, that comprised of a central pollen donor block (CPDB), containing blue aleurone triticale and purple pericarp wheat, which was surrounded by eight pollen receptor arms all orientated in the cardinal and intercardinal wind directions. Each arm was 66.25 meters in length, making the total area covered by the field trial 13788.65m^2. The F1 seed that was harvested from the *Nelder* wheel field trial was planted at Welgevallen experimental farm, Stellenbosch, South Africa, and screened for hybridization and outcrossing events. The F1 spring wheat progeny were screened as pooled samples using molecular marker, *Cfe53*. Individual F1 spring wheat samples were also screened using molecular marker (*Cfe53*) to confirm the pooled sample results, but technical difficulties were than encountered with the molecular marker. The molecular marker results were then confirmed through chromosome visualisations. The F1 spring triticale progeny was screened morphologically for outcrossing events by the expression of blue pigmentation within the seed.

Within this study a high amount of outcrossing and hybridization that was observed within the F1 wheat progeny (30-100%). The F1 spring triticale, outcrossing percentages was observed that ranged between 0.58% and 8.32%, with an average of 2.38%. Significant differences were calculated between the different wind directions, as well as between the different distances from the CPDB for the spring triticale. A correlation was also observed between the prevailing wind direction and speed with the pollen mediated gene flow (PMGF). PMGF at 66.25m from the CPDB indicates that pollen would have remained viable and that gene flow would have occurred past this point had the study extended past this distance from the CPDB. The 5% GM

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labelling threshold that is stipulated in Regulation 293 (2011), under the South African Consumers Protections Acts of 2008 (Acts online 2013) will be maintained within 66.25m from the pollen source. As the highest outcrossing percentage (OC%) that was obtained at 66.25m form the pollen source was 3.8% in the South-West arm. Isolation distances of more than 66.25m could be further investigated to obtain OC% of less than 1% in order for conventional farmers planting next to GM crops to be able to label their crops as GM free. The effect of temporal isolation and the use of physical barriers (i.e. other crops or bare ground) could also be further investigated and optimised to reduce PMGF and maintain the current GM thresholds.

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UITTREKSEL

Suid Afrika is een van die min lande in Afrika wat van geneties gemodifiseerde (GM) gewasse gebruik maak, en verbou eerste generasie GM gewasse al sedert 1997 (Gouse *et al*. 2005). In Suid Afrika is daar tot hede nog geen klem geplaas op die mede bestaan en ontwikkeling van bestuur praktyke vir die effektiewe isolasie van GM en nie-GM gewasse nie (Viljoen & Chetty 2011). Daar is tans geen GM koring of korog variëteite beskikbaar vir kommersiële produksie nie, maar daar is transgeniese koring en korog variëteite wat suksesvol ontwikkel en in verskillende gebiede getoets word (Doshi *et al*. 2007; Kavanagh *et al*. 2012; Loureiro *et al*. 2012; Wree & Sauer 2015). Die doel van hierdie studie is om die uitkruisbaarheid sowel as die potensiaal van stuifmeel bemiddelde geen vloei tussen koring en korog onder droëland toestande in die Wes-Kaap te bepaal.

'n *Nelder* wiel veld proef was uitgevoer by Mariondahl navorsing stasie, Stellenbosch, Suid Afrika, wat bestaan het uit 'n sentrale stuifmeel skenker blok (SSSB), wat blou aleurone korog en pers opperhuid koring bevat het, wat omring was deur agt stuifmeel ontvanger arms, wat almal georiënteerd was in die kardinale en inter-kardinale windrigtings. Elke arm was 66.25 meter lank, wat die totale gebied van die veld proef 13 788.65m^2 maak. Die F1 nageslag wat vanaf die *Nelder* wiel veld proef geoes is, was oor geplant op Welgevallen proefplaas, Stellenbosch, Suid Afrika, waar verbastering en hibridisering gebeure geidentifiseer was. Uitkruising in die F1 koring nageslag was geidentifiseer deur saamgevoegde monsters te toets met met behulp van 'n molekulêre merker, *Cfe53*. Individuele F1 koring monsters was ook getoets deur gebruik te maak van molekulêre merker (*Cfe53*) om die resultate van die saamgevoegde monsters te bevestig, maar die merker het begin probleme gee. Die resultate van die molekulêre merker was daarna bevestig deur chromosoom visualiserings. Hibridisering gebeure binne die F1 korog nageslag was morfologies geïdentifiseer deur die uitdrukking van blou pigmentasie binne die saad.

Binne hierdie studie is 'n groot hoeveelheid hibridisering en verbastering waargeneem in die F1 koring nageslag (30-100%). By die F1 korog, was hibridisering persentasies waargeneem wat gewissel het tussen 0.58% en 8.32%. Beduidende verskille was bereken tussen die verskillende windrigtings, sowel as tussen die verskillende afstande vanaf die SSSB. 'n Korrelasie was ook waargeneem tussen die stuifmeel bemiddelde geen vloei (SBGV) en die heersende windrigting en spoed. Waargenome SBGV was 66.25m vanaf die SSSB en dui daarop dat die stuifmeel lewensvatbaar sou gebly het en dat SBGV sou kon plaasgevind het

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verby hierdie punt vanaf die SSSB, indien die afstand vanaf die SSSB verder verleng was. Die vasgestelde 5% drumpel vir die etikettering GM materiaal in nie-GM produkte, wat gestipuleer is in Regulasie 293 (2011), onder die Suid Afrikaanse Verbruikers Beskermings Wette van 2008 (Acts online 2013) sal binne 66.25m vanaf die stuifmeel bron gehandhaaf word. Aangesien die hoogste persentasie hibridisering (OC%) wat waargeneem was in hierdie studie by 65.25m vanaf die stuifmeel bron 3.8% was in die Suid-Wes arm. Isolasie afstande van meer as 66.25m kan ook verder ondersoek word om OC% van minder as 1% te behaal en so konvensionele boere in staat te stel om langs GM gewasse te plant en steeds hulle gewasse as GM vry te kan etiketteer. Die effek van tydelike isolasie sowel as die gebruik van fisiese skanse (bv. ander gewasse of oop grond) kan verder ondersoek en geoptimaliseer word om SBGV te verminder om huidige drumpel waardes te handhaaf.

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LIST OF ABBREVIATIONS

AIA	Advanced Informed Agreement
AC	Advisory Committee
AFLPs	Amplified Fragment Length Polymorphisms
$AgNO_3$	Silver Nitrate
AOSCA	Association of Official Seed Certifying Agencies
APS	Ammonium persulphate
ARC	Agricultural Research Council
ASAP	Allele-Specific Associated Primers
Ba	Blue aleurone
bp	Base pairs
C:I	Chloroform:Isoamyl
CIMMYT	International Centre for Wheat and Maize Improvement
CGIAR	Consultative Group for International Agricultural Research
CLM	Cytogenetic Ladder Mapping
CBD	Convention on Biological diversity
CPB	Cartagena Protocol on Biosafety
CPDB	Central Pollen Donor Block
CTAB	Cetyl-trimethylammonium-bromide
dH_2O	Distilled water
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
DAFF	Department of Agriculture, Forestry and Fishery
DarT	Diversity Array Technology
E	East
EC	Executive council

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EDTA	Ethylene-diaminetetraacetate
Eml	Embryo lethality gene
EST	Expressed sequence tag
F	Forward
F1	Filial 1
FMs	Functional markers
g	Gram
GA	Gibberellins
GBS	Genotyping-by-Sequencing
GDD	Growing day degrees
GE	Genetically Engineered
GM	Genetically Modified
GMOs	Genetically Modified Organisms
H_2O	Water
HT	Herbicide Tolerant
HCl	Hydrochloride
Hz	Hertz
IARCs	International Agricultural Research Centres
IR	Insect Resistant
IRRI	International Rice Research Institute
km	Kilometers
Kr	Crossability genes
$K_2S_2O_5$	Potassium metabisulfite
L	Litre
LED	Light emitting diode
MAAP	Multiple Arbitrary Amplicon Profiling

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MAP	Multiple Amplicon Profiling
MAS	Marker assisted selection
MES	Mariendahl Experimental Station
Mb	Megabases
m	Meter
ms^{-1}	Meter per second
m^2	Square Meter
µM	Micromolar
µm	Micrometer
µl	Microliter
ml	milliliter
mm	millimeter
mM	millimolar
MITE	Retrotransposon/Miniature-Inverted Repeat Transposable Element
min	Minutes
MP-PCR	Microsatellite-primed polymerase chain reaction
N	North
NaOAc	Sodium Acetate
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NE	North-East
ng	Nanogram
nm	Nanometer
NW	North-West
OC%	Outcrossing percentage
OECD	Organisation for Economic Co-operation and Development

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PAGE	Polyacrylamide gel electrophoresis
%	Percentage
PCR	Polymerase chain reaction
PMGF	Pollen Mediated Gene Flow
Pp	Purple pericarp
Pty Ltd	Private Company limited
QTL	Quantitative trait locus
R	Reverse
RAPDs	Random amplified polymorphic DNAs
RFLP	Restriction fragment length polymorphisms
Rht	Height reduction genes
rpm	Revolutions per minute
s	Second
S	South
SADC	South African Development Community
SANSOR	South African National Seed Organisation monitor
SBGV	Stuifmeel Bemiddelde Geen Vloei
SE	South-East
SKr	Crossability genes
SNP	Single nucleotide polymorphism
SSAP	Sequence-Specific Amplified Polymorphism
SSRs	Simple sequence repeats
SSSB	Sentrale Stuifmeel Skenker Blok
STS	Sequence-tagged sites
SU-PBL	Stellenbosch University's Plant Breeding Laboratory
SW	South-West

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TAC	Total Anthocyanin Content
TBE	Tris Borate EDTA buffer
TEMED	Tetramethylethylenediamine
v/v	Volume to volume
W	West
w/v	Weight to volume

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CHAPTER 1: INTRODUCTION

Wheat is one of the most important food crops for the world population as it is a primary source of both carbohydrates and proteins, and contributes to one-fifth of the calories consumed (Langridge 2012). There are two primary polyploidy species that exist within modern wheat, these are: hexaploid bread wheat (*Triticum aestivum*, 2n = 6x = 42, AABBDD) and durum wheat (*T. turgidum*, 2n = 4x = 28, AABB) (Gustafson *et al*. 2009). As bread wheat is widely planted it was estimated in 2013 that wheat was grown on more than 219 million hectares of land in both the Northern and Southern hemispheres within temperate, Mediterranean-type and sub-tropical regions (Langridge 2012; FAOSTAT 2015). In the 2014/2015 season it was estimated that wheat in South Africa was grown on 476 570 hectares of land and yielded an average of 3.73 tons per hectare (SAGIS 2015). Within South Africa wheat is currently the winter cereal crop that is the most widely planted (South African Grain Laboratory 2013). Wheat has experienced a drastic increase in yield since the ‘Green Revolution’ in the 20th Century, with a doubling in the average yield and total production in the past 50 years (Baenziger *et al*. 2006). At the same time it is predicted that the world population is expected to surpass 9 billion by 2050 (James 2009), where Africa is projected to contribute the biggest proportion of this increase (Liagova 2014).

Triticale (x *Triticosecale* Wittmack ex. A. Camus, 2n = 6x = 42) is a synthetically developed crop that has a high yield potential and a higher stress tolerance than other small grains (Mergoum & Gómez-Macpherson 2004; Alheit *et al*. 2012). The name *triticale* was derived by combining the scientific classifications of the two genera involved in developing triticale (Mergoum et al. 2009). Hybridization between bread wheat and rye (RR) was attempted for the first time in 1875 by Wilson (1875) who reported it to the Botanical Society in Edinburgh. The first commercially available triticale cultivars, “Triticale no. 57” and “Triticale no.64”, were released in 1968 from a Hungarian breeding program (Ammar *et al*. 2004). A year after their release, 40 000 hectares were grown on Hungarian farmers’ fields (Ammar *et al*. 2004). Triticale is mainly utilized as animal feed and forage, but small amounts do enter the human food chain through either the intentional or unintentional mixing with other small grain (Hills *et al*. 2007). In 2013 it was estimated that triticale was grown on over 3 million hectares worldwide, with an average yield of 4.18 tons per hectare (FAOSTAT 2015). Hexaploid triticale cultivar improvement has been pursued since the 1970’s within South Africa by the Stellenbosch University’s Plant Breeding Laboratory (SU-PBL) (Roux *et al*. 2006). The focus

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of this breeding program has largely been on the development of cultivars for grazing, hay and silage feed (Manley *et al*. 2011).

One of the fastest adopted innovations in agriculture is genetically modified organisms (GMOs) or transgenic crops. There are many benefits offered to farmers by the many innovations in transgenic crops, but these innovations also pose uncertain risks to society (Wree & Sauer 2015). In 2014 the total production area of genetically modified (GM) crops increased to 181.5 million hectares from 1.7 million hectares in 1996 (James 2014). Where, engineered traits such as herbicide tolerance and insect resistance resulted in a significant reduction in the use of herbicides and insecticides (James 2014).

South Africa is one of the few countries within Africa that have introduced GM crops, and have been growing first generation GM crops since 1997 (Gouse *et al*. 2005). The commercialisation of transgenic crops in South Africa has mainly been focussed on four crops: soybean, maize, cotton, and canola. The genetic engineering of these crops has included two traits: insect resistance (IR) and herbicide tolerance (HT) (Keetch 2014). Given the large range of biotech crops that are researched and developed, the trend for an increase in the global planted area of transgenic crops seem set to continue (Keetch 2014). In 2012 four African countries - South Africa, Burkina Faso, Egypt and Sudan - grew biotech crops and together planted 3.2 million hectares of biotech crops (Keetch 2014). Official national approval has to be obtained for the commercial use of a biotech crop in all countries, whether the biotech crop will be used for planting and growing, for human use, or animal foods (Keetch 2014). The approval of biotech crops are based on a safety assessment by national authority, these assessments are based on scientific information regarding the crop, the specific trait and the receiving environment (Keetch 2014). Efficient biosafety systems are needed to protect agriculture, animal biodiversity, and minimise unintended effects from influencing agriculture productivity as well as human health (Keetch 2014).

Currently there are no GM wheat or triticale varieties available for commercial production, but there are transgenic wheat and triticale varieties that are currently being successfully developed and field tested (Doshi *et al*. 2007; Kavanagh *et al*. 2012; Loureiro *et al*. 2012; Wree & Sauer 2015). Before these transgenic crops can be commercially used, the environmental and economic risks have to be evaluated (Kavanagh *et al*. 2012). Environmental concerns include the risk of the GM crops becoming agricultural weed, becoming invasive, or outcrossing with wild and weedy relatives resulting in more invasive and weedy hybrids (Warwick *et al*. 2009).

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The economic risks include the potential of the GM crops seed mixing with conventional crop seeds (Kavanagh *et al*. 2012). This could occur through the unintentional mixing during grain handling, the harvest of persistent volunteers, or through pollen mediated gene flow (PMGF) from GM crops to nearby related conventional crop species (Demeke *et al*. 2006; Kavanagh *et al*. 2012). Even though seed movement is seen as an important factor in structuring the genetic composition of plant populations, PMGF is considered the main mode of gene flow in flowering plants as it provides a direct mechanism for inter- or intraspecific gene flow (Levin & Kerster 1974).

The aim of this study is to determine the crossability as well as the potential of pollen mediated gene flow between wheat and triticale under typical dry-land farming conditions within the Western Cape. Results obtained through this study could be useful in future risk assessments of pollen mediated gene flow between transgenic wheat and triticale cultivars as well as between conventional wheat and triticale cultivars.

In order to achieve the aim of this study the following objectives have been identified:

i) The optimisation of phenotypic and molecular screening methods for blue aleurone and purple pericarp seed traits that will be used as pollen donors in the field trial.

ii) Conducting a Nelder wheel field trial that comprises of a central pollen donor block, containing blue aleurone triticale and purple pericarp wheat that will be surrounded by eight pollen recipient arms, containing normal spring wheat and triticale, which will all be orientated to the cardinal and intercardinal wind directions.

iii) The planting of the F1 progeny harvested from the field trial in a single greenhouse, under standard greenhouse conditions.

iv) The phenotypic and genotypic screening of the progeny.

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CHAPTER 2: LITERATURE REVIEW

2.1. WHEAT

2.1.1. Wheat production and consumption

Cereal grain crops, which include: wheat, rye, rice, maize, barley, sorghum, oats and millets, have founded the staple of the world since their domestication about 10 000 years ago (Varshney *et al*. 2006). These grain crops are the most important plants cultivated for food production, as they provide more than 60% of the calories and proteins consumed in our everyday diet (Varshney *et al*. 2006). Wheat forms an important part in the daily diet of millions of people worldwide, as it provides up to 35% of the world population with a staple food diet (IDRC 2015). The viscoelastic properties that are found within wheat dough, is the distinctive feature that makes wheat unique in comparison to other cereals. These viscoelastic properties determine the capacity to give a specific-end product (Hoseney & Rogers 1990). Bread wheat is an important source of proteins, vitamins, and minerals (Brenchley *et al*. 2012); and accounts for 20% of the calories consumed by humans' worldwide (Wheat Initiative 2013), and 16.5% of the daily calories consumed in South Africa (Leete *et al*. 2013).

Wheat (*Triticum spp.*) is possibly the most important plant to humans, with a total production of 716 million metric tons per year, of which 75% is utilized as food (Langridge 2012; Lupi *et al*. 2013; United States Department of Agriculture 2014). Modern wheat cultivars belong primarily to two polyploid species: hexaploid bread wheat (*T. aestivum*), and tetraploid hard or durum-type wheat (*T. durum*), where durum wheat is mainly utilized for the production of pasta, couscous, and variety of bread types in Mediterranean countries (Gustafson et al. 2009; Gazza *et al*. 2011). Hexaploid bread wheat makes up 95% of wheat grown worldwide, while tetraploid durum wheat, makes up the remaining 5% (Shewry 2009). Within South Africa approximately 80% of the total winter cereal crop production is contributed by wheat, where the other winter cereal crops produced, include barley (*Hordeum vulgare* L.) for malting purposes and Rapeseed (*Brassica napus*) (The South African Grain Laboratory NPC 2015). South Africa is made up of nine provinces that are divided into 36 crop production regions, in which about 28 of these regions wheat is planted (The South African Grain Laboratory NPC 2015). In the 2014/2015 season it was estimated that wheat was grown on 476 570 hectares of land in South Africa and yielded an average of 3.67 tons per hectare (The South African Grain Laboratory NPC 2015). The total wheat production in South Africa for the 2014/2015 season

was 1.75 million tons, where about 72.6% was produced under dryland conditions, which is mainly in the Western Cape and the Free State areas, and about 27.4 % came from irrigation areas, largely in the Northern Cape (The South African Grain Laboratory NPC 2015). The Western Cape, that mainly includes the Namakwaland, Swartland, and Rûens regions, contributed 51% of the total wheat production in the 2014/2015 season (The South African Grain Laboratory NPC 2015).

Wheat is classified as either spring or winter wheat - this usually refers to the season in which the crop will be grown (Curtis 2002). Within winter wheat a period of cold winter temperatures (0 to 5°C) are required to initiate heading. Winter wheat is usually sown in autumn, after which they germinate and grow into juvenile plants that remain vegetative during the winter and resume their growth in early spring. Thus, winter wheat is utilizing the advantages of the moisture in autumn for the germination of the seeds, as well as the sunshine, warmth, and rainfall of early spring. Spring or summer wheat, on the other hand, is planted in spring and is harvested late in summer, but spring wheat can also be sown in autumn in countries that have mild winters (Curtis 2002).

Within the Western Cape, spring wheat is usually sown from the second half of April until the middle of June and is harvested during October to December (The South African Grain Laboratory NPC 2015). This is usually done in countries that have milder winters that are associated with a Mediterranean climate and do not experience the colder temperatures (0-5°C) that are needed to initiate heading of winter wheat (Curtis 2002). The winter rainfall of the Western Cape, provide enough soil moisture for the germination of the spring wheat. As a result of planting the spring wheat in autumn the spring wheat seedlings, that are sensitive to soil temperature, are also protected from the elevated temperatures of summer often experienced in the Western Cape (Curtis 2002).

2.1.2. Wheat origin and the 'Green Revolution'

The earliest cultivated wheat cultivars included diploid wheat (einkorn, AA) and tetraploid wheat (emmer, AABB) and their genetic relationship, indicated that they originated from the south-eastern part of Turkey (Heun *et al*. 1997; Dubcovsky & Dvorak 2007; Shewry 2009). The key changes that occurred in wheat during its domestication, include: i) an increase in grain weight (Curtis & Halford 2014), ii) the loss of shattering of the spike at maturity, and iii) the change in glumes from hulled forms to free-threshing forms (Dubcovsky & Dvorak 2007). Figure 2.1 illustrates the hybridization events that occurred between wild species in the past to

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produce new polyploidy species during domestication and cultivation of wheat by human civilisations through the centuries. Hexaploid bread wheat was developed by two hybridization and allopolyploidization events: the first hybridization event formed the polyploidy species, *T. turgidum* - that was developed when *T. urartu* (AA), as the male parent, was hybridized with *Aegilops speltoides* (BB), as the female parent. The second and more recent hybridization event was when primitive tetraploid wheat (*T. turgidum,* AABB), as the female parent, was hybridized with *Aegilops tauschii* (goat grass, also known as *T. tauschii* and *Ae. squarosa*, DD), as the male parent (Gustafson *et al*. 2009; Shewry 2009; Hernandez *et al*. 2012; Curtis & Halford 2014). Since hexaploid bread wheat evolved it has been undergoing intense selection aimed at developing improved, high yielding cultivars that are adapted to a wide range of environments and agricultural practices (Cavanagh *et al*. 2013). The improvements of wheat that was selected for included: modification of vernalization and photoperiod requirements of wheat; the adaptability of wheat to water-limiting conditions, low temperature, salinity, and to toxicities within the soil (Cavanagh *et al*. 2013). The semi-dwarf photoperiod-insensitive wheat varieties that were developed in the 1940's formed the basis of the "Green Revolution" (Hedden 2003).

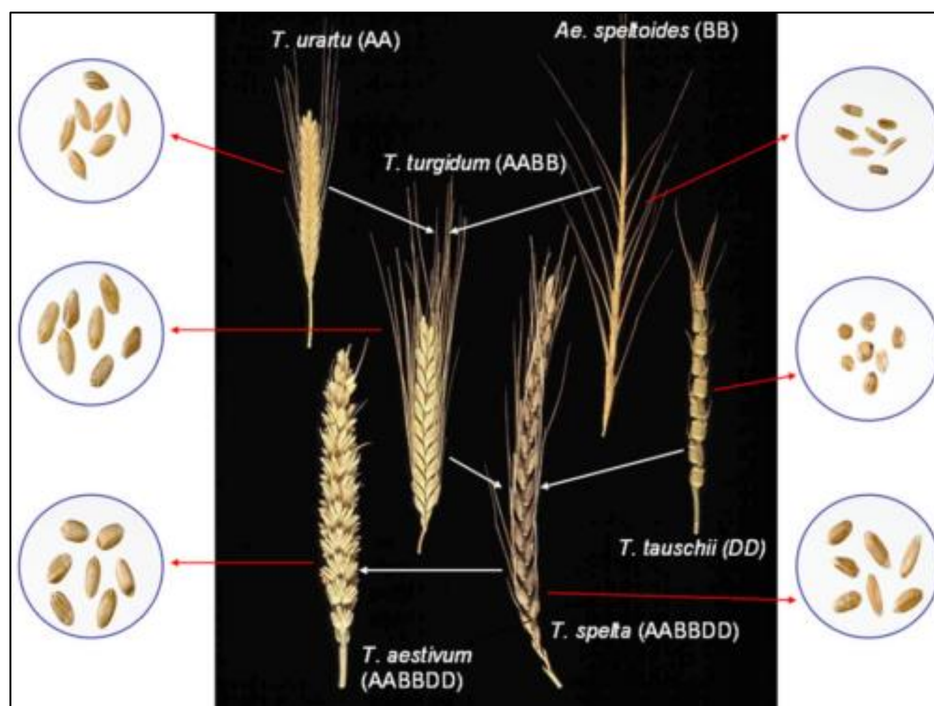

Figure 2.1: Diagrammatic representation of hybridization events that occurred during the evolution of wheat (Snape & Pankova 2006).

Within the late 1950's the development of high yielding wheat and rice varieties for developing countries began as a determined trend. By the mid-1960's these high yielding rice and wheat

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varieties that were developed by scientist, were released to farmers in Latin American and Asia (Evenson & Gollin 2003). The term "Green Revolution" was used to characterize the success of these high yielding varieties (Evenson & Gollin 2003). These early high yielding varieties were rapidly utilized within areas which had reliable irrigation and received adequate rainfall within tropical and subtropical regions (Evenson & Gollin 2003). The developed high yielding varieties were associated with IRRI (International Rice Research Institute) and CIMMYT (International Centre for Wheat and Maize Improvement), the World's first two major IARCs (International Agricultural Research Centres) of the CGIAR (Consultative Group for International Agricultural Research) consortium (Evenson & Gollin 2003).

The rapid increase in wheat yield was associated with the shortening of the wheat stature (Gooding *et al*. 2012). The reduction in the height of wheat plants resulted in the plants having a higher lodging resistance and a higher yield per plant, due to the greater proportion of the assimilate partitioned into the grain (Hedden 2003). The reduction in height of the high yielding varieties was achieved by either: the combined effect of many minor genes, and/or the inclusion of major genes to reduce height (Gooding *et al*. 2012).

The most common semi-dwarf habit genes in modern wheat are found on the *Rht*-B1b and *Rht*-D1b loci, previously known as *Rht*1 and *Rht*2, on chromosomes 4B and 4D respectively (McVittie *et al*. 1978; Lanning *et al*. 2012). These dwarfing genes are successful in reducing height in wheat and have been widely adopted in wheat breeding programmes since their introduction in the 1960s (Ellis *et al*. 2004). These semi-dwarf alleles reduce height by 15% (Addisu *et al*. 2010), by affecting the elongation of the internode cells in the response to endogenous Gibberellins (GA) (Appleford & Lenton 1991), which results in lowered stem growth rates (Nagel *et al*. 2013). This also lead to improved photosynthetic capacity, due to the increased concentration of soluble proteins, chlorophyll and Rubisco (Morgan *et al*. 1990). The more potent alleles that cause extreme dwarfing (more than 30% reduction in height) that are available include *Rht-Blc* (previously designated as *Rht3*). These alleles originate from "Tom Thum", but are not yet commercially used (Börner *et al*. 1997; Gooding *et al*. 2012).

2.1.3. Wheat Genetics

Bread wheat's genome is at 17 gigabases (six times that of the human genome). Its genome also has a hexaploid nature which makes wheat the species with the largest genome among cereals (William *et al*. 2007; Langridge 2012). It contains 21 linkage groups and is known to possess low levels of polymorphism for marker systems, such as: restriction fragment length

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polymorphisms (RFLP), simple sequence repeats (SSRs) or microsatellites (William *et al*. 2007). Through wild hybridisation many important genes have been introduced into wheat from wild relatives, as well as through chromosome manipulation that result in inter-chromosomal translocation between wheat chromosomes and the homologous chromosomes of related species (Jiang *et al*. 1994).

Wheat provides a model system for studying polyploid cytogenetics, because of the ease of chromosome manipulations within wheat (Gupta *et al*. 1999). Cytogenetic studies have been made possible within wheat through the availability of extensive genetic/cytogenetic stocks (Gupta *et al*. 1999). Wheat is a segmental polyploid that is comprised of three individual but homologous genomes (Gupta *et al*. 1999). Where each of these homologous genomes include seven metacentric or sub-metacentric chromosomes (Akhunov *et al*. 2003; Manickavelu *et al*. 2009). Bread wheat's genome contains approximately 1.7×10^{10}bp haploid DNA (deoxyribonucleic acid) (Arumuganathan & Earle 1991), and an average of 810 Mb per chromosome (10µm) (Gupta *et al*. 1999). It is estimated that the average wheat chromosome is 25-fold longer than the average rice chromosome (Gupta *et al*. 1999). Three average wheat chromosomes are thus equal to the haploid maize genome, and one half of an average wheat chromosome is thus equal to a haploid rice genome (Gill & Gill 1994). This large genome for bread wheat is the result of polyploidy and extensive duplications – resulting in over 80% of the bread wheat's genome consists of repetitive DNA sequences (Gupta *et al*. 1999). It has been observed, by using the cytogenetic ladder mapping (CLM) strategy, that the majority of wheat genes are present as clusters and that the small chromosome regions surrounding these gene clusters are highly recombinogenic (Gupta *et al*. 1999). These chromosome regions are suitable for molecular manipulations and these molecular manipulations are similar to those that are possible in other crops with small genomes, (i.e. rice) (Gill *et al*. 1996).

2.2. TRITICALE

2.2.1. Triticale origin and history

Triticale is a manmade crop that is classified within the family Poaceae, subfamily *Pooidea*e and tribe *Triticeae* (Kavanagh *et al*. 2010; Goyal *et al*. 2011). It was developed by crossing durum wheat (T. *durum*) or common bread wheat (*T. aestivum*), as the female parent, with rye (*Secale sp*., RR), as the male parent (Oettler 2005; Kuleung *et al*. 2004; Goyal *et al*. 2011). Triticale was developed with the objective of combining the resistance and tolerance that rye

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has to biotic and abiotic stresses, with the yield potential and good grain quality of wheat (Mergoum *et al*. 2009).

Hybridization between bread wheat and rye was attempted for the first time by Wilson (1875) who reported it to the Botanical Society in Edinburgh, where the resulting hybrid plant was an octaploid triticale (2n = 8x = 56, AABBDDRR) and was found to be sterile (Oettler 2005; Thiemt & Oettler 2008). The sterility of these hybrids were attributed to their dysfunctional pollen grains (Ammar et al. 2004). The first fertile hybrid hexaploid triticale (2n = 6x = 42, AABBRR) plant was developed by Rimpau (1891) in Germany, by crossing hexaploid wheat with rye, which was followed by spontaneous chromosome doubling of the F1 progeny (Lelley 1992; Mergoum *et al*. 2009).

It was only in 1968 that the first triticale cultivars were released and made available for producers (Ammar *et al*. 2004). These triticale cultivars originated from an Hungarian breeding program, where they were developed from an octaploid-hexaploid cross (Ammar *et al*. 2004). From 1991 to 2001 th e production of triticale increased with 50% worldwide, and in 2013 triticale was grown on over 3 million hectares worldwide, with an average yield of 4.18 tons per hectare (Hills *et al*. 2007; FAOSTAT 2015). Within South Africa the Stellenbosch University's Plant Breeding Laboratory (SU-PBL) have been pursuing to improve triticale cultivars since the 1970's (Roux *et al*. 2006). Most research and plant breeding efforts are mainly focused on improving hexaploid triticale's (2n = 42 = AABBRR), they are also the most commonly grown triticale cultivars, though octaploids (AABBDDRR) and tetraploids also occur (Kuleung *et al*. 2004; Hills *et al*. 2007; Goyal *et al*. 2011). This is due to the fact that hexaploid triticale is more vigorous and fertile, and also has a greater meiotic stability than octaploid triticale (Cheng & Murata 2002). The majority of octaploid triticale have poor seed development and are generally much more unstable than hexaploids (Salmon *et al*. 2004).

Triticale can be produced in various ways that result in two types – primary and secondary triticale (Thiemt & Oettler 2008). Primary triticale are developed by the chromosome doubling of the wheat-rye cross (Thiemt & Oettler 2008). Secondary triticale are all the genotypes that are developed from intercrossing primary triticale or crossing primary triticale with wheat, rye and secondary triticale (Thiemt & Oettler 2008). Two ploidy levels exist in primary triticale: primary octaploid triticale that is synthesized by crosses between *Triticum* species with rye, and primary hexaploid triticale that is synthesized by crossing tetraploid *Triticum* ssp. with rye. Various chromosome constitutions [2n = 6x = 42, AABBRR, AABB(DR)(DR)] are observed in secondary triticale at the hexaploid level (Thiemt & Oettler 2008). These hexaploid triticale

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are classified as complete or substitute: complete hexaploid triticale contains all the rye chromosomes (7 pairs), and ii) substitute hexaploid triticale is where some of the rye chromosomes are substituted with wheat D-genome chromosomes (Fox *et al*. 1990). The frequent cytogenetic imbalances that primary triticale undergo make them unsuitable for commercial production (Thiemt & Oettler 2008). Primary triticale, which include the octaploid and hexaploid triticale, are used in breeding programmes to enlarge the genetic basis of the commercially grown hexaploid secondary triticale and/or introgress traits via crosses (Thiemt & Oettler 2008). Triticale could also be used as a valuable genetic resource to transfer desirable genes (i.e. disease resistance) from rye to wheat (Kuleung *et al*. 2004).

The use of chromosome translocations between wheat and various other species are important within wheat breeding and cytogenetic research programs (Lukaszewski & Gustafson 1983). The wild and cultivated relatives of wheat offer fast pools of germplasm for agronomic and quality characteristics as well as resistance and tolerance to pests, disease and unfavourable environmental conditions (Lukaszewski & Gustafson 1983). The transfer of desirable genes from wild and cultivates relatives into wheat background has generally not been an easy process, as it requires a large input of labour and resources and produces mixed results (Lukaszewski & Gustafson 1983). Amphiploids that are produced between wheat and alien species do not necessarily combine the best characteristics of the parents involved. Of the amphiploids developed, only triticale is beginning to compete as a commercial cereal - after a great deal of effort (Lukaszewski & Gustafson 1983).

Triticale cultivars are also classified according to the season they are grown, that is: spring, winter and intermediate (facultative) triticale (Mergoum *et al*. 2009; Goyal *et al*. 2011). Spring triticale cultivars generally have an upright growth and produce much more foliage in the early stages of growth (Mergoum *et al*. 2009). They also have limited tillering and are generally insensitive to photoperiod (Mergoum *et al*. 2009). Spring triticale does not require vernalization to initiate the reproductive phase (Salmon *et al*. 2004). Winter triticale cultivars are generally planted in autumn, but some are also planted in spring in some situations - this is due to the vernalization that is required to initiate heading (Mergoum *et al*. 2009). They also produce more forage than the spring types - this is mainly attributed to their long growth cycle (Mergoum *et al*. 2009). Intermediate triticale cultivars are intermediate to spring and winter triticale cultivars, but do not require the vernalization to initiate the reproductive phase (Salmon *et al*. 2004).

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In South Africa the south Western Cape Province is climatically best suited for spring type cereal grains (Roux & Marais 1996). The south Western Cape Province also includes large areas of marginal, shallow and acid soils where the yields obtained for wheat are usually very low and unprofitable (Marais 1985; Roux & Marais 1996). Triticale in the south Western Cape Province is mainly utilized for: human consumption (2%), cover crop (8%), forage food (30%), and feed grain (60%). Triticale has largely replaced rye as a cover crop between vineyards and is favoured as a forage crop due to its ability to be grazed early in winter and to be cut later in the season as silage, hey, or grain crop. The grain yield obtained is mainly utilised to substitute maize in animal feed (Roux & Marais 1996).

2.2.2. Triticale uses

Wheat is considered a more important crop than triticale in terms of its grain production, but the yields obtained from triticale in field experiments often exceeds that of wheat, depending on growing conditions (Bassu *et al*. 2011). Triticale is well adapted to harsh environmental conditions, such as high elevations, acidic soils, salinity and aluminium toxicity, drought, and water logged soil conditions (Kuleung *et al*. 2004). Triticale is also more competitive with weeds and has a greater tolerance to common wheat diseases and pests than wheat (Horlein & Valentine 1995; Beres *et al*. 2010). The grain obtained from triticale is rich in essential amino acids, which makes it nutritiously more valuable than wheat, but the baking quality is inferior to that of bread wheat (Horlein & Valentine 1995). These traits make triticale an important crop and a suitable alternative to other small grain crops and it is thus likely that its productivity and area planted will increase in the future (Bassu *et al*. 2013). The traits for higher yield and better tolerance to environmental stresses of triticale could also contribute to the better adaptability of wheat to future drier and warmer climates in both the optimal and unfavourable conditions within Mediterranean-type of environments (Bassu *et al*. 2013).

Triticale is used for grazing, silage, hay, grain for feed, food and industrial use, protection from soil erosion and nutrient uptake to prevent water pollution (Salmon *et al*. 2004). But, has also been used as: a food-grade, bio-product, and biofuel grain (Wang *et al*. 1997; Boros 2002; Raatz *et al*. 2012). Triticale is also considered a less expensive alternative for biofuel production than bread wheat (Wang *et al*. 1997). Through the use of genetic engineering, triticale is being developed into a crop for novel bio-based products (CTBI 2015). Before these genetically modified (GM) triticale cultivars can be used commercially, the environmental and economic risks has to be evaluated (Kavanagh *et al*. 2012). Environmental risks include: i) that these GM triticale cultivars become agricultural weeds - in that they become more invasive or

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persistent; and ii) that they outcross with wild or weedy species resulting in more invasive or persistent weedy hybrids that could impact the community diversity (Kavanagh *et al*. 2010; Kavanagh *et al*. 2012). Gene flow has been reported from herbicide resistant crop species to wild relatives, in crops such as: bread wheat, rice (*Oryza sativa L.*), and canola (*Brassica napus L.*) (Seefeldt *et al*. 1998; Warwick *et al*. 2003; Shivrain *et al*. 2007; Sanchez Olguin *et al*. 2009). The economic risks include that the seeds of these GM triticale mix with conventional triticale or other small grain crops, either by: the accidental mixing during grain handling, by harvesting persistent triticale volunteer plants, or through pollen mediated gene flow (PMGF) from the GM triticale to nearby related commercial crop (Demeke *et al*. 2006; Kavanagh *et al*. 2012).

2.3. GENETICALLY MODIFIED ORGANISMS

2.3.1. What is Genetically Modified Organisms?

Genetically modified organisms (GMOs) or transgenic crops has become one of the fastest adopted innovations in modern agriculture (Gong & Wang 2013; Wree & Sauer 2015). Genetically modified (GM) crops, or Genetically Engineered (GE) crops, or GMOs are obtained by using recombinant DNA biotechnological procedures that allow the genetic makeup of a crop or organism to be altered (Schneider *et al*. 2010). The 'recombination' of the crop or organism, that results in the expression of attributes not in the original organism, can be obtained by moving genes from one organism to another or by changing genes in an organism that are already present (Schneider *et al*. 2010). These transferred genes or gene sequences are referred to as transgenes, and biotech plants are therefore also known as transgenic plants (Obonyo *et al*. 2014). Some examples of GM crops include: delayed-ripening tomatoes, pest-resistant crops, and herbicide tolerant crops (Schneider *et al*. 2010). The many innovations in GM crops offer potential benefits to farmers, such as: improving crop yields, reducing insecticide use, or increasing the nutritional value of foods; but also pose certain risks to society (Schneider *et al*. 2010; Wree & Sauer 2015). The transgene integration and/or transformation of tissue culture during transgenic progress may induce unintended genomic alterations in GM plants such as: deletions, insertions, and rearrangements, which may generate secondary or pleiotropic effects (Gong & Wang 2013). These unintended effects associated with genomic alterations are unpredictable and are one of the most controversial issues in debating the biological safety of GM crops (Garcia-Canas *et al*. 2011; Gong & Wang 2013).

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2.3.2. Genetically Modified Crops Production

In 2010 genetically modified (GM) crops exceeded, for the first time in 15 years, the 1 billion hectares accumulated global planted area of GM crops (Xia *et al*. 2012). There are up to date more than 30 different GM crops cultivated in 29 countries, where most of these are associated with first generation GM benefits (Evans & Ballen 2013). GM crops are classified into one of three generations, where: i) first generation crops are crops that have enhanced input traits, such as herbicide tolerance (HT), insect resistance (IR), and have tolerance to environmental stresses; ii) second generation crops include crops that have added-value output traits, such as nutrient enhancement for animal feed; and iii) third generation crops that produce pharmaceuticals, improve the processing of bio-fuels, or produce products other than food and fibre (Fernandez-Cornejo & Caswell 2006).

South Africa is one of the few countries within Africa that have introduced GM crops, and have been growing first generation GM crops since 1997 (Directorate: Genetic Resources Management 2005; Viljoen & Chetty 2011). In 2014 statistics South Africa was ranked ninth in terms of global commercial GM area planted, with GM crops in South Africa being planted on a total area of 2.7 million hectares (James 2014). It has been estimated that in South Africa 100% of cotton (IR and HT), 92% of soybean (HT), 90% of yellow maize (IR and HT) and 83% of white maize (IR and HT) produced are GM (ISAAA 2015). GM crops have been accepted rapidly in South Africa for more than a decade, yet no emphasis has been given on establishing management practices for the effective isolation of GM and non-GM crops (Viljoen & Chetty 2011). Despite this lack in establishing management practices, there are requirements for non-GM crops in term of exporting commodities, especially to countries in Africa, Asia, and Europe (Viljoen & Chetty 2011).

Within modern wheat breeding there have been numerous innovations (Wree & Sauer 2015). Wheat breeding techniques have developed from weak forms of selection, to the more precise selection in combination with mutation, inbred, hybrid and biotechnology or GMO (Wree & Sauer 2015). GMOs in wheat raise broad concerns and that is why no developed GM wheat varieties have been commercialized (Wree & Sauer 2015). Some of the GM wheat developed and field tested include: glyphosphate-tolerant wheat, powdery mildew resistant wheat, and novel winter wheat lines in Germany with high yield potential (Zhou *et al*. 2003; James 2014; Saalbach *et al*. 2014).

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Spring triticale is being considered as a feedstock for bio-ethanol production, monomer and polymer production, as well as bio-refining for chemical production (Bona 2004); these end uses may require GM triticale (Raatz *et al*. 2014). New technologies for the development of GM crops have been tested on triticale and have been successful (Ziemienowicz *et al*. 2012).

2.3.3. Biosafety of Genetically Modified Organisms

Biosafety is defined as “the avoidance of risk to human health and safety, and the conservation of the environment, as a result of the use for research and commence of infectious or genetically modified organisms” (Zaid *et al*. 2001). Concerns on GMO crops can be broadly placed in four categories: i) environmental, ii) food or feed safety, iii) legal/policy, and iv) socio-economic; but there are some cross-cutting issues that can span over more than one of these categories. (Obonyo *et al*. 2014).

Environmental concerns on GMO crops are linked to the potential impacts on the ecosystem from the introduction of GMOs (Obonyo *et al*. 2014). Some of these impacts include: the development of a resistance/tolerance by target organisms, consequences of gene flow, and negative impacts on non-target organisms (Thies & Devare 2007).

Food and/or feed safety concerns are that, other than the intentional introduced improvements by genetic modification, additional unintended differences may also occur (Obonyo *et al*. 2014). The unintended effects are defined as those differences that go beyond the primary expected effect(s) of introducing the target gene(s) (Andersson *et al*. 2011). Some of the possible adverse effects of GE food and/or feed that have raised concerns are: toxicity of GE food and/or feed, allergenicity to GE food and/or feed, changes in the nutritional value of GE food and/or feed, and emergence of resistant strains of bacteria (Key *et al*. 2008).

There are two international organisations that are directly involved in the international regulation of GMOs: the Convention on Biological diversity (CBD) and the World Trade Organisation (WTO) (Obonyo *et al*. 2014). The Cartagena Protocol on Biosafety (CPB) that was adopted in 2000 as supplement for the CBD and was the first specific international legal framework for biosafety regulation (Obonyo *et al*. 2014). The CPB has specifications regarding the safe use and transport of GMOs, with the exception of GMO products that are used for pharmaceutical production and those that are not intended for either food or feed (Obonyo *et al*. 2014). The main requirements of the CPB include: i) establishing procedures for the advanced informed agreement (AIA) between trading parties; ii) risk assessments; iii)

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handling, transport, packaging and identification of GMOs; iv) a biosafety clearing house; v) capacity building, particularly for parties from the developing world; vi) Public awareness and participation, that include socio-economic considerations in decision making, liability and compensation; and vii) compliance (Gupta *et al*. 2008).

The CPB gives parties the option to decide whether or not they want to include socio-economic considerations in their decision-making processes (Obonyo *et al*. 2014). The socio-economic concerns that are linked to GMOs go beyond the strict interpretation of the term, and involve ethical, philosophical, and even religious issues relating to GMOs (Obonyo *et al*. 2014). Regulatory systems that include socio-economic considerations in their decision-making processes include the 2006 GMO Amendment Act 23 of South Africa (Southern African Legal Information Institute 2010) and the African Union Model Law (African Union 2011; Falck-Zepeda & Zambrano 2011). The socio-economic concerns of biotech crops in agriculture focus on the possibility of making farmers more vulnerable to market forces (Obonyo *et al*. 2014). Some of these market forces could be as a result of: i) changes in cost of agriculture and agricultural practices; ii) monopoly control of seed supply by trans-national companies; iii) profit margins for farmers being squeezed between seed cost and declining world prices; iv) challenging market dynamics; and v) the fear of losing entire portions of foreign markets (Sengooba *et al*. 2009).

2.3.4. Genetically Modified Crops Legislation

The European Commission published a document in which they defined co-existence as: “The choice of consumers and farmers between conventional, organic, and GM crop production, in compliance with the legal obligations for labelling defined in Community legislation. The possibility of adventitious presence of GM crops in non-GM crops cannot be excluded. Therefore, suitable measures are needed during cultivation, harvest, transport, storage, and processing to ensure co-existence” (European Commission 2015).

Co-existence thus deals with the measures to prevent mixing between GM and non-GM crops in order to minimize economic losses and negative impacts on human health, trade and environment (Viljoen & Chetty 2011). Even though the plants containing authorized transgenes are regarded as safe for human and animal health, as well as for the environment, the labelling threshold will require the segregation of GM and non-GM crops (Beckie & Hall 2008). Transfer of genes from one population to another through pollen mediated gene flow is one of the considerations of co-existence (Viljoen & Chetty 2011).

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In South Africa all activities regarding GMOs are conducted in accordance to permits issued in terms of the Genetically Modified Organisms Act of 1997 (Directorate: Genetic Resources Management 2005). The Act is administered by the Directorate Genetic Resources and makes provisions for the appointment of a Registar, two regulatory bodies - that include the Advisory Committee (AC) and the Executive council (EC), and inspectors (Directorate: Genetic Resources Management 2005). The AC assesses the risk assessment and risk management data of the proposed activity and subsequently makes a specific recommendation to the EC (Jaftha 2014). The AC consist of ten members appointed by the minister of Agriculture, Forestry and Fisheries, after recommendations of the EC (Jaftha 2014). The EC is the ultimate decision body with respect to GMOs in South Africa and consist of eight governmental departments: Agriculture, Forestry and Fisheries; Science and Technology; Environmental Effects and Tourism; Health; Labour; Trade and Industry; Arts and Culture; and Water Affairs. The Council is chaired by the Department of Agriculture, Forestry and Fisheries which works on the basis of consensus (Prince & Black 2010).

On 14 August 2003 South Africa ratified the Cartagena Protocol on Biosafety and implemented it on 11 November 2003. South Africa had to review its existing national biosafety framework, the GMO Act, 1997 (Act No. 15 of 1997), in order to comply with the obligations of Parties to the CPB and to accommodate the provisions stipulated in the CPB (Directorate: Genetic Resources Management 2005).

Additional to the provisions in terms of the CPB, other legislation that may impact the regulation of GMOs in South Africa were also considered by the Directorate Genetic Resources. The legislative items relating to the safe use of GMOs in South Africa administered by the Department of Environmental Affairs and Tourism include: the Environmental Conservation Act, 1989 (Act no. 73 of 1989), the National Environmental Management Act, 1998 (Act no 107 of 1998) and the National Environment Management Biodiversity Act, 2004 (Act no 10 of 2004). The legislative item administered by the Department of Health includes the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act no. 54 of 1972 (Directorate: Genetic Resources Management 2005).

The proposed amendments were made to the GMO Act 1997 (amended by Act 23 of 2006) by considering the provisions of the CPB, the above mentioned legislation, and further developments in the field of biotechnology. On 8 October 2004 the draft Bill was published in the Governments Gazette (R1266 of Government Gazette No.26848). The second draft of the

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amended Bill was presented to the Cabinet Committee on Governance and Administration in the 2005/2006 financial year (Directorate: Genetic Resources Management 2005).

The South African Consumers Protections Acts of 2008 (Acts online 2013) was passed into law in 2009 (Viljoen & Marx 2013). This Act aims to protect consumers in South Africa from unfair trade practices, to improve consumer awareness and confidence through a legal framework, which also provides a system for consumer redress (Acts online 2013). Various aspects of fundamental consumer rights are included in the Act, these include: the right of consumers to equality, privacy, choice, the disclosure of information, fair and responsible marketing, fair and honest dealing, just and responsible terms and conditions, and fair accountability (Acts online 2013). An important inclusion in the Act is the mandatory labelling of GM products or ingredients in food (Viljoen & Marx 2013). The Act states in section D: 24 that, "any person who produces, supplies, imports, or packages any prescribed goods must display on, or in association with the packaging of those goods, a notice in the prescribed manner and form that discloses the presence of any GM ingredients or components of these goods in accordance with applicable regulations" (Acts online 2013). South Africa is one of about 39 countries that have implemented mandatory GM labelling (Gruère & Roa 2007). Canada, Argentina and the USA are the three major GM producers and all follow a voluntary GM labelling approach (Gruère & Roa 2007).

The thresholds that allow a tolerance for the adventitious presence of approved GMOs with mandatory labelling range from 0% (China), 0.9% (EU and Russia), 1% (Brazil, Austria, New Zealand, and Saudi Arabia), 3% (South Korea) to 5% (Japan, Indonesia, Taiwan, and Thailand) (Viljoen & Marx 2013). The above mentioned thresholds are not based on health and food safety considerations, but rather on consumer perceptions, practical limits and cost implications (Bansal & Ramaswami 2007).

The application of GM labelling in South Africa was considered due to the extent of GM crop production and GM crop imports (Viljoen & Marx 2013). Regulation applies to goods approved for commercialisation by the EC for GMOs (Acts online 2013). Regulation 293 (2011), under SASCA, facilitates GM labelling under the following conditions that: i) "ingredient or component" "containing at least 5% of genetically modified organisms", the "good or ingredient or component" must be labelled "contains Genetically Modified Organisms"; ii) food ingredient or components that are "intentionally and directly produced using genetic modification processes" must be labelled "produced using genetic modification"; iii) if ingredients or components contain less than 5% GM it may voluntarily "state that the

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level of genetically modified organisms contained in the good or ingredient or component" "is less than five percent"; iv) A product may be labelled "does not contain genetically modified organisms" as long as the "good or ingredient or component contains less than one percent genetically modified organisms"; v) A cost effective option to label food products may be used by companies with "may contain genetically modified ingredients" if "it is scientifically impractical or not feasible to test goods" "for the presence of genetically modified organisms or ingredients" (Acts online 2013). This is also intended to address the issue of traceability and is applicable to products derived from but not containing GM material as a result of processing (Viljoen & Marx 2013).

2.4. CROSSABLILITY BETWEEN CEREALS

2.4.1. Crossability genes

A trait or behaviour of interest of an animal or plant that is closely linked to the animal or plants evolution or specification, is the characterization of crossability (Zhang *et al*. 2011). The evolution of genetic exchange barriers between previously interbreeding populations is the characterization of speciation between animals or plants (Zhang *et al*. 2011). Plant speciation is characterised by the evolution of genetic exchange barriers between previously interbreeding populations (Riesenberg & Willis 2007). Animals and plant species can cross with their close relatives that results in the exchange of genes, this is defined as hybridisation, and can lead to the development of new species (Zhang *et al*. 2011). Crossability is thus important for crop improvement (Zhang *et al*. 2011). As the genetic diversity of modern crops has decreased as consequence of crop domestication and improvement (Zhang *et al*. 2011). This leads to the increased vulnerability of these crops to biotic and abiotic stresses and thus also to the prevention of the potential for sustainable genetic improvement of elite cultivars on the long term (Tanksley & McCouch 1997). The natural biological processes of hybridisation and introgression occur among closely related species (Zaharieva & Monneveux 2006). Wide hybridisation between crops and their relatives is a useful tool in plant breeding for enriching genetic diversity, creating new species, gene transfer and induction of haploids (Zenkteler & Nitzsche 1984). The successful hybridisation or gene transfer between crops and their wild relatives depend on a number of factors, that include: the proximity of the parental plants, their mating system (outcrossing rate), the synchronous flowering, and the genetic compatibility (ploidy level, genomic similarity, and chromosome pairing) of the crop and the wild species, as well as the viability of the cross progenies produced (Zaharieva & Monneveux 2006).

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All crop plants used today have been domesticated from their wild relatives (Brozynska *et al*. 2015). These wild relatives are the primary source of diversity that could be used in plant breeding (Brozynska *et al*. 2015). Crop wild relatives were defined by Maxted *et al*. (2006) as "a wild plant taxon that has an indirect use derived from its relatively close genetic relationship to a crop". In some cases the wild relative from which a crop plant was domesticated may be well known and easily identified in current wild plant populations (Brozynska *et al*. 2015). But not all species from which crop plants were domesticated are known, or the species may have gone extinct (Brozynska *et al*. 2015). Some cases, such as wheat, the plant is of hybrid or polyploid origin and several species may have contributed to the genome of the domesticated plant (Brozynska *et al*. 2015). Species that are related to the immediate wild relatives of domesticated crops could also be used as gene pools or reservoirs of genetic diversity for crop improvement (Brozynska *et al*. 2015). The pool of genetic diversity that is available for crop improvement depend on the relative crossing ability between that crop itself and the wild relative (Maxted *et al*. 2006). Harlan & de Wet (1971) categorised the wild relatives as primary, secondary or tertiary gene pools.

The primary gene pools include spontaneous races (wild and/or weedy) and cultivated races. Crossing with the primary pool is easy, the hybrids are generally fertile with good chromosome pairing, gene segregation is normal and gene transfer is generally easy. The different gene pools and the effort needed to transfer traits from them are illustrated in Figure 2.2. The secondary gene pool includes species that will cross with the crop and would approach an co-enospecies (Harlan & de Wet 1971). A co-enospecies is defined as a group of related species that more or less frequently hybridize (Ogden 1989). The hybrids produced in this crosses tend to be sterile, chromosome pairing is poor or absent, some hybrids may be weak and difficult to bring to maturity, and the recovery of desired types in advanced generations may be difficult (Harlan & de Wet 1971). This gene pool is available to breeders or geneticists who are willing to infest the required effort (Harlan & de Wet 1971). The tertiary gene pool includes species that can cross with the crop species, but the hybrids produced tend to be anomalous, lethal or completely sterile (Harlan & de Wet 1971). Gene transfer is either not possible with known techniques or where possible requires the use of sophisticated techniques, such as embryo rescue, somatic fusion or genetic engineering (Harlan & de Wet 1971; Maxted *et al*. 2006).

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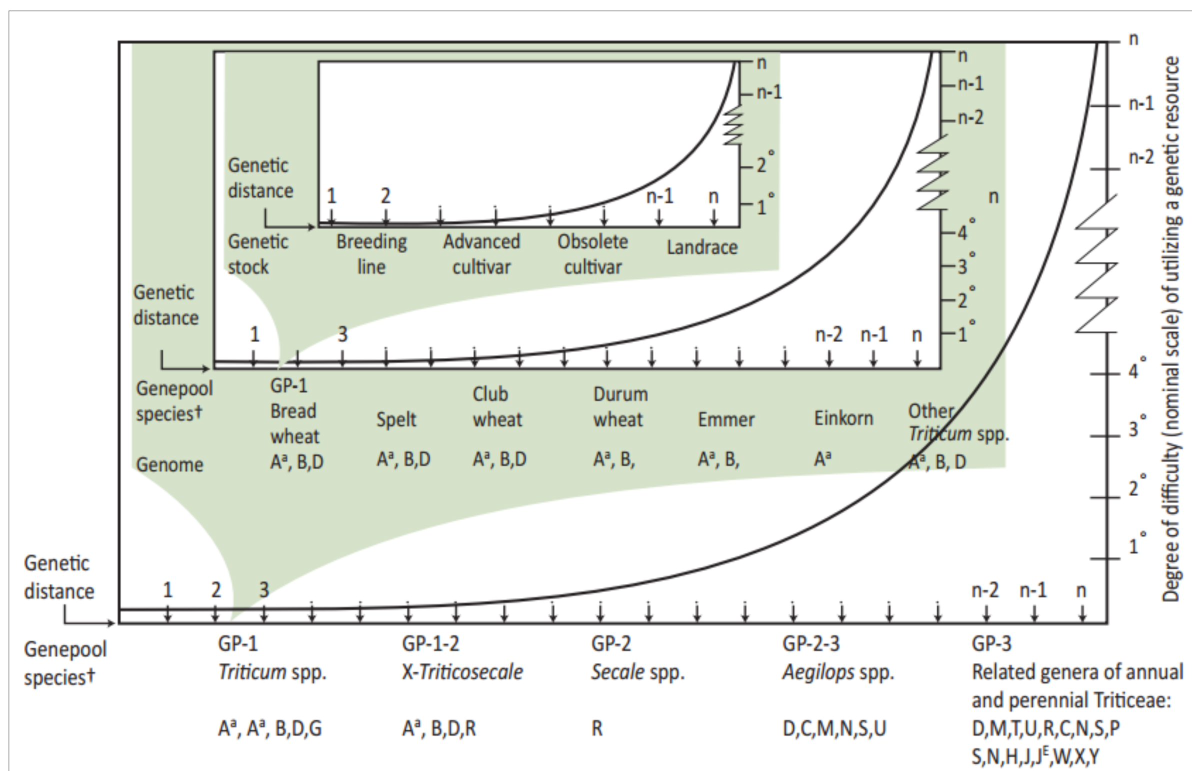

Figure 2.2: The different gene pools and the effort needed to transfer traits from them (Payne *et al*. 2012).

The crossability between crops has also raised the concerns for biosafety of GMOs (Zhang *et al*. 2011). One pathway in which transgene movement can take place is through pollen mediated gene flow (Hills *et al*. 2007). Thus, investigating the crossability of wheat with its relatives will be beneficial for crop improvement and for biosafety as well (Zhang *et al*. 2011). The study in crossability date back to the work done by Backhouse (1916), who investigated the crossability between wheat and rye. Four genes are known to have a major effect on the crossability of hexaploid wheat with rye: *Kr*1/*kr*1 that is located on chromosome 5B (Riley & Chapman 1967), *Kr*2/*kr*2 located on 5A (Riley & Chapman 1967), *kr*3 located on 5D (Krolow 1970), and *kr*4 located on chromosome 1A (Zheng *et al*. 1992). Where *kr*1 gene has the greatest effect and *kr*4 is stronger than *kr*2, but still weaker than *kr*1 gene, and *kr*3 seems to have a weakest effect (Deng-cai *et al*. 1999). A cross between bread wheat and rye will only be successful if the wheat parent caries the recessive allele(s) at the crossability genes (*kr*) (Riley & Chapman 1967; Sitch *et al*. 1985). If the wheat parent caries the dominant alleles hybrid seeds may be formed, but only in low frequencies (Tikhenko *et al*. 2011).

In 1998 a new locus, known as *SKr*, was discovered that controls the crossability between wheat and rye (Tixier *et al*. 1998). Tixier *et al*. (1998) detected *Skr* by using a mapping

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population of 187 double haploid lines that were produced from anther culture from the F1 hybrids of the cross between "Courtot", a non-crossable French wheat cultivar, and "Chinese Spring", a Chinese crossable cultivar. The new locus was identified as a major quantitative trait locus (QTL) on the distal end of chromosome 5B and had a confidence interval from 8.7 to 20.9 cM (Lamoureux *et al*. 2002). Lamoureux *et al*. (2002) found within the study population that the effect of *Skr* (22.1% heritability) was stronger than that of the QTL identified on 5BL (presumably *Kr*1, 5.5% heritability), and no significant effect was detected on 5AL (*Kr*2) (Lamoureux *et a*l. 2002).

Crossability is reduced by the *Kr* alleles that inhibit the entry of pollen tubes into the micropyle of the maternal tissue (Fedak & Jui 1982). Manickavelu *et al*. (2009) stated that it seemed that the *Kr* genes were expressed in the floral part of the plant. That the *Kr1*gene along with and two important genes, *Vrn1* and *Ph1*, were identified and finely characterized on chromosome 5B (Barrett *et al*. 2002; Griffiths *et al*. 2006). The *Vrn1* genes role was related with the vernalization requirement of wheat that is needed for flowering (Manickavelu *et al*. 2009). Where the *Ph1* gene has an important role in regulating the correct pairing of homologous chromosomes, and is also responsible for preventing pairing of related chromosomes (Manickavelu *et al*. 2009).

2.4.2. Embryo lethality genes

Wide hybridization often results in one of the following developments: non-crossability, embryo or seedling lethality, death at a later developmental stage, and morphological abnormalities (Tikhenko *et al*. 2005). Baum *et al*. (1992) stated that the viability and fertility of interspecific cross hybrids can be limited by: i) hybrid sterility, ii) the failure of endosperm development causing the death of a potential viable embryo, and iii) the selective elimination of parental chromosomes during the development of the embryo.

When crossing species that are relatively closely related it usually leads to i) sterile filial 1 (F1) hybrids - this is usually overcome by chromosome doubling (Tikhenko *et al*. 2008), and ii) Abnormal embryo and endosperm development - this is usually linked to crosses between rye and tetraploid wheat (*T. durum*) (Raina 1984). Most of the seeds produced by such a crossing fail to germinate (Tikhenko *et al*. 2008). This failure of endosperm to develop can be overcome by cultivating the seeds on artificial medium (Sirkka & Immonen 1993). Thirdly, the cross between hexaploid wheat and maize (*Zea mays*) is one of the known examples of where selective chromosome elimination has been observed (Laurie & Bennett 1988). Selective

chromosome elimination has not yet been observed in a cross between hexaploid wheat and rye (Tikhenko *et al*. 2008).

The embryo lethality that is expressed within wheat-rye hybrids, and not within rye itself, is an indication of genome interaction between wheat and rye genes (Tikhenko *et al*. 2011). The *Eml-R1b* (embryo lethality) gene, mapped to the chromosome arm 6RL, thus interacts with the corresponding gene(s) of wheat. If this interaction is negative it leads to embryo lethality (Tikhenko *et al*. 2011).

2.4.3. Wheat-triticale crosses

The major concern regarding the introduction of transgenic crops is the successful production of hybrid seed by pollen from transgenic crops with similar or related crops (Hills *et al*. 2007). During the process of pollen drift transgene introgression could occur, which could enter the food chain that may possibly harm the conventional market (Hills *et al*. 2007). Hybridization depends on: i) the sympatry of species involved, ii) the synchronous pollen production of the pollen donor and the stigmatic receptivity species, iii) the viability of the hybrid seeds produced, and iv) the genetic compatibility of the species (Kavanagh *et al*. 2010).

Small grain, which includes: spring wheat (*T.aestivum*), durum wheat (*T. durum*), rye (*S. cereale L.*) and triticale (*X Triticosecale Wittmack*) are closely related (Kavanagh *et al*. 2013). Spring wheat and durum wheat provided the initial maternal components and rye the initial paternal components of the triticale genome (Oettler 2005). Triticale is primarily self-fertilizing, but considerable outcrossing may occur, as fertilization was recorded as far as 30m from the pollen source (Yeung & Larter 1972). Triticale has been reported to outcross with common wheat and rye (Guedes-Pinto *et al*. 2001; Lelley 1992), but crossability with wheat was higher than with rye (Lelley 1992). Hills *et al*. (2007) observed that outcrossing between triticale with common wheat and durum wheat was significantly higher when triticale acted as the pollen donor, with outcrossing ≥75% and ≥69.5% respectfully, and observed outcrossing ≤23.9% and ≤3% when triticale was used as the pollen receptor. It was also observed that although outcrossing between triticale and common wheat occurred at a higher frequency with triticale as the pollen donor, these outcrosses rarely produced viable seed (Hills *et al*. 2007). Similar results were obtained in a study conducted by Coetzee (2011) where outcrossing between spring common wheat and spring triticale was higher, 48.7 to 66.4% when triticale was the used as the pollen donor, than when triticale was used as the pollen receptor, 5.4 to

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24.2%. A lower frequency F1 hybrid emergence was obtained with triticale as the pollen donor (<4%), compared to wheat as the pollen donor (12.5% - 44%).

Kavanagh *et al*. (2013) quantified pollen mediated gene flow from triticale to parental crop species under Canadian field conditions. They observed that the frequency of hybrids between triticale and spring wheat (0.0001% and 0.0005%), as well as triticale and durum wheat (0.0006%) was very low. Kavanagh *et al*. (2013) concluded that the occurrence of hybrids does not pose a considerable risk to the development of GM triticale. Badiyal *et al*. (2014) investigated the effect of different climatic regiments in the Himalayas on the crossability of diverse genotypes of triticale and wheat. Within their study they found significant genotypic and environmental variations for seed set for the two agroclimatic zones, with seed set being 39.53% and 45.37% for short and long day climates respectively, indicating the influence of environmental factors on crossability. Badiyal *et al*. (2014) found the variable frequencies of seed set among the different crosses indicated that the crossability of wheat and triticale is greatly influenced by the genetic composition of the parental species involved.

2.5. BLUE AND PURPLE GRAIN AS PHENOLOGICAL MARKERS

Anthocyanins present in the pericarp layer causes the purple grain colour whereas the blue grain colour is caused by anthocyanins in the aleurone layer (Zeven 1991). Figure 2.3 illustrates the differences in grain colour of purple wheat and blue triticale in comparison to common red hard triticale. The anthocyanin predominantly present in the purple pericarp include cyanidin 3-glucoside (Abdel-Aal & Hucl 2003), and is caused by genes for purple pericarp (*Pp*) (Martinek *et al*. 2013). The blue colour of grain is determined by the gene for blue aleurone, *Ba* (Martinek *et al*. 2013). The anthocyanins most abundant in the aleurone layer of blue grain wheat included: cyanidin 3-glycoside and delphinidin 3- glucoside (Abdel-Aal *et al*. 2006), where cyanidin 3-glycoside and cyaniding 3-rutinoside were present is much lower quantities, in comparison to purple grain (Knievel *et al*. 2009). The total amount of anthocyanin within blue grain generally seems to be higher than for purple grain (Jaafar *et al*. 2013). The anthocyanin content in wheat is significantly influenced by environmental conditions and thus subjected to year x site x management interactions (Jaafar *et al*. 2013). Abdel-Aal & Hucl (2003) stated that anthocyanins in the pericarp of purple grained wheat are more prone to environmental effect due to their location.

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Figure 2.3: Visualising seed colour from left to right: purple wheat, common red hard triticale, and blue aleurone triticale.

To wheat producers, millets and bakers, as well as food processors, blue and purple grained wheat are not well known. In contrast with blue and purple wheat - white and red hard common wheat as well as amber durum wheat are well known (Abdel-Aal & Hucl 1999). Purple grain wheat is considered to be good for human health as it contains and is rich in natural pigments, vitamins, amino acids, proteins, as well as beneficial micro-elements (Li *et al*. 2010). The blue grained wheat can be used for speciality foods and food colorants, due to the active roles that anthocyanins play in human metabolic activities, i.e. anti-oxidant activity, anti-inflammatory, and hypoglycemic effects (Abdel-Aal *et al*. 2006).

Purple grain wheat was first reported by Ludwig Wittmack in 1879 (Jaafar *et al*. 2013). The genes for purple pericarp (*Pp*) were transferred to common hexaploid wheat from tetraploid wheat (*Triticum turgidum* L. subsp. *abyssinicum* Vavilov), that originated in the Abyssinian region in Ethiopia (Martinek *et al*. 2014). Several genes have been identified for purple pericarp (Martinek *et al*. 2014). Arbuzova & Maystrenko (2000) identified two genes: *Pp1* and *Pp2,* on chromosomes 7B and 7A respectfully in a variety named “Purple Feed”, with the use of monosomic analysis. In another variety named “Purple” the genes *Pp1* and *Pp3* were identified by Dobrovolskaya *et al*. (2006). Dobrovolskaya *et al*. (2006) discovered that Pp3 comprised of two alleles that were named *Pp3a* and *Pp3b*, that are located at the centromeric region of chromosome 2A. They also described a complementary effect for the *Pp1* and *Pp3* genes (Dobrovolskaya *et al*. 2006). Within their study Li *et al*. (2010) found two genes for purple grain in “Jinzi 439” that was located on 2AS and 3AL respectfully. Where the purple grain gene on chromosome 2AS was linked to the SSR marker *Xgwm47* at a distance of 34.7cM, and the purple grain gene on chromosome 3AL was linked to the SSR marker *Xgwm155* with a distance of 14.7cM.

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No blue grained crops were reported prior to the first half of the 20th century, where blue grained wheat types were developed by interspecific crosses between wheat and wheat relatives (Zeven 1991; Jaafar *et al*. 2013). The fist blue grained wheat was reported by Kattermann (1932). These crosses were originally carried out to transfer genes for disease resistance, winter hardiness, perennial habit, forage traits and yield components (Jaafar *et al*. 2013). The European "Blaucorn" germplasm source was shown to be einkorn wheat (*Triticum boeoticum* Bioss.) or *Triticum monococum* L (Zeven 1991). Other blue grained wheat types were shown to have resulted from interspecific crosses with *Agropyron elongatum* (syn. *Thinopyrum ponticum*), a decaploid tall wheatgrass (Zheng *et al*. 2009), and other wheat wild relatives (Zeven 1991). The blue aleurone gene in *T. boeoticum* was located on chromosome 4A and displays a form of incomplete dominance (Dubcovsky *et al*. 1996). In wild relative, *Th. Bessarabicum*, the blue grained gene was located on chromosome 4J (Shen *et al*. 2013). Two blue grained genes have been identified for the blue pigmentations in the aleurone layer in grain: *Ba1* and *Ba2* (Martinek *et al*. 2014). The *Ba1* gene was transferred by an entire chromosome arm from *Th. ponticum* (2n = 10x = 70) to wheat and was incorporated into chromosome 4B. Zheng *et al*. (2006) located the locus (loci) confer the blue grain trait in the region 0.71 to 0.80 on 4AgL using a set of translocations that showed different seed colours. The *Ba2* gene was transferred from *Tr. monococcum* L. (2n = 2x = 14, genome AA) to wheat (Shen *et al*. 2013). Dubcovsky *et al*. (1996) mapped the Ba2 gene near the centromere on chromosome arm 4AL. The *Ba2* gene is incompletely dominant to non-blue aleurone alleles present in white grained cultivars, thus resulting in a hybrid that exhibit an intermediate phenotype of seed colour, this differs from the *Ba1* gene that is completely dominant (Shen *et al*. 2013).

Historically cross pollination within wheat cultivars has not been of much concern to wheat producers (Hanson *et al*. 2005). But recently with the introduction of new legislation on genetically modified wheat cultivars there is a renewed interest within wheat production in how to ensure genetic purity within wheat (Loureiro *et al*. 2012). Within the risk assessments of genetically modified (GM) crops, the use of molecular markers could be a valuable tool (Kavanagh *et al*. 2013). The quantification of rare interspecific gene flow between GM crops and their wild and weedy relatives or other crops, continues to be a major concern within the risk assessment for regulation of GM crops (Kavanagh *et al*. 2013). Inter- and intraspecific gene flow is generally quantified by using a variety of phenotypic markers (i.e. herbicide resistance and seed colour) (Kavanagh *et al*. 2013).

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The blue aleurone trait, when crossed with a male-sterile line, has also been useful in determining the amount of selfing and/or outcrossing in lines (Keppenne & Baenziger 1990). Assuming that apomictic forms in wheat may exist at rare rates, the xenia effect of blue aleurone wheat cultivar, "Sebesta Blue", could be used as a visible marker to detect apomixis (Morrison *et al*. 2004).

The anthocyanin colouring of the grain aleurone and/or pericarp has been used in many studies to quantify pollen mediated gene flow with wheat and/or triticale on field scale (Jaafar *et al*. 2013). Griffin (1987) used the purple pericarp trait as a pollen donor to determine outcrossing levels in New Zealand wheat cultivars. Some of the examples where the blue aleurone trait used, include: i) using blue aleurone wheat as a pollen source to reassess currently recommended minimum isolation distances in Canadian spring wheat (Hucl & Matus-Cadiz 2001); ii) determine pollen mediated gene flow among winter wheat cultivars in the United States (Hanson *et al*. 2005); iii) determine pollen mediated gene flow in spring wheat at distances within 10km radius from the pollen source in Canada (Matus-Cádiz *et al*. 2007); iv) determine pollen mediated gene flow among spring wheat cultivars in South Africa (Coetzee 2011); and iv) assess the potential for pollen mediated gene flow between spring wheat and triticale in Canada (Kavanagh *et al*. 2012; Kavanagh *et al*. 2013).

2.6. MOLECULAR MARKERS AND PLANT BREEDING

In plant breeding the main objective is to improve existing cultivars, which are deficient in certain a trait or traits, by crossing them with cultivars that possess the desired trait or traits (Varshney *et al*. 2007). Molecular markers and molecular breeding strategies offer plant breeders and geneticist possibilities to overcome many of the problems faced during conventional breeding (Varshney *et al*. 2007). Molecular markers have been used in recent years to examine the relationship between individuals, map useful genes, and construct linkage maps (Kalia *et al*. 2011). These markers have also been used in marker assisted selections (MAS) and backcrosses, population genetics and phylogenetic studies (Kalia *et al*. 2011).The progress that has been made in DNA based marker systems has advanced our understanding of genetic resources (Kalia *et al*. 2011).

Molecular markers are broadly classified into six groups: i) Hybridization-based DNA markers; ii) PCR-based DNA markers; iii) microsatellite-based DNA markers; iv) Expressed sequence tag (EST)-based DNA markers and v) Retrotransposon/miniature-inverted repeat transposable element (MITE)-based markers. An example of Hybridization-based DNA markers include

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restriction fragment length polymorphism (RFLP). PCR-based DNA markers are categorised based on the type of primers used as either multiple arbitrary amplicon profiling (MAAP) markers or multiple amplicon profiling (MAP) markers. An example of a MAAP marker is random amplified polymorphic DNAs (RAPDs). A MAP maker example is amplified fragment length polymorphism (AFLP). Microsatellite-based DNA markers include microsatellite-primed PCR (MP-PCR). Examples of Expressed sequence tag (EST)-based DNA markers include expressed sequence tag—single nucleotide polymorphism (EST-SNP). Retrotransposon/miniature-inverted repeat transposable element (MITE)-based markers include Sequence-Specific Amplified Polymorphism (SSAP). Microsatellite- and minisatellite-based markers include simple sequence repeats (SSRs). Other markers include: sequence-tagged sites (STS), allele-specific associated primers (ASAP), cDNA-AFLP, and diversity array technology (DArT) (Sehgal *et al*. 2008). The most of the above mentioned molecular markers are developed from genomic DNA libraries (i.e. RFLPs and SSRs) or random PCR amplification of genomic DNA (RAPDs) or both (i.e. AFLP) (Varshney *et al*. 2007).

The above mentioned molecular markers can also be classified as i) low throughput (RFLPs), ii) medium throughput (RAPDs, SSRs and AFLPs), iii) high throughput (SNPs and DArt), and iv) ultra-high throughput marker systems (genotyping-by-sequencing, GBS) (Mir *et al*. 2013). Recent advances in next-generation sequencing technology have provided an unmatched discovery and characterisation of molecular polymorphisms. GBS is an approach that identifies and genotypes the molecular polymorphisms simultaneously (Mir *et al*. 2013).

Available sequence data for genes that are obtained through genome- and/or EST (expressed sequence tag) sequencing projects make the development of molecular markers possible (Andersen & Lubberstedt 2003). These markers are developed from the transcribed region of the genome and are commonly revered to as ‘genic’ or ‘functional’ markers (FMs) as the assumed function can generally be deduced from these markers (Andersen & Lubberstedt 2003). Because of the complete linkage that FMs form with potential trait-locus alleles they are seen as superior to random DNA markers, that are obtained from genomic DNA (Varshney *et al*. 2006). ESTs have been used to identify SSRs and SNPs and to develop genetic molecular markers in several small grain species (Varshney *et al*. 2006). FMs are also used as an important source for estimating, through comparative mapping, the functional variation in natural and breeding populations (Varshney *et al*. 2006). Molecular markers have been used extensively in characterising traits within cultivated species and are thus considered valuable for crop improvement (William *et al*. 2007). When charactering genes associated with target

traits it is important to have dense linkage maps with evenly distributed markers available (William *et al*. 2007).

Within plant breeding microsatellite or SSRs markers have been used most extensively, among the different classes of molecular markers, as they can be readily amplified by PCR as well as the large amount of allelic variation at each locus (Miah *et al*. 2013). Microsatellites are tandem repeated motifs of 1-6bp and have a frequent occurrence in all prokaryotic and eukaryotic genomes analysed to date (Zane *et al*. 2002). They offer hyper-variability, reproducibility, have a multi-allelic nature, co-dominantly inheritance, chromosome specificity, capable to automation and high throughput genotyping, relative abundance and have good genome coverage (including organelle genomes) (Parida *et al*. 2009; Kalia *et al*. 2011). These markers have also been utilized in localizing genes to chromosomes, identifying quantitative trait loci (QTL) for yield and quality traits, characterization of wheat varieties and germplasm, as well as marker assisted selection and backcrossing (Siddig *et al*. 2013).

The parental material, within plant breeding, can be better characterised through the use of markers (William *et al*. 2007). This will then lead to an increased efficiency and effectiveness of parental selection for crossing, as well as tracking of the genes in the segregating progenies within the selection process (William *et al*. 2007). An increasing number of plant breeders are utilizing MAS in the public and private sector, but the main restriction to the wide-spread use of MAS is the cost that is associated with the use of it (William *et al*. 2007). The nature of the breeding program determines the traits of interest, if markers are available for the specific traits of interest MAS could be a useful tool in breeding plants for the specific trait of interest. The utilization of MAS is thus dependent on the availability of markers for traits of interest (William *et al*. 2007).

The development and commercialization of new wheat cultivars, in particular those that are GM, has caused great interest in information about the distance and amount of gene flow that may occur in wheat (Waines & Hegde 2003). This information could be used to manage gene flow in wheat breeding, identity preservation, or other applications (Waines & Hegde 2003). The accurate identification of rare hybridization events is critical in risk assessment and relying solely on phenotypic markers is thus problematic, especially if the trait is subject to incomplete penetrance or variable expression (Kavanagh *et al*. 2013). It is generally assumed that hybrids are morphologically intermediate (Kavanagh *et al*. 2013), but hybrids can be unpredictable (Rieseberg & Ellstrand 1993). The utilization of molecular markers in assessing environmental

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biosafety could supplement existing screening methods and thus overcome such limitations (Kavanagh *et al*. 2013).

2.7. SMALL GRAIN POLLEN DISTRIBUTION AND GENE FLOW

Gene flow is defined as the movement between populations that results in genetic exchange (Hedrick 1995). 'Potential gene flow' refers to pollen and seeds deposition from the source as a function of distance (Levin & Kerster 1974). The 'actual gene flow' refers to the occurrence of fertilization and the establishment of reproductive individuals as a function of the distance from the pollen source (Levin & Kerster 1974). Pollen movement and hybridization or the direct movement of seed or vegetative propagules are all causes of gene flow (Ennos 1994). The main mode of gene flow in flowering plants is through pollen dispersal (Levin & Kerster 1974). Pollen dispersal can also provide the mechanism of gene flow into populations of the same species or sexually compatible relatives (Levin & Kerster 1974). The rate at which gene flow occurs varies greatly among crops due to their different modes of pollination, which can range from highly autogamous (self-fertilizing) to completely allogamous (cross-fertilizing) (Hanson *et al*. 2005). The flowering biology of the species as well as the environmental conditions during and before flowering determines the dispersal of wind-borne pollen (Waines & Hegde 2003). Pollen-mediated gene flow within wheat is influenced by: air temperature, relative humidity, rainfall, and light intensity (Hanson *et al*. 2005). The above mentioned environmental factors as well as other stress factors influence: the degree of floret opening, the length that the stigma will stay receptive, the number of anthers that are extruded, the amount of pollen that is available during the flowering period, and the time span that the pollen will stay viable (Hanson *et al*. 2005).

During flowering or pollination, the flowers open or remain closed (self-pollenating) and three anthers open and release pollen (anthesis) (Waines & Hegde 2003). In wheat, flowering normally begins midway up the spike and then proceeds up- and downwards with the opposite spikelets on the rachis usually undergoing synchronised flowering (Waines & Hegde 2003). The first floret of the spikelet flowers first, followed by the successive florets on successive days (Percival 1921). Two to six days is required for a spike to finish blooming (Waines & Hegde 2003). After the first round of flowering, the unfertilized flowers open again and expose their stigmas for another two to three days, and the ovaries stay receptive for an additional two to three days as well (Hoshikawa 1960). The genotypic differences among wheat cultivar flowering is due to the deliberate selection by plant breeders (Waines & Hegde 2003).

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Wheat is mainly a self-pollinating crop (Willenborg & Van Acker 2008), but varying levels of outcrossing can occur (Beckie *et al*. 2011). The anthers in wheat mainly shed pollen within the florets before or just after the flowers open (Nguyen *et al*. 2015). Rye, in contrast to wheat, is a cross-pollinating crop with differing degrees of self-incompatibility that has long anthers that extrude from the florets that shed large amounts of pollen grains (Nguyen *et al*. 2015). Wheat anthers have been reported to range between 2.99mm and 4.41mm in length (de Vries 1972), where rye anthers are reported to be as long as 8mm (Immonen & Anttila 1998). See Table 2.1 for summary. In a study conducted by Yeung & Larter (1972) they found the anther length as well as the amount of pollen grains per anther to differ significantly within the triticale cultivars used within their study, where the longest anther size recorded was 7.1mm. The number of pollen grains produced is positively correlated to anther size (Milohnic & Jost 1970), where rye produces an average of 42×10^5 pollen grains per spike, which is ten times the amount of pollen grains produced in wheat per spike (42×10^4) (Waines & Hegde 2003). The average amount of pollen grains produced per anther in rye is approximately 19 000 (Reddi & Reddi 1986), compared to wheat where between 856 to 3 022 pollen grains produced per anther (Waines & Hegde 2003). In a study conducted by Yeung & Larter (1972) they found pollen grains per anther that are produced in triticale ranged between 15 000 to 21 000. The quantity of pollen shed can be increased by unfavourable conditions. High stress conditions and low moisture can lead to increased open flower flowering, and thus increase the incidence of outcrossing (Waines & Hegde 2003).

About 90% of wheat pollen from the spike is dispersed by gravity and falls within 6m from the pollen source (Jensen 1968). Small amounts of wheat pollen has travelled as far as 65m from its source (Coetzee 2011), and have been detected as far as 2.75km from the pollen source (Matus-Cádiz *et al*. 2007). This is evidence that the pollen densities outside the florets are enough to lead to outcrossing (Willenborg *et al*. 2010), but it should also be kept in mind that the presence of viable pollen does not always ensure successful pollination and gene flow (Levin & Kerster 1974). In a study conducted by D'Souza (1970) it was reported that rye pollen, with moderate wind speed conditions, could be carried about 70m further and 0.4m higher than wheat pollen. Rye pollen can remain viable after release for up to 72 hours or more under optimal field conditions, where wheat pollen loses viability within 15 to 30 minutes after release (D'Souza 1970). Within triticale no viable pollen can be detected after 120 minutes (Fritz & Lukaszewski 1989). Wheat pollen viability is mainly attributed to the crop being self-pollinating (Waines & Hegde 2003). As soon as any viable pollen grains come in contact with

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the stigma surface they germinate (Chandra & Bhatnagar 1974). The location of pollen landing generally does not affect pollen germination, as the entire length of the stigma is receptive to pollen grains in wheat (Waines & Hegde 2003).

Table 2.1: A summary of the different flowering characteristics of cereal grain.

Crop	Mating System	Pollen Viability	Anther length (mm)	No. pollen grains per anther
Wheat	self-pollinating	15 to 30min	2.99 to 4.41	856 to 3 022
Triticale	self-pollinating	≤120min	7.1	15 000 to 21 000
Rye	cross-pollinating	≥72 hours	8	19 000

An important factor limiting the yield of a wheat crop is the temperature present during ear formation and anthesis (Chakrabarti *et al*. 2011). The absence of grain in any floret grown sufficiently large to contain grain is regarded as sterility within wheat (Rawson 1996; Chakrabarti *et al*. 2011). The number of viable florets that are formed as well as the effective fertilization of these florets during anthesis determines the amount of grain that develops in an ear (Evans & Wardlaw 1976). The optimum temperature for fertilization of wheat ranges between 18 to 24°C, with a minimum at 10°C and a maximum at 32°C (Hoshikawa 1960). The maximum temperature, at or below, at which development during anthesis will be zero, is 8.1°C (Slafer & Rawson 1995). For successful seed to set the following phases, which are all temperature sensitive, are necessary: i) the production and transfer of viable pollen to the stigma, ii) germination of the pollen and growth of the pollen tubes down the style, and iii) effective fertilization (Chakrabarti *et al*. 2011). High temperature during the grain filling period in wheat can results in a significant drop in kernel dry weight at maturity, thus reducing the yield and quality of wheat (Stone & Nicolas 1995; Wardlaw & Moncur 1995). Cold temperatures cause agricultural losses in especially sub-tropical and temperate grain crops (Thakur *et al*. 2010). Plants that are exposed to cold temperatures during reproduction display reduced metabolic rates that lead to low yields (Thakur *et al*. 2010). The combination of boron deficiency and cold temperatures during the reproductive development of wheat cause the failure of grain to set, in that cold temperatures reduced the plants response to boron (Subedi *et al*. 1998). Subedi *et al*. (1998) found the principle effect of cold temperature in spring wheat to be the impairment of pollen viability, in that anthers develop poorly, are small, shrivelled and rarely released pollen. They also found the adverse effect of cold temperatures was irreversible, even if ear emergence and anthesis of the stressed plant was in ambient temperatures (Subedi *et al*. 1998).

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The duration of pollen viability can be adversely influenced by temperature and relative humidity (Waines & Hegde 2003). Extremely cold or hot temperatures are unfavourable for pollination and fertilization (Waines & Hegde 2003). Where, low temperatures reduce the duration of pollen shed and high temperatures reduce both the duration of pollen shed as well as pollen viability (Major 1980). Humid weather makes the pollen grains heavy and thus reduces the distance the pollen can disperse from the parent plant. Whereas, dry weather causes the pollen to dehydrate and also reduce the viability of the pollen, thus reducing the chance of effective gene flow (Waines & Hegde 2003). However, the combination of humid weather and light wind may favour gene flow (Dowding 1987).

Outcrossing rates within wheat are usually lower than 1%, but exceptions with higher outcrossing rates have been recorded (Hucl & Matus-Cadiz 2001). The rate of outcrossing is related to genotypes and environmental conditions ((Waines & Hegde 2003). Within large commercial fields high outcrossing rates are of little concern as most of the outcrossing that will occur will be intra-varietal (Hucl & Matus-Cadiz 2001). But, with small plots it is the complete opposite, where there are different genotypes planted in close proximity and high outcrossing rates could thus result in off-types (Hucl & Matus-Cadiz 2001). The outcrossing frequencies within 12 winter wheat varieties was found by Martin (1990) to vary from 0.1% to 5.6 % over 30cm. Pollen-mediated gene flow, with the use of blue aleurone spring wheat, was found by Hucl & Matus-Cadiz (2001) to have frequencies ranging of 3.8% adjacent to the 25m^2 source plot to a frequency of 0.2%, 30m form the source plot. Thus even though pollen-mediated gene flow occurs naturally under field conditions and at considerable distances, distance can still be used to limit pollen-mediated gene flow (Willenborg *et al*. 2010).

2.8. MINIMIZING GENE FLOW

Populations can be reproductively separated either spatially or temporarily, as to ensure that they are not affected by pollen-mediated drift (Brûlé-Babel *et al*. 2006). ‘Temporal isolation’ or ‘temporal separation’ is the deliberate disruption of flowering synchronization between plots or fields, which is a prerequisite for hybridization within plants (Halsey *et al*. 2005). This disruption of the flowering synchronization is usually achieved by moving sowing dates with the aim to accomplish reproductive isolation (Halsey *et al*. 2005). Even with temporal isolation, it will not be possible to ensure the complete absence of transgenic impurities in non-transgenic wheat (Willenborg *et al*. 2010). But, with the incorporation of sufficient temporal [emergence offset by 100 growing day degrees (GDD)] and spatial isolation it should be possible to meet

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tolerance levels of between 1 to 5% (Willenborg *et al*. 2010). In order to prevent pollen contamination between crops, the following will need to be taken into consideration: i) the flower characteristics of the crop, ii) the compatibility of the crop with neighbouring crops, iii) the pollen quality and viability, iv) mode of pollen dispersion, as well as v) environmental conditions (Loureiro *et al*. 2007).

Plants that are sown around a pollen source or recipient field can function as a physical barrier (Devos *et al*. 2005). A physical barrier will result in the reduction of pollen from the airflow by impaction and filtering of the pollen due to turbulence (Treu & Emberlin 2000). In a study conducted by Foetzki *et al*. (2012) they found a border crop as a pollen barrier as well as isolation distances to commercial fields and seed propagation fields to be very effective measures in reducing PMGF to an undetectable level in spring wheat. Foetzki *et al*. (2012) used two spring wheat cultivars that were enhanced for fungal resistance and herbicide tolerance as the pollen donors, and a conventional wheat variety "Friscal" as pollen receptor plots in the border crop and around the experimental field up to a distance of 200m. Only three outcrosses was observed by Foetzki *et al*. (2012) in the 185 000 seedlings tested, even though the environmental conditions were favourable and flowering synchrony was observed.

Increasing the plant population density in spring wheat to more than the recommended planting density of 300 plants m^2 has no benefit in reducing plant-mediated gene flow further (Willenborg *et al*. 2009). Within their study Willenborg *et al*. (2009) found a critical planting density in spring wheat that ranges from 174 to 200 plants m^2. Below this critical planting density the PMGF will increase exponentially with a decrease in planting density. The frequency of gene flow that was observed by Willenborg *et al*. (2009) was consistently low and lower than the 0.9% threshold required by the European Union for labelling transgenic content within products. Willenborg *et al*. (2009) concluded that planting density will thus be very important for non-transgenic wheat producers to maintain genetic purity, due to the low frequencies of PMGF observed within their study.

To ensure genetic purity the distance at which genetic drift takes place will need to be determined as well as the frequency at which this occurs (Hanson *et al*. 2005). This knowledge will then be used to determine isolation distances that could be used to ensure a certain degree of genetic purity; as well as develop management practices within commercial wheat production to prohibit pollen-mediated genetic drift (Hanson *et al*. 2005). The use of isolating distances is one of the most effective methods in preventing outcrossing and pollen contamination between compatible genotypes (Loureiro *et al*. 2007).

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For a similar set of isolating distances between different experiments the outcrossing rates varied greatly, this could be due to differences in: i) weather conditions during the time of blooming, ii) day and night temperatures that differ between the different experiments, iii) light intensity, iv) relative humidity as well as v) wind direction and speed. These are all the factors that have a major influence on pollen dispersal (Loureiro *et al*. 2007).

Seed production at all registered establishments in South Africa are monitored by the Department of Agriculture, Forestry and Fishery (DAFF) and the South African National Seed Organisation (SANSOR) monitor (African Center for Biosafety 2012). Since 1989, SANSOR has been the designated authority that manages and executes all functions concerning seed certification on behalf of the government (SANSOR 2015). This includes the National Seed Certification Scheme, and international seed schemes, such as: Association of Official Seed Certifying Agencies (AOSCA), Organisation for Economic Co-operation and Development (OECD) and South African Development Community (SADC) (SANSOR 2015). The South African seed industry is regulated under the Plant Improvements Act 1976 (Act No.53 of 1976), to ensure the orderly trade in seed (DAFF 2005). SASOR administers seed certification schemes under the Plant Improvements Act 1976. In South Africa the participation in seed certification is voluntary. The main objective of seed certification is to release seed of superior quality and to guarantee the genetic quality (true-to-type, germination, and physical purity) of the seeds by means of certificate, seal, and label (DAFF 2005).

South African seed units have to be registered within 28 days of planting, where an authorized inspector conducts a series of quality checks at the various physiological stages of the production process (African Center for Biosafety 2012). These quality checks ensure that all the quality standards are met, isolation distances are correct, that the fields are free of weeds, and that all ‘off type’ plants are removed from the fields (Setimela *et al*. 2006). The early season inspection checks isolation distances as to ensure no cross-contamination. Middle season inspection checks the uniformity, tillering, and other physiological traits of the plants. At the end of season inspection samples are taken to test for germination and other laboratory tests (Tinsley 2009), where half of the seed must be saved as reserve stock (African Center for Biosafety 2012).

The isolation requirements for the production of basic, pre-basic, and certified wheat seed in South Africa requires an isolation distance of 5m (SANSOR 2008). The wheat seed certification requires a maximum allowed threshold of mixing with other seed at 0.3%, no more than 0.02% weed seeds be present, and a maximum of 1% other material present within the

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seed (i.e. plant material, insects, etc.). Triticale basic, pre-basic, and certified seed production also requires an isolation distance of 5m. The certification of triticale seed requires a maximum threshold of 0.5% other seed present, no more than 0.2% weed seed present, and a maximum of 2% other material present within the seed (SANSOR 2008). With rye basic and pre-basic seed production an isolation distance of 500m is required, while the production of certified rye seed requires an isolation distance of 300m. The certification requirement for rye seed requires a maximum threshold of 0.5% other seed present, no more than 0.2% weed seed present, and a maximum of 2% other material present within the seed. Within triticale and rye certified seed a physical limit of 1 seed common wild Oats (*Avena fatua*) is allowed in 400g seed (SANSOR 2008).

Hucl & Matus-Cadiz (2001) found in their study that the isolation distance of 10m for the production of select seed, as well as the isolation distance of 3m for the production of Foundation, Registered, and Certified seed, in Canada was satisfactory for spring wheat cultivars with low outcrossing rates. These isolation distances were found not to be satisfactory for cultivars with high outcrossing rate, where contamination was detected at 27m from the pollen source (Hucl & Matus-Cadiz 2001). Hucl & Matus-Cadiz (2001) concluded that a minimal isolation distance of 30m was required within spring wheat cultivars that exhibited higher than normal outcrossing rates, for the production of pedigreed seed. In the study conducted by Hanson *et al*. (2005) they concluded that an isolation distance of 45m would be more satisfactory for winter wheat cultivars grown in the United States than the 30m found by Hucl & Matus-Cadiz (2001) for spring wheat cultivars in Canada. Loureiro *et al*. (2007) conducted a field trial on spring wheat in semi-arid conditions in Spain, they observed viable pollen at 14m from the pollen source, and the maximum distance at which cross pollination decreased below 1% at 8m from the pollen source. Beckie *et al*. (2011) conducted a study on spring wheat in Canada and observed 0.2% PMGF at the common border that declined exponentially with increasing distance, where 80m from the donor field was the maximum distance PMGF was detected.

Kavanagh *et al*. (2012) quantified PMGF in spring triticale in Canada and observed the highest average PMGF (3.5%) adjacent to the donor block (blue aleurone triticale) that rapidly declined to 0.09% at 50m from the donor block, where PMGF corresponded to the prevailing wind direction during flowering. They concluded that PMGF in triticale is similar to spring wheat and should not prevent the co-existence of GM and conventional triticale.

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CHAPTER 3 – MATERIALS AND METHODS

3.1. INTRODUCTION

A topic that is of considerable global interest is the use of genetic engineering to improve crops and the accompanying cultivation of these transgenic crops (Willenborg *et al*. 2010). When considering the cultivation of GM crops or determining the biosafety requirements for the experimental release of GM crops, the possible gene flow between GM and non-GM crops remain a major concern (Foetzki *et al*. 2012).

Bread wheat is widely planted and is considered one of the most important food crops, as it is a primary source of proteins and carbohydrates for the world population (Langridge 2012; United States Department of Agriculture 2014). Triticale is mainly used as animal feed, silage or cover crop (Roux & Marais 1996); but is also considered a valuable genetic resource for crop improvement of wheat (Bassu *et al*. 2013). GM wheat and triticale cultivars are in the process of being developed and field tested (Doshi *et al*. 2007; Kavanagh *et al*. 2012; Wree & Sauer 2015). When these GM crops are developed and released for cultivation it is likely that they will be grown next to conventional (non-GM) wheat and triticale cultivars (Waines & Hegde 2003). The understanding of PMGF between commercially used South African wheat and triticale cultivars will thus be useful in the development of crop management practices as to obtain an acceptable level of crop purity within both GM and non-GM cultivars.

Figure 3.1 that was drawn using Lucid Software (2015), illustrates the workflow for this study. For the first objective of this study: blue aleurone and purple pericarp markers was sourced out of literature and optimised. Anthocyanin quantification procedures were also sourced and optimized. This was followed by second objective of this study that included the measuring, planting, and harvesting of the field trial. The third objective involved the planting of the F1 seeds that were harvested from the field trial. The F1 triticale seeds were planted in a single greenhouse and the F1 wheat seeds were planted in a growth chamber. This was followed by the fourth objective: that included the genotypic (wheat) and phenotypic (triticale) screening of the progeny to identify crossing events.

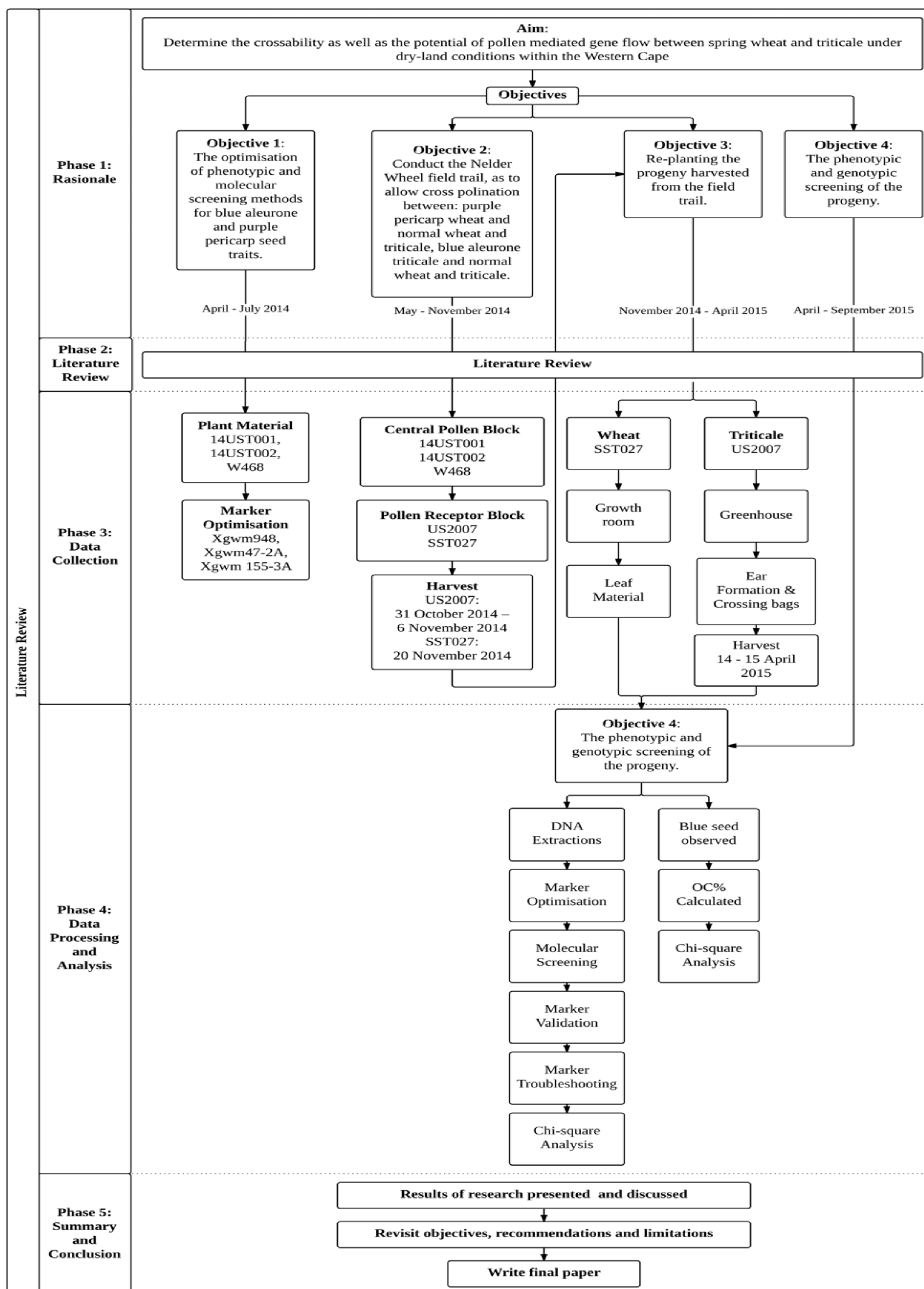

Figure 3.1: An illustration of the workflow of this study. The work flow of this study was drawn using © 2015 Lucid Software Inc.

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3.2. OPTIMIZATION OF SCREENING METHODS FOR BLUE ALUERONE AND PURPLE PERICARP TRAITS.

The following methods were optimised to screen the F1 progeny, which will be harvested from the Nelder wheel field trial, and be able to detect and determine the distance and rate at which PMGF and outcrossing occurred within the Nelder wheel field trial.

3.2.1. Phenotypic screening

i. Plant material

The plant material used to optimise the phenotypic screening methods for purple and blue seeds included: purple pericarp wheat, cultivar “W468”, which was used as a pollen donor in the Nelder wheel field trial, and blue aleurone wheat, cultivar “Cltr1202STR(Ba)”, which was used to develop the blue aleurone triticale cultivars, “14UST001” and “14UST002” that was also used as pollen donors in the Nelder wheel field trial. “14UST001” and “14UST002” was developed by Ntladi (2011) by making the following crosses: for “14UST001” the following crosses were conducted Cltr1202STR(Ba)*US2007, and for “14UST002” Cltr1202STR(Ba)*US2008. The blue and purple seeds were sourced out of bags that were stored in a seed store room at Welgevallen Experimental farm, Stellenbosch, South Africa (33°56’34.61”S, 18°51’59.11”E).

ii. Anthocyanin Quantification Protocol

The method that was developed by Syed Jaafar et al. (2013) was adjusted and used to quantify the Total Anthocyanin Content (TAC) within the grain samples: 20g of the blue and purple seed samples was milled into wholemeal flour using a model Twister cyclone mill at 12 000rpm and equipped with a 0.5mm sieve. Eight millilitre solvent (Methanol/ 1M HCl, 85:15, v/v, pH 0.95 ± 0.05) was added to 1g wholemeal flour in a 50ml centrifuge tube. The sample was premixed and put on a shaker for 60 minutes (min) at 150rpm, and was then centrifuged at 4 000rpm for two minutes and 30 seconds (s) using a Universal 32 centrifuge (Hettich Zentrifugen, Labrotech, Cape Town, South Africa). The solution was then mixed using a Vortex mixer and centrifuged again for two minutes and 30s at 4 000rpm. Samples were then decanted, as to separate the extracts from the solids. The extraction was performed three times for each sample. Supernatants was collected in a volumetric flask and the final volume adjusted to 25ml with the extract solvent (Figure 3.2a). Two replicas were prepared for the blue (“Cltr1202STR”) and purple (“W468”) seed samples. TAC was determined using a spectrophotometer (Milton Roy, Spectronic 501/601): where the extracted anthocyanins were

diluted (3:1) for the samples to fit onto the standard curve. Measurements were taken at 525nm against a reagent blank (solvent) and TAC was expressed as cyaniding 3-glucoside equivalents (ppm) (Figure 3.2b).

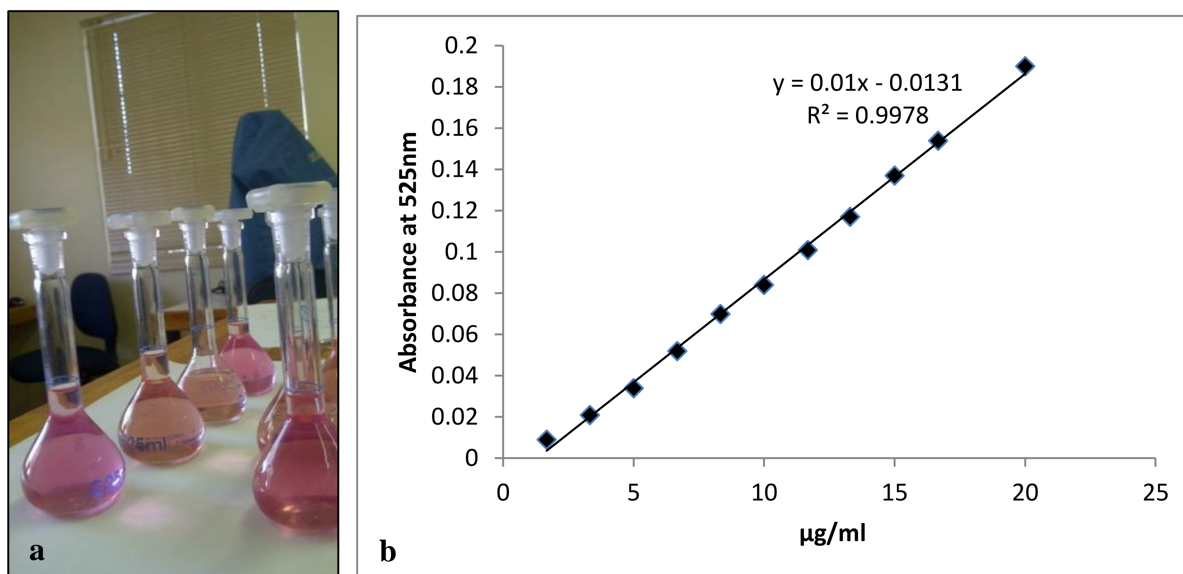

Figure 3.2: a) Supernatants collected in volumetric flasks, where the pink solution is the blue aleurone wheat and the orange solution the purple wheat seeds, **b**) Standard curve for cyaniding 3-glucoside.

3.2.2. Molecular screening

i. Plant material

The plant material used to optimise the molecular screening methods for purple and blue seeds includes the plant material that was used as pollen donors and pollen receptors in the Nelder wheel field trial. The pollen donors include the purple pericarp wheat "W468" and blue aleurone triticale cultivars "14UST001" and "14UST002". The pollen receptors included spring wheat, cultivar "SST027", and spring triticale, cultivar "US2007". Purple pericarp spring triticale "Amethyst*US2007" as well as the triticale parent that was used to develop the blue aleurone triticale - "AgBeacon" (US2008) was also used to optimise the molecular screening methods.

Four seeds were planted separately for each cultivar in plastic planting pots (5L) that were filled with crusher dust (AFRIMAT, Smalblaar Quarry, Rawsonville) within a single greenhouse at Welgevallen experimental farm. The plants were irrigated daily through an automated hydroponic system with a nutrient solution of 164g Sol-u-fert-T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa), 77ml potassium nitrate diluted in 100L H_2O, 0.05% Jik (household detergent containing 3.5% sodium hypochlorite, Reckitt and Colman South Africa Pty Ltd.,

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Elandsfontein, South Africa), and tap water. Growth conditions within the greenhouse were standard greenhouse conditions and under natural daylight length, where temperatures above 25°C were cooled using a water cooling system.

ii. DNA Extraction

The DNA was extracted using the CTAB (cetyltrimethylammonium bromide) DNA extraction method from Doyle and Doyle (1990), with a few adjustments: where the leaves of the seedlings were cut, when sufficient leave material had grown, into 2ml microcentrifuge tubes containing 3 stainless steel bearings. Five hundred microlitres 2% (m/v) CTAB extraction buffer [1.4 M NaCl, 20 mM Na_2EDTA (pH 8), 100 mM Tris-HCl (pH 8)] was added and the samples were grind using a Qiagen®TissueLyser (Qiagen (Pty) Ltd, Southern Cross Biotechnology, Claremont, South Africa) three times for 60s at 30Hz. The samples were then incubated in water bath for 20min at 55 to 60°C. Five hundred microliters Chloroform:Isoamyl (C:I::24:1) was then added and the samples were centrifuged in a Microfuge® 18 Centrifuge (Beckman Coulter™, Pinelands, South Africa) for 10min at 14 000rpm. The supernatant was transferred to a clean 1.8ml microcentrifuge tube and 400µl C:I was added. This mixture was then slowly mixed by inversion and then centrifuged in a Microfuge® 18 Centrifuge for 5min at 14 000rpm. The supernatant was transferred to a clean 1.8ml microcentrifuge tube, where 50µl 7.5M Ammonium Acetate was added followed by 500µl ice cold 100% ethanol. The microcentrifuge tubes were then inverted slowly several times to precipitate the DNA, the precipitate was then isolated by centrifuging the microcentrifuge tubes at 14 000rpm for 2min in an Allegra™ X-22R Centrifuge (Beckerman Coulter™, Pinelands, South Africa). The precipitate was washed twice with 70% ethanol, where the pellet was then left to air dry. Once dry the DNA was then re-suspended in DNase free water, where 30µl DNase free water was used for a small pallet and 50µl for a large pallet. The re-suspended DNA and was then stored at 4°C for a day before determining the quality and quantity of the DNA extracted. The quality and quantity of the extracted DNA was determined by using a Nanodrop ND-100® spectrophotometer (Thermo Scientific, Gauteng, South Africa). The DNA samples were then diluted and adjusted to a final concentration of 100ng/µl and stored at 4°C, while all the stock DNA was stored at -20°C until needed.

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iii. SSR Validation

Two microsatellite markers specific to purple pericarp wheat coloration were selected out of the study conducted by Li *et al*. (2010), which included: *Xgwm47*-2A and *Xgwm155*-3A (Table 3.1). Microsatellite marker, *Xmwg948*, which have previously been completely linked to the blue aleurone locus in *T. monococcum*, DV92 x G3116 (McIntosh 1995; GrainGenes 2015), was selected for the detection of the blue aleurone coloration (Table 3.1).

The molecular markers were screened and optimized to be able to screen spring wheat and triticale, cultivars "SST027" and "US2007", harvested from the field trial, for possible hybridization events. Out of all the molecular markers that were tested, A-genome markers *Xgwm47*-2A and *Xgwm155*-3A performed the best at making distinctions between purple spring wheat and triticale with normal spring wheat and triticale (Figure 3.3). Molecular marker, *Xmwg948*, did not give consistent results, and it was decided to optimise molecular markers that was used in a previous study conducted by Botes & Bitalo (2013) for blue aleurone triticale crossing events (see section 3.4.2).

For the molecular markers the following PCR reaction was conducted: each sample contained 2GFastmix (X1), Forward Primer (0.2µM), Reverse Primer (0.2µM), DNA (100ng), with a final volume of 12.5µl. The mixture was then run with a Thermal Cycler 2720 (Applied Biosystems, Johannesburg, South Africa) with following the time regime:

i) *Xgwm47*-2A and *Xgwm155*-3A, an initial denaturation of 94°C for 3 minutes, followed by 45 cycles at 94°C for 1min, 60°C for 1min, and final extension period at 72°C for 10min;

ii) *Xmwg948*, an initial denaturation of 94°C for 3min, followed by 45 cycles at 94°C for 1min, 60°C for 1min, 72°C for 2min, and final extension period at 72°C for 10min

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Table 3.1: Microsatellite marker used to screen for hybridization events

Genome	Marker	Forward/Reverse	T_A (°C)	Expected Length (bp)
A-genome	*Xgwm47*-2A F	TTGCTACCATGCATGACCAT	60	±153
	Xgwm47-2A R	TTCACCTCGATTGAGGTCCT		
	Xgwm155-3A F	CAATCATTTCCCCCTCCC	60	±147
	Xgwm155-3A R	AATCATTGGAAATCCATATGCC		
	Xmwg948 F	GCATTCCGTTTGGACTATGG	55-58	283
	Xmwg948 R	CGTCGCCGATATTTGTAAGC		

iv. Polyacrylamide (PAGE) Gels

All the samples were run on a 6% poly-acrylamide (PAGE) gel that was run at 770 volts for 290 to 300min. Before loading the samples on the PAGE gels, the SSR products were loaded with equal volumes (10µl) of AFLP loading buffer (98% formaldehyde, 10mM EDTA pH8, 0.05% w/v bromo phenol blue, 0.05% w/v xylene cyanol FF) and denatured at 95°C for 5min and immediately quenched on ice. The samples were loaded onto the gel by firstly pre-running the gel at 770 volts for 30min. Twelve microliters of each sample was then loaded onto the gel and 4.9µl of the ladder (0.9 µl GeneRuler 50bp, 3µl dH_2O, 1µl loading dye) was loaded in the centre of the gel. Electrophoresis was then performed at a constant power of 770 volts for 327min. The band sizes for the markers *Xgwm47*-2A and *Xgwm155*-3A ranged between 147bp and 153bp (Figure 3.3).

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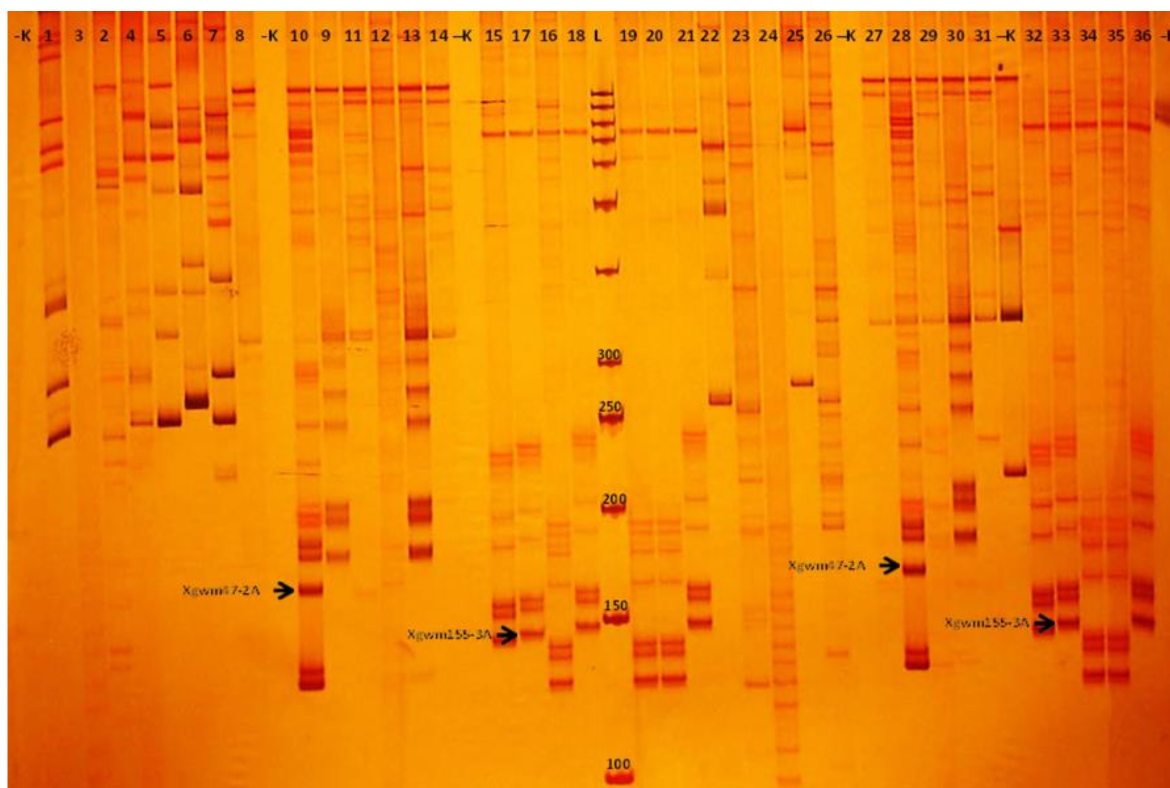

Figure 3.3: Polyacrylamide gel of *Xgwm47*-2A and *Xgwm155*-3A, where 10, 17, 28 and 33 is represents purple wheat, cultivar "W468".

The 6% PAGE gel mix preparation and casting was done as follows: 800µl of 10% Ammonium persulphate (APS), 160µl of Tetramethylethylenediamine (TEMED), and 160ml of 6% gel mix was added and mixed in a glass beaker. The mixture in the glass beaker was mixed well and the gel was cast between a long (330mm x 416mm) and a short (330mm x 394mm) glass plate, both 4mm thick. The mix was then left for an hour to set. The 6% gel mixture (500ml) was prepared by adding 71.4ml of 40% Acrylamide/bis-acrylamide solution (75g Acrylamide, 4g Bis-acrylamide) and 100ml 5X TBE (121.1g Tris, 61.83g Boric Acid, 61.83g EDTA, bring to volume (2L) with dH_2O) to 180.1g Urea.

The glass plates on which the gel was cast was prepared using the following procedure: the long and short plates were cleaned using 70% ethanol and a paper towel. The long plate was then prepared by applying a water proofing agent (c-thru) onto the entire plate using a paper towel. The water proofing agent on the plate was then left to dry for 3min and wiped dry with a paper towel until the plate was shining. The short plate was prepared by applying plate glue (± 2 ml) onto the entire plate using a paper towel. The plate glue was then left for 30s to dry and wiped dry immediately with a paper towel.

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After running the gel, it was stained to visualize the bands using the following procedure on a belly dancer: the gel was firstly treated with a fixing solution (210ml Ethanol, 10.5ml Acetic acid, 1879.5ml dH_2O) for 20min, followed by two 5min distilled water (dH_2O) rinses. The gel was then treated with a staining solution (2.1g $AgNO_3$, 2100ml dH_2O) for 20min, followed by a 10s dH_2O rinse. This was then followed by treating the gel with a developing solution (31.5g NaOH, 2100ml dH_2O, 8.505 Formaldehyde) on the belly dancer until the bands appeared. The gel was then rinsed with dH_2O and covered with a plastic film treated with 70% ethanol. A photograph was then taken of the gel and then scored on the band range for the specific marker.

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3.3. THE NELDER WHEEL FIELD TRIAL

3.3.1. Plant Material

The plant material used for the central pollen donor block included: blue aleurone spring triticale cultivars “14UST001” and “14UST002”, as well as purple pericarp spring wheat, “W468”. The pollen receptor arms comprised of spring triticale, cultivar “US2007”, and spring wheat, cultivar “SST027”. The spring triticale, cultivar “US2012”, was planted within the areas between the arms, as well as the area between the central pollen donor block and the receptor arms. The planting density used for this trial was 300 plants m^{-2} with a inter-row spacing of 17cm.

3.3.2. Field Trial Dimensions

The field trial design and location was similar to what was done in the previous study that was conducted by Coetzee (2011). The Nelder wheel field trial was conducted at Mariendahl Experimental Station (MES), Stellenbosch, South Africa (33°51'10.07"S, 18°49'11.75"E). Figure 3.4 was drawn using AutoCAD® software and illustrates the dimensions of the Nelder wheel field trial. The trial consisted of a central pollen donor block, with a diameter of 2.5 meters, which was surrounded by eight pollen receptor arms. The arms were all orientated in the cardinal and intercardinal wind directions: North (N), South (S), West (W), East (E), North-West (NW), North-East (NE), South-West (SW) and South-East (SE). Each arm contained sixteen blocks, where the first six blocks, those nearest to the central pollen donor block, were planted 1.5 meters apart and the remaining ten blocks were planted four meters apart. The last block of each arm was situated at a distance of 66.25m from the central pollen donor block, thus making the total area covered by the Nelder wheel 13 788.65 m^2. The sixteen blocks within each arm, were one meter in width and two meters in length. Half of each block was planted with spring wheat cultivar “SST027”, whilst the other half of the block was planted with spring triticale, cultivar “US2007”. The areas between the arms were planted on 22 May 2014, three weeks prior to planting the pollen receptor arms and central pollen donor blocks, to avoid gene flow between “US2012” with pollen receptor arms (Figure 3.5a). The pollen receptor blocks were sown over a course of two days, where North, North-East, East and South-East were planted on 11 June 2014; and South, South-West, West and North-West were planted on 12 June 2014. The central pollen donor block was planted on three different dates (6 June 2014, 12 June 2014, and 23 June 2014), a week apart. This was done as to maximize the overlap in flowering between the pollinator block and recipient arms. The trial was also treated with

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appropriate herbicides, insecticides, fungicides and fertilizer when needed – refer to Addendum A for the application of chemicals.

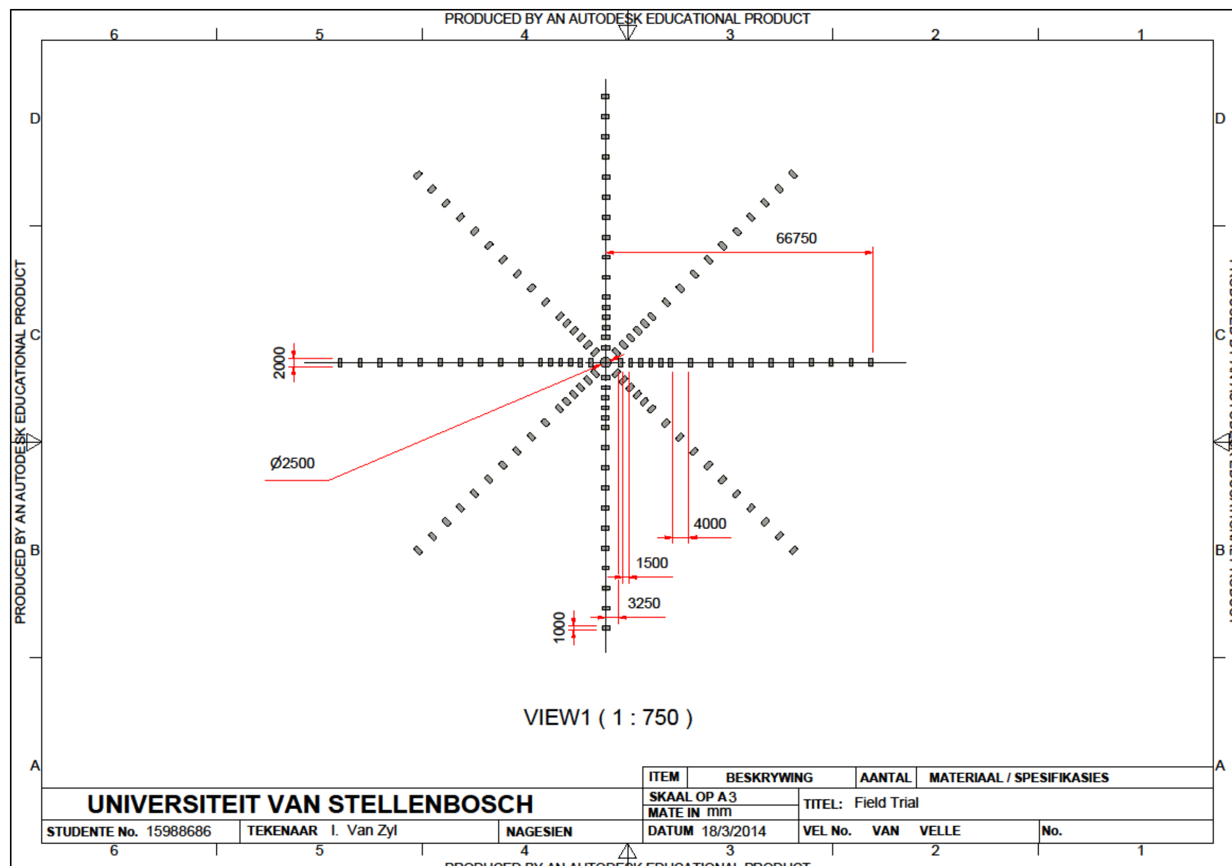

Figure 3.4: The layout and dimensions of the Nelder Wheel Field trial. The design of the field trial was drawn using AutoCAD® software.

3.3.3. Bird and Water Damage and the Re-planting of Blocks

In the same week that the central pollen donor block and the pollen receptor arms were planted, nets were cut and placed over them (Figure 3.5b). This was done to prevent the Guinnea fowl and Blue Crane birds from eating the planted seeds, as well as preventing the Egyptian geese from eating the younger plants. Some bird damage was found in one block in both the North and North-West arms and were re-planted on 24 June 2014.

Water damage due to rainfall occurred in fifteen blocks on 16 July 2014: five blocks in the North-West arm, one block in the North arm, six blocks in the East arm, one block in the South-East arm, two in the South arm, and one in the South-West arm. These blocks were re-planted on 17 July 2014, drainage paths were also made around these blocks as to prevent further water damage.

The purple seed that were planted in the central pollen donor block, cultivar "W468", failed to geminate and was re-planted on 30 June 2014, but still none of the purple seeds germinated.

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On 30 June 2014 the nets on all the blocks were raised to accommodate the larger plants. The nets of the pollen receptor arms were completely removed on 21 July 2014 and the central pollen donor block on 22 August 2014.

3.3.4. Flowering Synchrony

The heading dates of the pollen donor blocks and the pollen receptor blocks were recorded between 80 and 100 days after planting them, that was between 25 August and 2 October 2015. When a block had an ear formation of 50% the heading dates were recorded for that block. This was done to indicate whether there was sufficient overlap in flowering between the central pollen donor block and the pollen receptor arms (Figure 4.1 and Table 4.1). Heading synchrony was assessed as the number of days the receptor cultivars' heading dates were recorded together with the donor cultivar divided by the total number of heading dates recorded and multiplied by 100. After all the heading dates were collected, the area around each block was cleared using weed-eaters (Figure 3.5c and 3.5d) and an aerial photograph was then captured of the trial. This was conducted on 11 October 2014, by Gustav Ludick via drone (Figure 3.5e and 3.5f).

3.3.5. Weather Data

All the weather data was supplied by the ARC (Agricultural Research Council) and was collected from their weather station that is located at Elsenburg (33°50'47.18"S, 18°50'6.24"E), which is situated 2.1km from MES. The weather data on the relative humidity, wind speed, wind direction, temperature and rainfall was collected for the dates between 80 and 100 days after planting the pollen donor and receptor blocks (Figure 3.3). The exact time and rate of flowering is strongly influenced by the above mentioned meteorological conditions throughout the day (Leighty & Sando 1924). Where some flowers open in less than a minute and other require more or less three minutes to open (Waines & Hegde 2003). Flowering in wheat has been observed to occur typically between 04h30 and 19h00, where the most of the flowers (86%) reached their maximum opening between 09h00 and 11h30, and a second flush of flower opening between 15h00 to 17h00 (Virmani & Edwards 1983). It was decided to collect all the wind data for this study for each day a half an hour before and after sunrise and sunset. The weather data was collected to determine the viability of the pollen, as well as the possibility for the occurrence of pollen mediated gene flow over long distances.

3.3.6. Harvesting of the Nelder Wheel Field Trial

As soon as the plants reached maturity each block was harvested and bagged by hand (Figure 3.6a). Each of the 128 blocks harvested were given a number and each sample harvested from

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these blocks were labelled, threshed and bagged with the corresponding number in order to exclude mixing during harvesting. The spring wheat blocks were harvested from 31 October 2014 to 6 November 2014, and the central pollen donor block as well as the spring triticale blocks was harvested on 20 November 2014. After being harvested the ears were threshed with a stationary threshing machine (Wintersteiger (Pty) Ltd, LD 180) at the Welgevallen experimental farm, Stellenbosch, South Africa.

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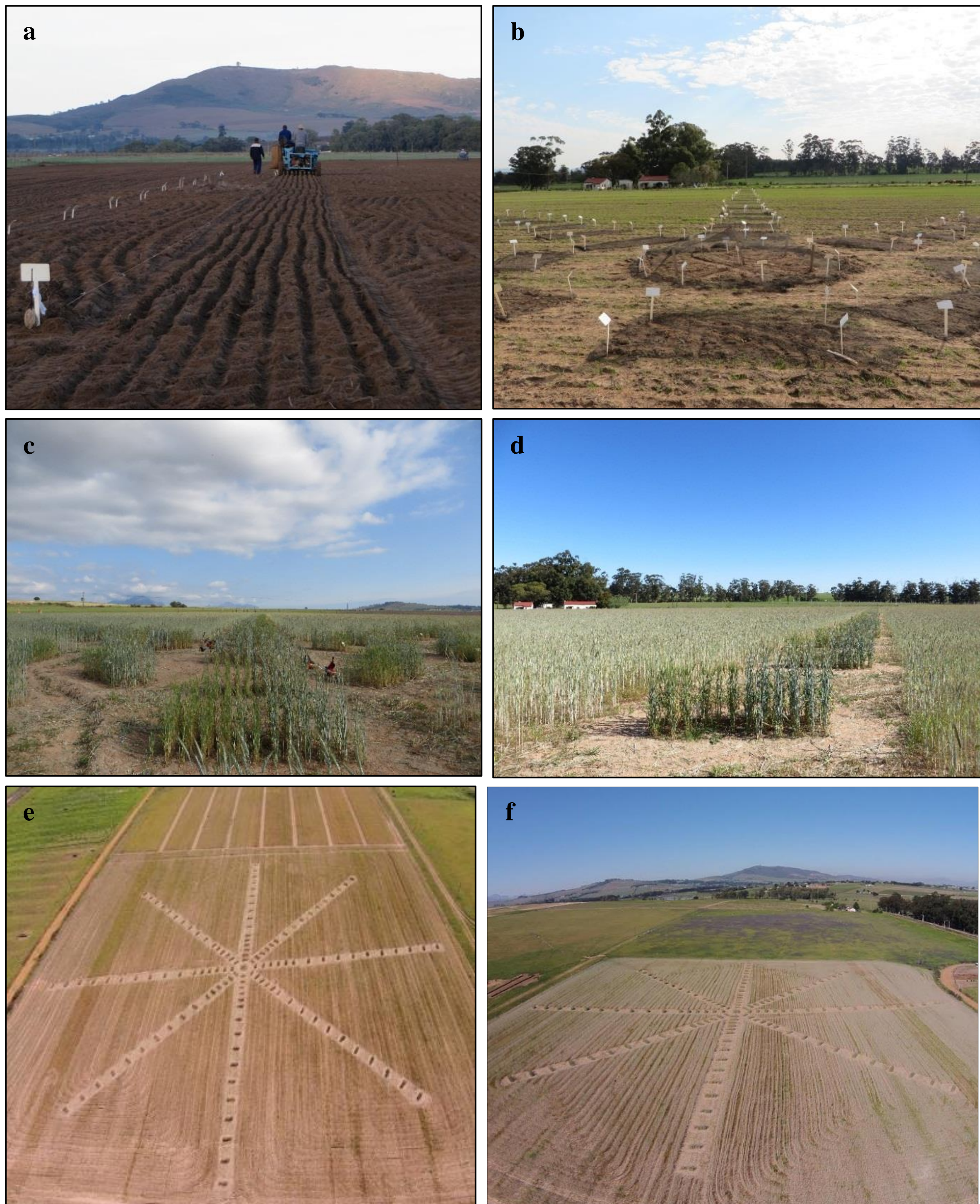

Figure 3.5: A photographic representation of the field trial, where **a**) Planting of the areas between the arms three weeks prior to planting the pollen receptor arms and the central pollen donor block; **b**) Nets placed over the central pollen donor block and pollen receptor arms as to prevent bird damage; **c**) The central pollen donor blocks and eight arms were cleared using weed-eaters; **d**) The North arm after blocks were cleared using weed-eaters; **e** and **f**) Aerial photographs of the field trial captured by Gustav Ludick via drone on 11 October 2014.

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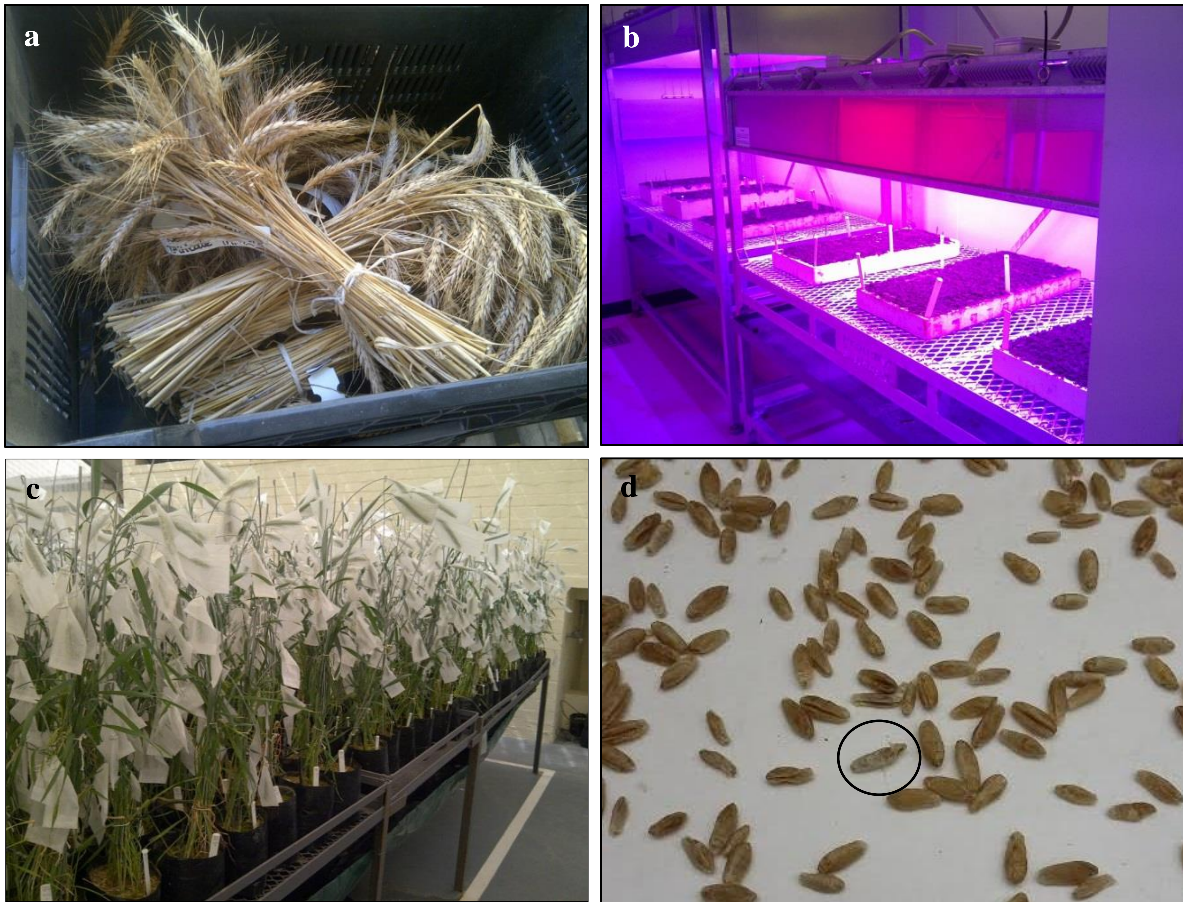

Figure 3.6: a) Triticale bunches harvest by hand and labelled with corresponding number; **b**) Spring wheat F1 progeny planted in trays within the germinating room; **c)** Triticale ears covered with crossing bags 4 weeks after ears started forming; **d**) Blue seed within the F1 triticale seeds harvested from the greenhouse.

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3.4. THE PHENOTYPIC AND GENOTYPIC SCREENING OF THE PROGENY.

After the F1 progeny was harvested from the field trial and planted at Welgevallen experimental farm. This was done in order to be able to screen and identify crossing events as well as to determine the distance and rate at which PMGF occurred within the field trial.

3.4.1. The planting of the F1 progeny harvested from the Nelder Wheel field trial

i. F1 Spring Wheat

The F1 spring wheat seeds that were harvested from the cardinal wind direction arms of the Nelder wheel field trial were planted in trays within a growth room at Welgevallen experimental farm (33°56'34.61"S, 18°51'59.11"E) between 14 November 2014 and 5 May 2015 (Figure 3.6b). The trays were filled with a potting soil (Reliance, Cape Town, South Africa) and were watered with the same nutrient solution mentioned in section 3.2.2. The growth room was temperature controlled at 18°C and had a day/night cycle that was maintained by red (640 to 680nm) and blue (430 to 450nm) LED lights that were automatically switched on at 06h00 and off at 19h00 on a time schedule switch as to optimally germinate the seeds and enhance vegetative growth. Hundred seeds were planted for each of the 16 blocks within each arm. The samples were then extracted as pooled samples of ten plants per extraction. A total of 6 400 seeds were planted and screened.

ii. F1 Spring Triticale

A representative sample of each block of the F1 spring triticale seeds that were harvested were replanted in eight plastic pots (5L) within a single greenhouse at Welgevallen experimental farm on 8 and 9 December 2014. The five litre plastic planting pots were filled with crusher dust (AFRIMAT, Smalblaar Quarry, Rawsonville). Growth conditions within the greenhouse were standard greenhouse conditions and under natural daylight length and temperature. When ambient temperature inside the greenhouse rose above 25°C a water cooling and a greenhouse misting system was used that could lower the ambient temperature by 5°C. The plants were irrigated every four hours daily through an automated hydroponic system with a nutrient solution of 164g Sol-u-fert-T3T (Kynoch Fertilizers Pty Ltd, Milnerton, South Africa), 2g Microplex (Ocean Agriculture Pty Ltd, Muldersdrift, South Africa), 77ml potassium nitrate diluted in 100L H_2O, 0.05% Jik (household detergent containing 3.5% sodium hypochlorite, Reckitt and Colman South Africa Pty Ltd., Elandsfontein, South Africa), and tap water. Insecticides and fungicides were also applied to the plants in the greenhouse when needed – refer to Addendum B for the application of chemicals.

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3.4.2. Molecular Screening of the F1 Spring Wheat

i. DNA Extraction

The DNA was extracted using the same protocol explained in 3.2.2.

ii. SSR Analysis

A set of six microsatellite markers were selected out of the study conducted by Botes & Bitalo (2013). The markers were selected based on their ability to make distinctions between wheat, triticale and rye. These markers included: *Xrms1266*, *Cfe53*, *Scm152*, *Scm159*, *Scm40* and *Scm9* (Addendum C). The PCR reaction conditions for markers were the same as the condition described by Botes & Bitalo (2013). All SSR-PCR reactions were performed using a Thermal Cycler 2720 (Applied Biosystems, Johannesburg, South Africa). The molecular markers were screened and validated to be able to screen spring wheat cultivar "SST027", harvested from the field trial, and be able to identify where hybridization events occurred with spring triticale. Out of all the molecular markers that were tested, A-genome marker *Cfe53*-2AL (Table 3.2) performed the best at making distinctions between spring wheat, spring triticale and rye (Figure 3.7).

The following PCR reaction was conducted: each sample contained 2GFastmix (X1), Forward Primer (0.2µM), Reverse Primer (0.2µM), DNA (100ng), with a final volume of 14µl. The mixture was then run with a Thermal Cycler 2720 (Applied Biosystems, Johannesburg, South Africa) with following the time regime for *Cfe53*-2AL: initial denaturation of 94°C for 4 minutes, followed by 45 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and a final extension period at 72°C for 10 minutes.

Table 3.2: Microsatellite marker used to screen for hybridization events

Marker	Forward/Reverse	Expected Length
Cfe53-2AL F	TGGACCGCAGAGACTTCG	88.97-273.68bp
Cfe53-2AL R	GTCCGCCCAAACCCTACC	

iii. PAGE Gels

The same PAGE gel protocol was used as explained in 3.2.2.

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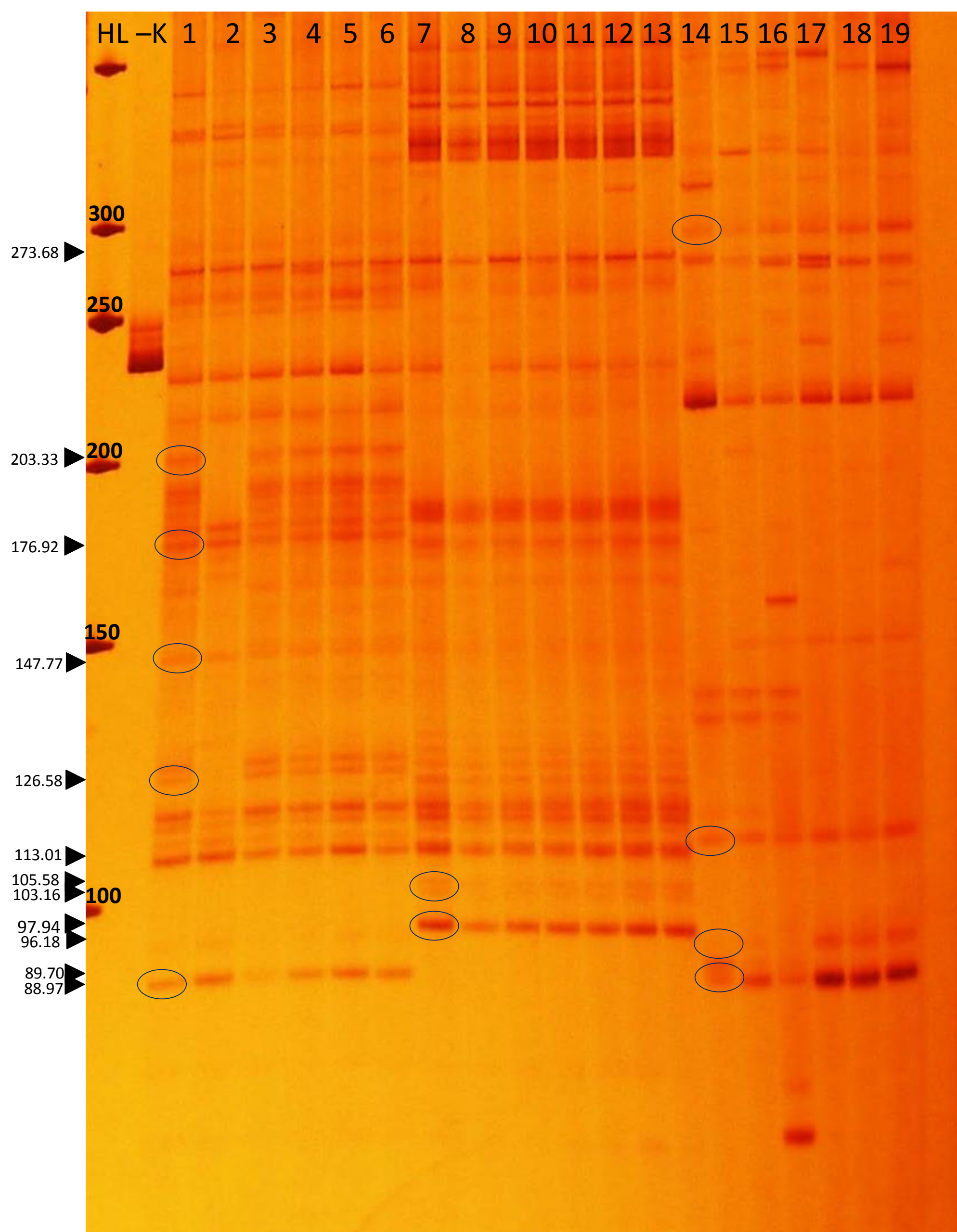

Figure 3.7: 6% Poly-acrylamide gel of the microsatellite marker *Cfe53*, where spring wheat cultivar "SST027" was represented by samples 1-6; spring triticale cultivar "US2007" represented by samples 7 – 13; and rye cultivar "Duiker Max" represented by samples 14 – 19.

iv. Molecular Marker Data Summarised and Visualised

The data that was obtained with molecular marker, *Cfe53*-2AL, was summarised in Table 4.2 to Table 4.5 using Microsoft Word. A Figure was also produced using the "Radar Chart" in Microsoft Excel (Figure 4.5) and represents the hybridization events that were recorded for each spring wheat block between 3.75m to 66.25m from the central pollen donor block in the cardinal wind direction arms (N, E, S, and W) within the Nelder wheel field trial.

v. Marker Validation

A hundred plants were re-planted for blocks North 12 and North 16 after conducting the molecular screening of the spring wheat, as to confirm the results obtained. In order to confirm the results it was decided to molecularly screen and to visualise the chromosomes during mitoses in the individual plants from North 12 and North 16, as well as in control plants: “SST027”, “US2007”, “Duiker Max”, “14UST001”, and “14UST002”.

The plant material was planted in trays within a growth room at Welgevallen experimental farm, on 26 June 2015. The trays were filled with a potting soil (Reliance, Cape Town, South Africa) and were watered daily. The temperature and lighting conditions, as well as the water solution used was the same as was described in section 3.4.1. The DNA of the samples were extracted as one plant per extraction.

As soon as enough leaf material had grown DNA was extracted using the method of Doyle and Doyle (1990), previously described in section 3.2.2, and the samples were screened with molecular marker *Cfe53*-2AL for hybridization events. The same plants that were used for molecular screening, were then cytologicaly screened. The plants roots were trimmed and given enough time to re-grow, after which the active growing root tips were cut off. The root tips were cut before 10h30 and were placed on ice in a glass tube that was filled with distilled water (dH_2O), this was then stored for 30 hours at 4°C. After 30 hours the root tips were fixated in a 4ml methanol:propioon (3:1) acid solution and was then stored at 4°C for one to two days. The fixing solution was replaced with 4ml dH_2O for 30 minutes, after which the root tips were then transferred into new glass tubes that were filled with 4ml 1N HCl for seven and a half minutes at 60°C. The root tips were then washed with dH_2O for one to two minutes. The dH_2O was then replaced with Feulgen and the glass tubes were left overnight at 4°C. The following day the Feulgen was replaced with dH_2O and the root tips were washed two times with dH_2O for five minutes. The dH_2O was replaced with a Sodium Acetate (NaOAc) buffer (pH 4.5) for five minutes. After five minutes the buffer was replaced with a fresh 2.5% peticlear solution (0.5g peticlear in a 20ml NaOAc buffer). The solution was then placed at 37°C for 30 minutes, after which the peticlear solution was replaced with dH_2O and stored at 4°C.

The Feulgen solution that was used to stain the root tips, was prepared as follows: 1g Fisher basic fuchsin was dissolved using 200ml boiling distilled water. This was then shook well and cooled to 50°C. The solution was then filtered and 30ml 1N HCl was added to the filtrate. Three grams Potassium metabisulfite ($K_2S_2O_5$) was then added. This was followed by adding 2g decolorizing carbon to the solution. The solution was then allowed to bleach for 24 hours in

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a tight-stoppered bottle (250ml) in the dark. The solution was then filtered again and stored in a tightly-stoppered bottle in the dark.

3.4.3. Phenotypic screening of the F1 Spring Triticale

As soon as the plants started forming ears, the ears were covered with crossing-bags. This was done before pollen-shed as to prevent cross-pollination between the samples (Figure 3.6c). When the ears reached maturity the seeds were harvested, and threshed with a stationary threshing machine (Winterteiger (Pty) Ltd, LD 180). Cross-pollination within the F1 progeny was identified by the expression of blue pigmentation within the seed (Figure 3.6d). The amount of seeds where blue pigmentation was observed were kept separate and counted. The distance and rate at which pollen mediated drift (PMDF) occurred was then determined by expressing outcrossing as a percentage of blue seed in a sample, this was calculated by using the following equation, developed by Hanson *et al*. (2005):

OC% = (number of blue seed in a sample ÷ total number of seed in a sample) x 100

The outcrossing percentages (OC%) obtained for all the blocks in the different arms were visually illustrated using a Java program, written by Alex Schwalbe. The Java program was written in a text file that was then compiled and ran in the terminal. All the OC% values were hard coded using the Java program. The program then produced a two dimensional Figure, using a nested loop and simple math, where the OC% obtained for each block in the different arms was represented by the size as well a

33s the colour intensity of the circle (Figure 4.16).

Pairwise correlation coefficients was calculated in Microsoft Excel using the "CHITEST" function on all the outcrossing percentages that were obtained. This was done as to determine whether the outcrossing percentages obtained for the eight arms differed significantly from one another. It was also conducted to determine whether the outcrossing percentages obtained for the different distances differed significantly from one another.

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CHAPTER 4 – RESULTS AND DISCUSSION

4.1. OPTIMISATION OF SCREENING METHODS FOR BLUE ALUERONE AND PURPLE PERICARP TRAITS.

The blue aleurone molecular markers that were sourced out of literature did not amplify within the blue aleuorone plant material that was used within this study. The purple pericarp molecular markers that were sourced out of literature were optimised, but none of the purple pericarp seeds planted within the central pollen donor block germinated, and the optimised purple pericarp molecular markers could thus not be used to screen for hybridization events.

It was decided to optimise the molecular markers that were used in a previous study that was conducted by Botes & Bitalo (2013), to screen the F1 spring wheat harvested from the Nelder wheel field trial for hybridization events with blue aleurone spring triticale.

For the F1 spring triticale it was decided to only phenotypically assess for hybridization events, by visually identifying blue seeds within the F1 spring triticale. As the amount of blue seeds obtained per block was too few to conduct the anthocyanin quantification protocol. As seen in Table 4.1 the amount of blue seeds per block ranged between two to 172 seeds per block.

Table 4.1: The amount of blue seeds obtained for the F1 spring triticale in the eight major wind directions in the Nelder Wheel at distances 2.5 to 65m from the pollen source.

		Blue seeds per block							
Block	**Mean Distance**	**N***	**NE**	**E**	**SE**	**S**	**SW**	**W**	**NW**
1	3.75	3	47	40	78	51	13	32	20
2	6.25	3	31	63	47	19	17	63	7
3	8.75	9	24	38	54	43	23	13	10
4	11.25	6	76	51	31	20	14	21	55
5	13.75	39	172	24	43	26	17	44	25
6	16.25	25	27	22	27	24	28	42	25
7	21.25	17	28	14	29	30	25	54	19
8	26.25	22	21	42	42	16	8	46	17
9	31.25	13	41	37	51	20	25	33	7
10	36.25	26	17	18	51	18	24	33	10
11	41.25	9	26	28	26	25	14	26	11
12	46.25	21	10	78	42	4	15	24	4
13	51.25	10	7	14	38	23	33	23	18
14	56.25	30	13	33	34	21	8	33	14
15	61.25	29	18	24	45	37	22	25	19
16	66.25	7	51	21	19	21	31	18	2

*N, North; NE, North-East; E, East; SE, South-East; S, South; SW, South-West; W, West; NW, North-West

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4.2. NELDER WHEEL FIELD TRIAL

4.2.1. Flowering Synchrony

The heading dates that were recorded between 10 and 29 September 2014 indicated a good overlap, with flowering synchrony exceeding 75% between the blue aleurone triticale pollen donor with the spring wheat and triticale pollen receptor cultivars (Table 4.1). Where, "US2007" and "SST027" had a 100% and 78% synchrony respectively. Within the central pollen donor block a 50% ear formation was recorded in 38% of the plants on 10 September 2014, 24% of the plants on 15 September 2014, and in 19% of the plants on both 22 and 29 September 2014 (Figure 4.1). The wheat pollen receptor blocks had a fifty percent ear formation in 56% of the plants on 10 September 2014, 39.1% of the plants on 15 September 2014 and 4.7% plants on 22 September 2014. With the triticale pollen receptor blocks a fifty percent ear formation was recorded for 93% of the plants on 10 September 2014, 3.9% of the plants on 15 September 2014, 2.3% of the plants on 22 September 2014, and the remaining 0.8% plants were recorded on 29 September 2014.

The flowering synchrony between male and female flowers has an important effect on the rate of cross-fertilization (DuPlessis & Dijkhuis 1967). Where, the probability of cross-fertilization is higher if the synchrony between the pollen source and recipient is closer (Della Porta *et al.* 2008). This is due to the fact that viable pollen must successfully enter the florets, germinate on the stigmas and fertilize the ovules (Waines & Hegde 2003). Anthesis in wheat has been reported to last up to ten days (De Vries 1973) and the stigmas to be receptive for a period of four to 13 days (De Vries 1971). In a study conducted by Yeung & Larter (1972) they found that anthesis in triticale was generally longer than that of wheat, with differences between stains, and this led to the promotion of outcrossing. Under optimal field conditions wheat pollen loses viability within 15 to 30 minutes after release, and rye pollen can stay viable for up to 72 hours after release (D'Souza 1970; Fritz & Lukaszewski 1989). Within triticale no viable pollen can be detected after 120 minutes (Fritz & Lukaszewski 1989). Wheat pollen viability is mainly attributed to the crop being self-pollinating (Waines & Hegde 2003). Cross-pollination in wheat can thus be affected by both genetic and environmental factors (Gaines *et al*. 2007).

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Table 4.2: Planting, heading, heading synchrony, and harvest dates for "SST027", "US2007" and the blue aleurone triticale (BA) at Mariendahl Experimental Station 2014.

Cultivar	Planting date	Heading date*	Heading date synchrony with CPDB**	Harvest date
SST027	11 June	10 - 22 September	78%	31 October - 6 November
US2007	11 June	10 - 29 September	100%	20 November
14UST001/2	6 June 12 June 23June	10 – 29 September	-	20 November

*Heading dates were recorded when a block had 50% ear formation
** Central pollen donor block, CPDB

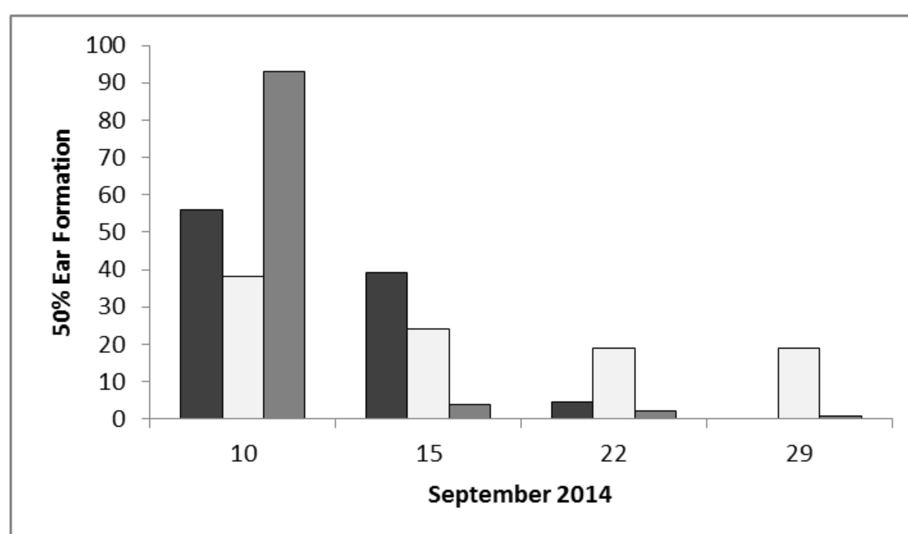

Figure 4.1: The percentage blocks within the Nelder Wheel field trial that reached 50% ear formation on the specified dates (Filled black square = spring wheat pollen receptor blocks; filled white square = central pollen donor block; grey brown square = spring triticale receptor blocks).

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4.2.2. Weather data

As indicated by Figure 4.2a the temperature range during the flowering period was between 5.8°C to 30.1°C. A total of 91.3mm precipitation was recorded during the flowering period (Figure 4.2b). Relative Humidity recorded during the flowering period averaged at 73.1% (Figure 4.2c). The prevailing wind direction, as indicated by Figure 4.4 and 4.5, during the flowering period was in the North-East direction with an average wind speed for the flowering period of 1.61ms^{-1}. The highest wind speed recorded during the flowering period was 4.87 ms^{-1} in the North-West direction. The wind speed recorded for the North-East, North-West, and North wind directions was statistically significantly higher than the other wind directions for the flowering period (data not shown). This was obtained by calculating the LSD values.

The duration of pollen viability is influenced by temperature and relative humidity (Waines & Hegde 2003). Extremely cold or hot temperatures are unfavourable for pollination and fertilization (Waines & Hegde 2003). Where, low temperatures reduce the duration of pollen shed and high temperatures reduce both the duration of pollen shed as well as pollen viability (D'Souza 1970; Major 1980). Humid weather makes the pollen grains heavy and thus reduces the distance the pollen can disperse from the parent plants. Whereas, dry weather causes the pollen to desiccate and thus also reduce the viability of the pollen, thus reducing the chance of effective gene flow (Waines & Hegde 2003). The optimum temperature for fertilization to occur in wheat ranges between 18 to 24°C, with a minimum at 10°C and a maximum at 32°C (Hoshikawa 1960). With, the maximum temperature, at or below, at which development during anthesis will be zero, being 8.1°C (Slafer & Rawson 1995). According to a study conducted by de Vries (1972), the most pollen appeared to be released at a relative humidity of 70 – 75%.

Criteria was established for ideal weather condition on which outcrossing events were more likely to occur within the 80 to 100 days after planting the pollen donor and receptor blocks, the criteria for ideal weather conditions included: temperature that ranged between 10 and 32°C, relative humidity at 70-75%, and no precipitation. Days that did not comply too any one of these criteria were ruled out as days where possible cross-pollination and fertilization events could take place. The average wind speed and direction was then calculated for the remaining days that did comply with these ideal conditions, see Figure 4.4 and 4.5. Where an average wind speed of 1.45 ms^{-1} was obtained and the prevailing wind direction was in the North-East direction. The highest wind speed recorded within these ideal conditions was 3.73 ms^{-1} in the North-East direction. Where the wind speeds that were recorded for the North-East, South-West, and North-West wind directions were statistically significantly higher than the wind

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speeds recorded for the other wind directions under ideal weather conditions (data not shown). Elevated outcrossing rates are expected to be associated with the prevailing wind directions (Matus-Cádiz *et al*. 2007).

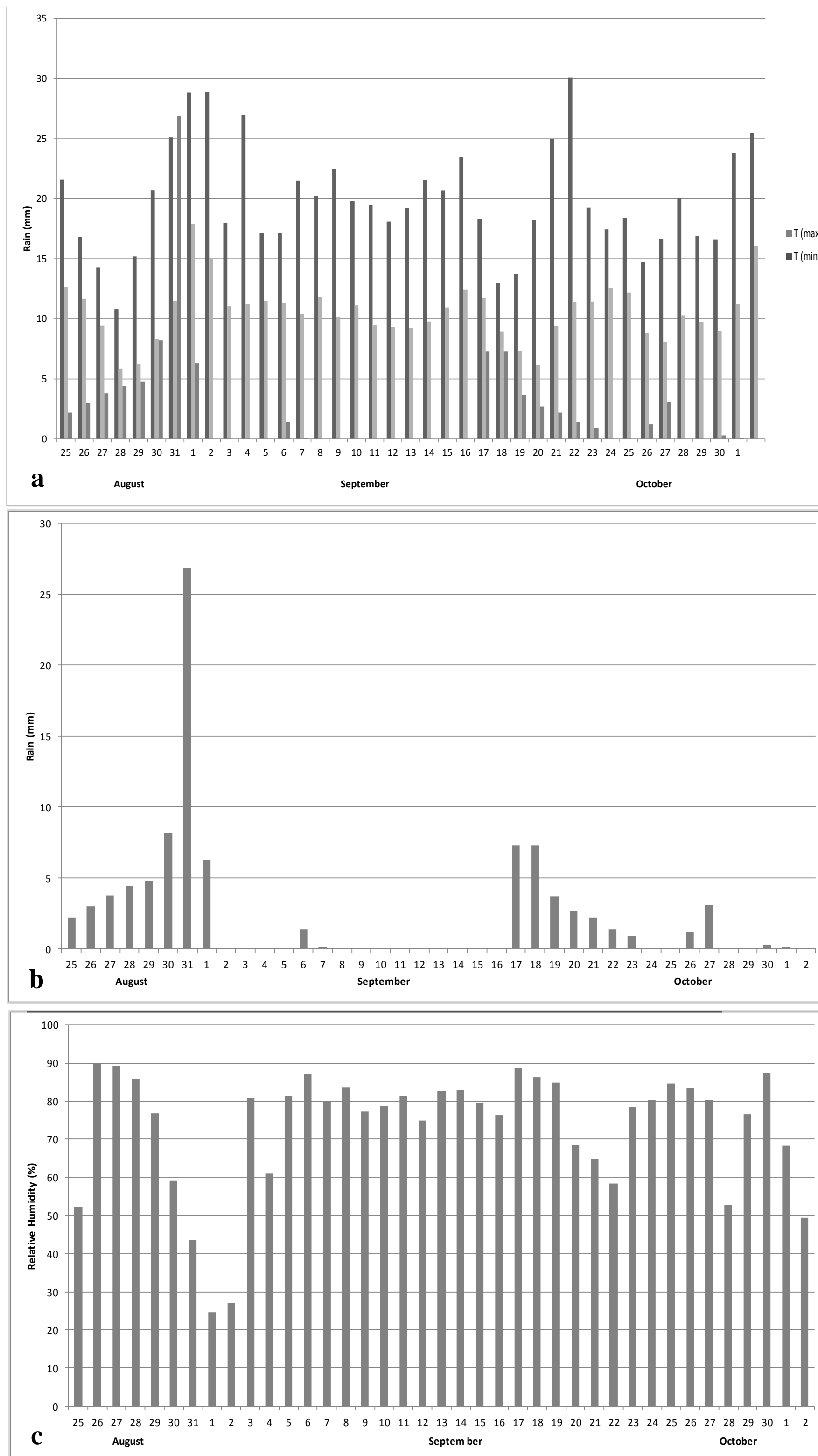

Figure 4.2: Weather data for the flowering period (25 August 2014 to 1 October 2014), for MES, where **a**) represents the maximum and minimum temperatures, measured in degrees celcius (°C); **b**) the rainfall, measured in millimeters (mm); and **c**) represents the relative humidity readings, measered in percentage (%).

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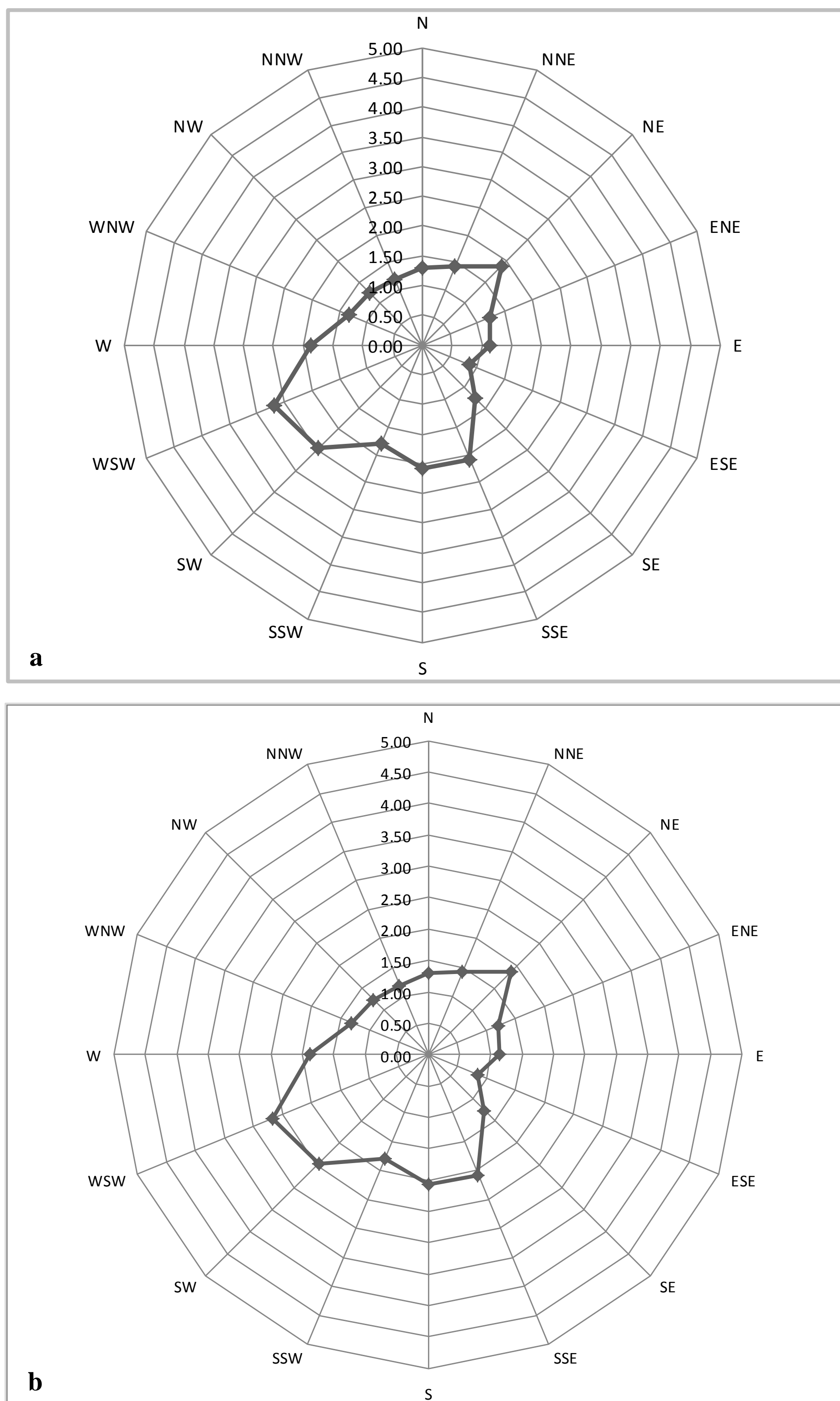

Figure 4.3: Wind data for the flowering period (25 August 2014 to 1 October 2014) for MES, where **a**) average wind speed and direction for the 2014 flowering period: and **b**) average wind speed and direction for the ideal days for pollen mediated gene flow between August and October 2014 - where the middle circle represents a wind speed of 0.5 ms^{-1} and the outside circle 5 ms^{-1}.

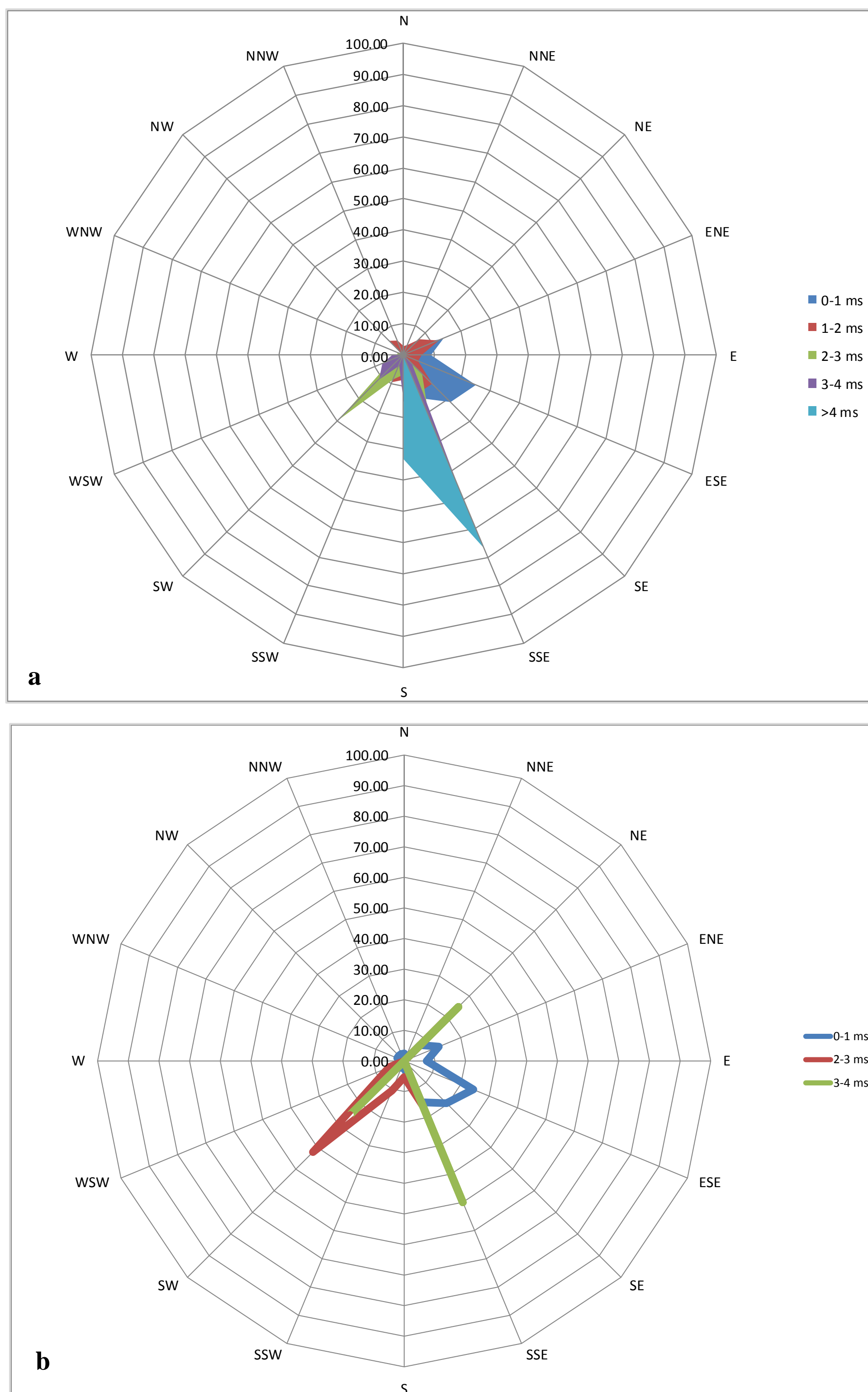

Figure 4.4: Wind data for the flowering period (25 August 2014 to 1 October 2014), for MES, where **a**) represents the frequency (%) of wind speeds between 0 ms^{-1} to wind speeds higher than 4 ms^{-1} at each cardinal and inter-cardinal direction; and **b**) the frequency of wind speed between 0 ms^{-1} to wind speeds higher than 4 ms^{-1} for ideal days for pollen mediated gene flow between August and October 2014.

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4.3. THE PHENOTYPIC AND GENOTYPIC SCREENING OF THE PROGENY

4.3.1. Spring wheat

i. Molecular screening

As indicated by Tables 4.2 to 4.5 the molecular screening of the wheat seed harvested from the field trial indicated a high potential frequency of hybrids. Within the pooled samples representing each block in the North arm, shared hybridization was observed that ranged between 3 of the 10 (30%) pooled samples having a heterozygous profile for North 13, to 10 of the 10 (100%) pooled samples having a heterozygous profile for North 8. The blocks within the South and East arms had pooled samples displaying heterozygous profile ranging between 5 of the 10 (50%) to 10 of the 10 (100%) pooled samples. While the blocks within the West arm ranged between 6 of the 10 (60%) and 10 of the 10 (100%) pooled samples displaying the heterozygous profile.

Hybrids are generally expected to be intermediate in morphology, but they can be unpredictable (Rieseberg & Ellstrand 1993), these limitations can be overcome through the use of molecular markers to supplement existing screening methods (Kavanagh *et al*. 2013). Simple sequence repeat (SSR) markers, such as *Cfe53*, are used more frequently than other markers due to the advantages that are associated with SSR markers that include: co-dominance, high accuracy, high repeatability, high level of polymorphism, chromosome specificity and the ease of manipulation (Naik *et al*. 2015). SSR markers are particularly useful in distinguishing between closely related cultivars and species because they have a high degree of polymorphism (Kavanagh *et al*. 2013).

Marker validation is defined as the process of examining the behaviour of markers and their associated polymorphism in different genetic backgrounds (Langridge & Chalmers 1998). The process of marker validation includes four steps: first, the identification of a potential marker or markers within literature that are linked to a specific trait of interest; second, the identification of the source for genetic material; third, the evaluation of the effectiveness of the identified marker or markers within the identified source genetic material; and finally, the critical discussion of the results obtained and suggestions on the decision to validate the specific marker or markers for a particular marker-trait combination (Gupta *et al*. 1999).

Within this study it was decided to further investigate the spring wheat samples using the microsatellite marker, *Cfe53*. This was done by running the marker on 100 individual plants

from the blocks North 12 (hybridization of 50% observed in the pooled samples) and North 16 (80%). It was also decided to visualise mitoses within the root tips of these 100 individual plants (Figures 4.8 and 4.9), as well as the control plants: "SST027", "US2007", "US2008/AgBeacon", "Duiker Max", "14UST001" and "14UST002" (Figures 4.7); as to confirm the results obtained from the molecular screening and thus validate this specific marker.

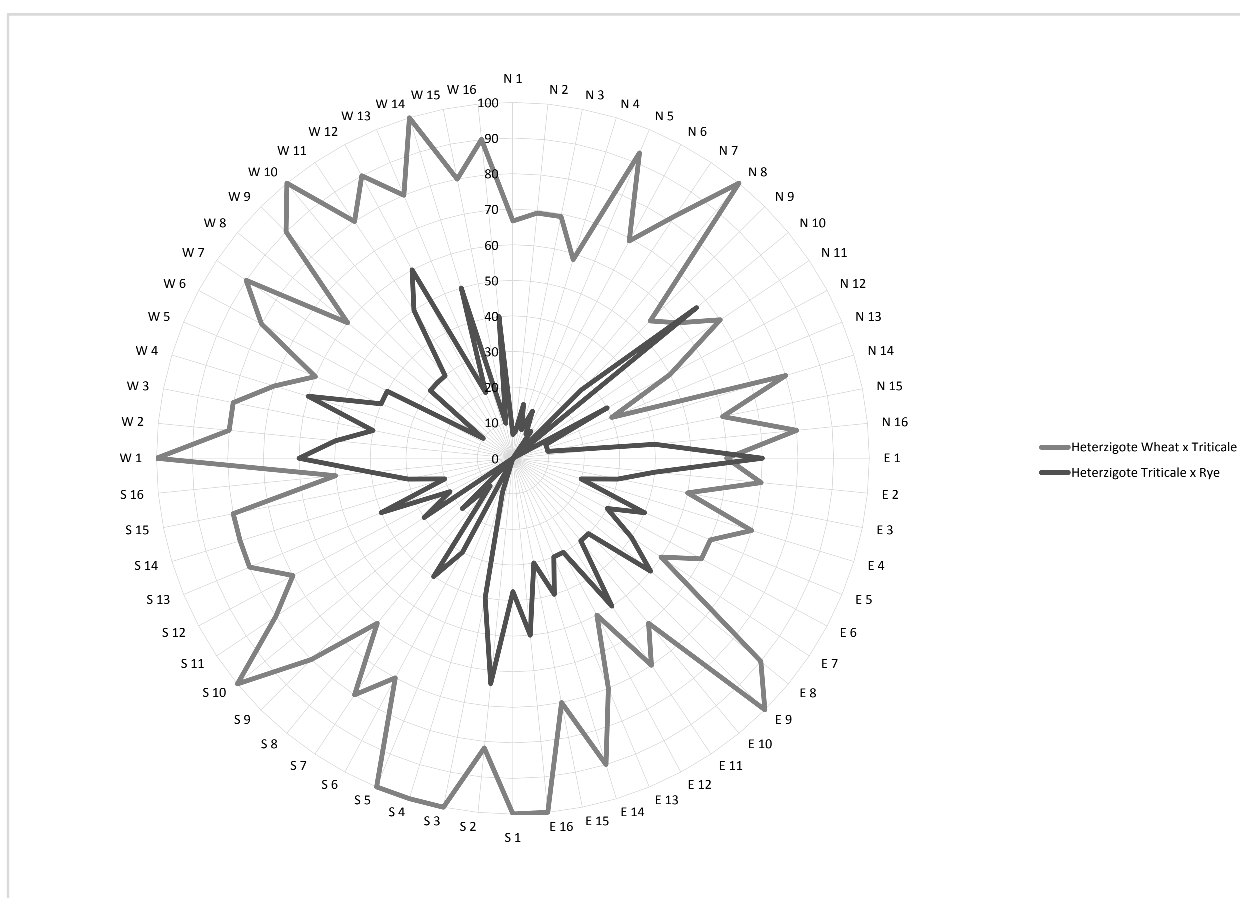

Figure 4.5: Visual representation of the heterozygotes that were recorded for each spring wheat block between 3.75m to 66.25m from the central pollen donor block in the cardinal wind direction arms within the Nelder Wheel.

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Table 4.3: The amount of heterozygotes detected in wheat of the North arm at distances 3.75 to 66.25m from the pollen source using molecular marker, *Cfe53* and 6% poly-acrylamide gels. The number of seeds screened and the scoring of the bands specific to wheat, triticale and rye are included.

Block	Mean Distance (m)	Seed Screened*	SST027					US2007			Duiker Max			Heterozygote	
			88.97	126.58	147.77	176.92	203.33	97.94	103.16	105.58	96.18	113.01	273.68	Wheat x Triticale	Triticale x Rye
North 1	3.75	100	0	0	7	7	67	100	93	93	0	0	7	67	7
North 2	6.25	100	0	8	8	15	54	100	69	69	0	23	8	69	8
North 3	8.75	100	0	8	0	0	69	100	77	77	0	23	0	69	15
North 4	11.25	100	0	8	25	8	58	92	58	58	0	8	25	58	8
North 5	13.75	100	0	14	14	7	86	100	57	57	0	21	0	93	14
North 6	16.25	100	0	8	31	0	62	100	69	69	0	8	8	69	8
North 7	21.25	100	9	27	27	9	45	100	73	73	0	18	9	82	9
North 8	26.25	100	0	82	18	0	73	100	91	91	0	73	0	100	0
North 9	31.25	100	0	36	0	0	36	100	82	82	0	36	9	55	27
North 10	36.25	100	0	30	30	10	20	100	90	90	0	50	30	60	67
North 11	41.25	100	0	20	10	0	40	100	50	50	0	0	0	70	0
North 12	46.25	100	0	10	10	20	40	100	80	80	0	30	0	50	30
North 13	51.25	100	0	10	10	0	10	100	70	70	0	10	0	30	10
North 14	56.25	100	10	50	10	30	60	100	90	90	0	0	10	80	10
North 15	61.25	100	0	40	10	0	50	90	90	90	0	0	10	60	10
North 16	66.25	100	0	60	10	0	60	100	80	80	10	30	0	80	40
		Mean	1.1	24.0	13.7	6.6	53.6	98.9	76.0	76.0	0.5	20.2	7.1	68.9	15.4

* The seeds screened were pooled samples of ten seeds per sample and ten samples per block.

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Table 4.4: The amount of heterozygotes detected in wheat of the South arm at distances 3.75 to 66.25m from the pollen source using molecular marker, *Cfe53* and 6% poly-acrylamide gels. The number of seeds screened and the scoring of the bands specific to wheat, triticale and rye are included.

Block	Mean Distance (m)	Seed Screened*	SST027					US2007			Duiker Max			Heterozygote	
			88.97	126.58	147.77	176.92	203.33	97.94	103.16	105.58	96.18	113.01	273.68	Wheat x Triticale	Triticale x Rye
South 1	3.75	100	11	33	11	11	89	100	78	78	11	0	22	100	38
South 2	6.25	100	0	0	36	9	64	100	91	91	0	18	45	82	64
South 3	8.75	100	0	0	40	10	100	100	100	100	0	0	40	100	40
South 4	11.25	100	0	0	80	10	80	100	100	100	0	0	20	100	10
South 5	13.75	100	0	0	70	0	70	100	100	100	0	0	0	100	0
South 6	16.25	100	0	0	40	10	30	100	100	100	0	0	20	70	30
South 7	21.25	100	0	0	50	0	40	100	100	100	0	0	40	80	40
South 8	26.25	100	0	0	40	0	40	100	100	90	0	0	10	60	10
South 9	31.25	100	0	10	30	10	40	100	80	80	0	0	20	80	20
South 10	36.25	100	10	20	30	0	70	100	90	90	0	0	0	100	0
South 11	41.25	100	10	10	50	20	40	100	100	100	0	0	30	80	30
South 12	46.25	100	10	0	50	0	70	100	80	80	0	0	20	70	20
South 13	51.25	100	0	0	50	10	50	100	90	100	0	0	40	80	40
South 14	56.25	100	0	10	80	0	60	100	70	70	0	0	30	80	20
South 15	61.25	100	0	0	60	20	40	100	80	80	0	0	30	80	30
South 16	66.25	100	0	0	30	0	50	100	100	100	0	0	40	50	40
		Mean	2.5	5.0	46.9	6.9	58.1	100.0	91.3	91.3	0.6	1.3	25.6	81.9	27.0

* The seeds screened were pooled samples of ten seeds per sample and ten samples per block.

Table 4.5: The amount of heterozygotes detected in wheat of the West arm at distances 3.75 to 66.25m from the pollen source using molecular marker, *Cfe53* and 6% poly-acrylamide gels. The number of seeds screened and the scoring of the bands specific to wheat, triticale and rye are included.

Block	Mean Distance (m)	Seed Screened*	SST027					US2007			Duiker Max			Heterozygote	
			88.97	126.58	147.77	176.92	203.33	97.94	103.16	105.58	96.18	113.01	273.68	Wheat x Triticale	Triticale x Rye
West 1	3.75	100	0	0	70	0	90	100	100	100	10	0	50	100	60
West 2	6.25	100	0	0	60	10	50	100	40	50	0	10	40	80	50
West 3	8.75	100	0	0	60	20	70	100	80	80	0	0	40	80	40
West 4	11.25	100	0	0	60	0	30	100	100	100	0	0	60	70	60
West 5	13.75	100	10	10	50	30	30	90	90	100	10	0	30	60	40
West 6	16.25	100	10	0	30	30	50	100	70	70	0	0	40	80	40
West 7	21.25	100	0	0	80	0	70	100	100	100	0	0	10	90	10
West 8	26.25	100	0	0	50	0	30	100	100	100	0	0	30	60	30
West 9	31.25	100	0	50	20	20	60	100	90	90	0	0	30	90	30
West 10	36.25	100	0	80	40	50	40	100	60	40	0	0	30	100	30
West 11	41.25	90	0	11	56	11	56	100	67	67	0	0	56	80	50
West 12	46.25	100	0	70	40	20	60	100	90	90	0	0	60	90	60
West 13	51.25	100	0	0	40	10	70	100	80	80	0	0	20	80	20
West 14	56.25	100	0	0	50	0	90	100	100	100	0	0	50	100	50
West 15	61.25	100	0	0	30	10	70	100	80	80	0	0	10	80	10
West 16	66.25	100	0	30	60	20	80	100	90	90	0	0	40	90	40
		Mean	1.3	15.7	49.7	14.5	59.1	99.4	83.6	83.6	1.3	0.6	37.1	83.1	38.8

* The seeds screened were pooled samples of ten seeds per sample and ten samples per block.

Table 4.6: The amount of heterozygotes detected in wheat of the East arm at distances 3.75 to 66.25m from the pollen source using molecular marker, *Cfe53* and 6% poly-acrylamide gels. The number of seeds screened and the scoring of the bands specific to wheat, triticale and rye are included.

Block	Mean Distance (m)	Seed Screened*	SST027					US2007			Duiker Max			Heterozygote	
			88.97	126.58	147.77	176.92	203.33	97.94	103.16	105.58	96.18	113.01	273.68	Wheat x Triticale	Triticale x Rye
East 1	3.75	100	0	30	50	0	50	100	80	80	0	0	70	60	70
East 2	6.25	100	0	0	40	20	60	100	80	80	0	0	40	70	40
East 3	8.75	100	0	10	10	0	40	100	80	80	0	0	30	50	30
East 4	11.25	100	0	0	40	20	60	100	80	80	0	0	20	70	20
East 5	13.75	100	0	10	40	10	40	100	70	70	0	0	40	60	40
East 6	16.25	100	0	10	10	10	40	100	70	70	0	0	30	60	30
East 7	21.25	90	0	0	30	30	30	100	80	90	0	0	40	50	40
East 8	26.25	100	0	0	30	10	90	90	70	70	0	0	60	90	50
East 9	31.25	100	0	0	50	60	80	100	70	70	0	0	40	100	30
East 10	36.25	90	0	10	30	20	30	90	90	90	0	0	40	60	30
East 11	41.25	90	0	10	50	0	40	90	60	60	0	0	40	70	50
East 12	46.25	80	0	0	30	10	40	80	60	60	0	0	40	50	30
East 13	51.25	80	0	20	30	10	40	80	50	50	0	0	40	70	30
East 14	56.25	100	0	10	40	0	70	100	90	90	0	0	40	90	40
East 15	61.25	100	0	0	30	10	50	100	90	90	0	0	30	70	30
East 16	66.25	100	0	20	50	40	80	100	90	90	0	0	50	100	50
		Mean	0.0	8.1	35.0	15.6	52.5	95.6	75.6	76.3	0.0	0.0	40.6	70.0	38.1

* The seeds screened were pooled samples of ten seeds per sample and ten samples per block.

ii. Molecular Marker Technical Difficulties

When the newly extracted DNA from North 12 and North 16 was screened with molecular marker, *Cfe53*, the marker started encountering technical difficulties, with smears that formed within the 6% poly-acrylamide gels (PAGE), see Figure 4.6 for the first gel where the marker technical difficulties were encountered. The smears formed in both the control DNA that previously worked, and the newly extracted DNA. The PCR was firstly re-run as to eliminate human error, a new marker mix was made, all reagents were replaced, new DNA extraction were made, different PCR machines were used; but the resulting gels still had the development of smears.

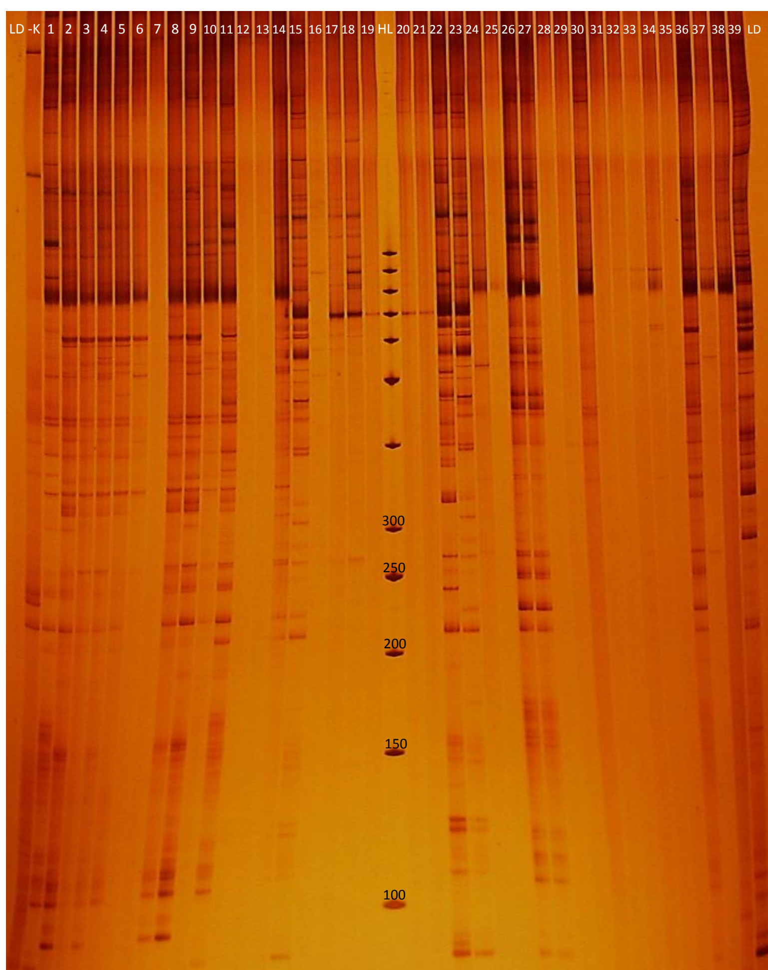

Figure 4.6: The first control gel of the newly extracted DNA where molecular marker technical difficulties were encountered, where **i**) wheat cultivar, "SST027" is represented by 1-8; **ii**) triticale cultivar, "US2007" is represented by 9-14; **iii**) rye cultivar, "Duiker Max" is represented by 15-23; **iv**) triticale cultivar, "AgBeacon/US2008" is represented by 24-31,and 38; **v**) blue aleurone triticale cultivar "14UST002" is represented by 32-36; and **vi**) blue aleurone triticale cultivar, "14UST001" is represented by 37.Where samples 1, 9, 15, and 23 are control DNA from the previous DNA extractions that did amplify.

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iii. Chromosome Visualisation

The results obtained for the chromosome visualisations of the control plant material as well as the plant material from blocks 12 and 16 from the North arm of the Nelder Wheel field trial are listed in Table 4.6 and photographed in Figure 4.10 to Figure 4.12. Duiker Max had a somatic chromosome number of 14, and the normal wheat and triticale cultivars, as well as the blue aleurone triticale cultivars had a somatic chromosome number of 42. Within the North 12 block 50% (5/10 plants) of the plants were heterozygous, this was indicated by the abnormal somatic chromosome number within the plant material that ranged between 32 and 39 chromosomes. The North 16 block had 42.8% (3/7 plants) plants that were heterozygous, with somatic chromosome numbers that ranged between 33 and 46 chromosomes.

The results obtained for the chromosome visualisations of North 12, 50% heterozygous (Table 4.6), correlated perfectly with the results that were obtained through the molecular screening of the same material, 50% heterozygous (Table 4.2). For North 16 the results did not correlate, with the hybridization with chromosome visualization at 42.8% and hybridization with molecular screening at 80%.

Spring wheat, durum wheat, and triticale are closely related species, with the initial maternal material of triticale being provided by either spring or durum wheat, and the paternal component of the triticale genome being provided by rye (Oettler 2005). Wheat and triticale are both primarily autogamous, which limits gene flow, but under natural growing conditions interspecific gene flow does occur (Kavanagh *et al*. 2013). Interspecific gene flow in wheat and triticale can be increased by environmental stress that may cause pollen sterility and promote floret opening (Waines & Hegde 2003). Open pollinated or chasmogamous grass species have florets that are open during anthesis, this allows pollen to be distributed via wind and/or animals (Hegde & Waines 2004). Bread and durum wheat are closed pollinated or cleistogamous grass species that are primarily serf-fertilizing and the florets of these species usually open after anthesis (Kavanagh *et al*. 2010). Florets of wheat may open under environmental stress conditions that increase the opportunity for cross-fertilization (Hegde & Waines 2004). Cleistogamous floral structure is exhibited by triticale, but it also exhibits some tendency for chasmogamous (Yeung & Larter 1972). Both wheat and triticale possess *Ph1* and *Ph2* genes that suppress the homologous pairing and recombination of alien chromosomes, thus reducing the viability of the F1 progeny (Kavanagh *et al*. 2013). Both also possess crossability genes *Kr1-4* that inhibits interspecific outcrossing to rye and rye relatives (Guedes-Pinto *et al*. 2001; Oettler 2005). When introducing new traits from related species through

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introgression in wheat and triticale breeding programs, these genes have been problematic (Kavanagh *et al*. 2010). But, in the development of GE wheat the function of the *Ph* and the *Kr* genes make them desirable to prevent the movement of transgenes (Weissmann *et al*. 2008; Manickavelu *et al*. 2009). However, seeds may be produced, some viable, through the manual crossing of triticale and spring wheat and durum wheat using emasculation (Hills *et al*. 2007).

Sequence changes and deletions are results of allopolyploidization in many species, including bread wheat (Ozkan *et al*. 2002). Triticale's hybridization potential with its relatives can be influenced by many factors, including: the genome compatibility and constitution with its relatives, possible genetic barriers, the floral structure, the distance between the pollen donor and a compatible pollen receptor, the size of the compatible pollen receptor population, physical and temporal barriers, as well as environmental conditions (Kavanagh *et al*. 2010).

Within a study conducted by Hills *et al*. (2007) under greenhouse conditions they observed outcrossing between triticale and common wheat to be significantly higher when triticale acted as the pollen donor (86±4% to 89.4±5.7%), than when triticale acted as the pollen receptor (<23%). But although outcrossing between triticale and common wheat occurred at a higher frequency with triticale as the pollen donor, these outcrosses rarely produced viable seed. Similar results were obtained in a study conducted by Coetzee (2011) under greenhouse conditions where it was observed that outcrossing was higher (48.7 to 66.4%) when triticale was the male parent, than when wheat was used as the male parent (5.4 to 24.2%). Coetzee (2011) also observed that although the outcrossing between triticale and wheat was at a higher frequency with triticale as the male parent, that the F1 hybrid emergence was very low (<4%), compared to when wheat was used as the male parent (12.5 to 44%). The results observed by Hills *et al*. (2007) together with the results observed by Coetzee (2011), support previous observations that if hybrid seeds are produced from triticale and common wheat crosses, are only viable when triticale acts as the pollen receptor in the cross (Khanna 1990; Nkongolo *et al*. 1991; Bizimungu *et al*. 1998).

Kavanagh *et al*. (2013) quantified interspecific gene flow from blue aleurone triticale to spring wheat and durum wheat under field conditions in Canada, where F1 hybrid seeds were collected and screened morphologically and molecularly. Within their study 14 hybrids were identified, 11 of the hybrids were identified molecularly and five were identified morphologically, making the number of hybrids identified within their study below the threshold for approved events in

Canada. Kavanagh *et al*. (2013) concluded that the interspecific gene flow from triticale to conventional wheat crops may not be a barrier for GM triticale development.

Genetic instability during initial hybridization that includes the loss of entire chromosomes is known to occur (Oettler 2005; Dou *et al*. 2006; Ma & Gustafson 2008). This suggests that within hybrids the transgene retention and genome position may be unpredictable (Kavanagh *et al*. 2013). Single polymorphisms may also have been attributable to random mutation, examples include: point mutations or structural changes to chromosomes (Ozkan *et al*. 2002; Kavanagh *et al*. 2013). Within the study conducted by Kavanagh *et al*. (2013) only two of the five hybrids that were identified morphologically were confirmed molecularly. They concluded that it may be that too few markers were selected for the molecular screening and that they did not encompass the genome portion containing triticale fragments in the other three plants.

Within this study the pooled samples representing a heterozygous profile that was observed within the F1 wheat progeny (30 to 100%) from the North, South, West and East arms was in range, with a few higher than with what has previously been reported in greenhouse experimental conditions: 48.7 to 66.4% (Coetzee 2011) and 86±4% to 89.4±5.7% (Hills *et al*. 2007). Where, within greenhouse experimental conditions, pollen competition gets removed through emasculation, enhancing the potential for hybridization to occur (Hills *et al*. 2007). The higher values obtained in this study could be due to the fact that the samples were pooled and plants within the pooled samples could thus not be individually assessed. As observed with the chromosome visualisation for North 16 where three of the seven plants screened were heterozygous, while eight of the ten pooled samples screened molecularly were heterozygous. Thus a positive in the pooled sample can represent one of the plants in the pooled sample, or more than one. The limitation in this part of the study is thus that the pooled samples could not assessed individually using the molecular marker as the molecular marker encountered technical difficulties with the individual assessment of North 12 and North 16 plants.

A study that was conducted by Yeung & Larter (1972) on three hexaploid triticale cultivars was found that they had a wide range of variability in their pollen production and distributing properties. That differences in cultivar pollen production and distributing properties, as well as differences in environmental conditions could be the reason for the differences in the results of the field studies conducted. The fact that higher outcrossing percentages were obtained could also be due to the fact that the wheat plants within the blocks were surrounded by triticale: triticale within the same block and the blue aleurone triticale pollen donor block, and that the pollen donor and receptors were temporarily planted to optimally cross pollinate. Where within

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their study Kavanagh *et al*. (2013) isolated the triticale and wheat blocks with barley to reduce pollen movement between the treatments. The few samples where higher outcrossing values were observed (within this study than on previous greenhouse studies) could possibly be due to the fact that unfavourable or stressful environmental conditions increased flower opening and thus increased the amount of outcrossing and PMGF.

What is important to conclude out of this part of the study is that a definite high amount of outcrossing (30 to 100%) was observed between triticale and wheat, with triticale as the pollen donor in open field conditions within the Western Cape. Future research can thus be conducted by using other crops as physical barriers of PMGF between the pollen donor crop and the pollen receptor crops, temporal isolation of the pollen donor and pollen receptor plants can further be investigated, as well as the effect of fire brakes on PMGF in wheat and triticale. This will help to develop isolation distances and strategies that will maintain the 5% GM labelling threshold that is stipulated in Regulation 293 (2011), under the South African Consumers Protections Acts of 2008 (Acts online 2013). The differences in the success of the pollen donor and/or pollen receptor will also be relevant in future hybridization assessments (Hills *et al*. 2007; Kavanagh *et al*. 2010).

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Table 4.7: The chromosome number obtained for the control plants: “Duiker Max”, “SST027”, “US2007”, “US2008”, “14UST001”, and “14UST002”; and the plant material from blocks 12 and 16 of the North arm from the Nelder Wheel field trial.

Plant	Chromosome Number
Duiker Max	14
SST027	42
US2007	42
US2008	42
14UST001	42
14UST002	42
North 12-1	42
North 12-2	42
North 12-14	42
North 12-23	32
North 12-36	42
North 12-37	35
North 12-7	42
North 12-39	38
North 12-43	39
North 12-48	35
North 16-59	42
North 16-2	33
North 16-49	42
North 16-35	42
North 16-8	46
North 16-78	42
North 16-7	35

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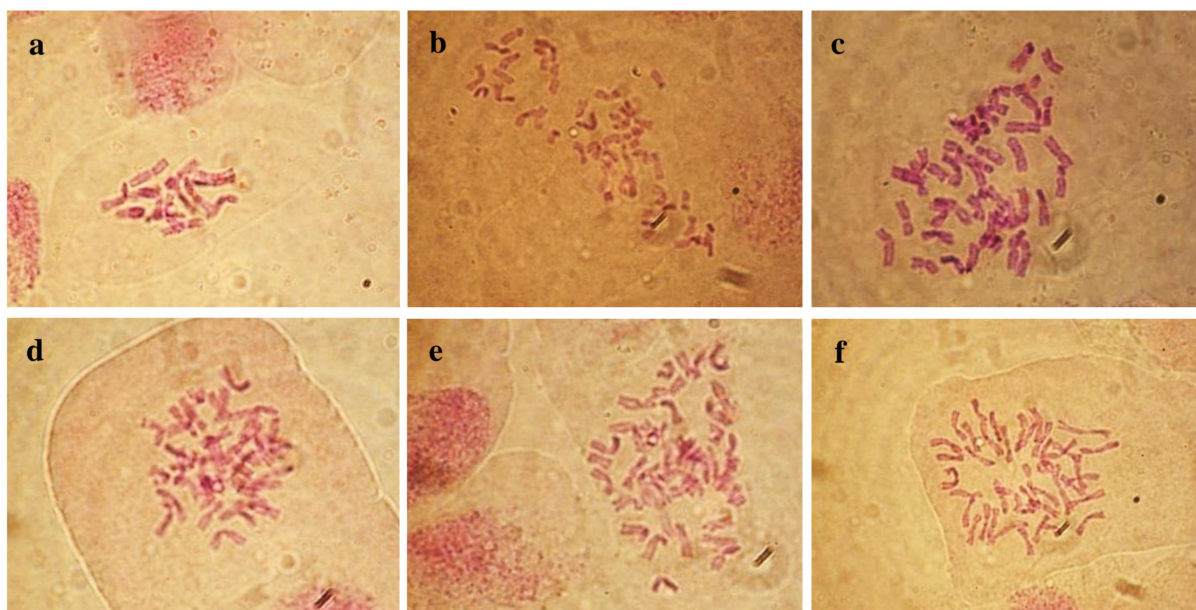

Figure 4.7: Stained somatic chromosomes and the visualisation thereof for selected material, where: **a**) represents rye cultivar, “Duiker Max”, with 14 somatic chromosomes; **b**) spring wheat cultivar, “SST027”, with 42 somatic chromosomes; **c**) spring triticale cultivar, “US2007”, with 42 somatic chromosomes; **d**) spring triticale cultivar, “AgBeacon”, with 42 somatic chromosomes; **e**) blue aleurone spring triticale cultivar, “14UST001”, with 42 somatic chromosomes; and **f**) blue aleurone spring triticale cultivar, “14SUT002”, with 42 somatic chromosomes.

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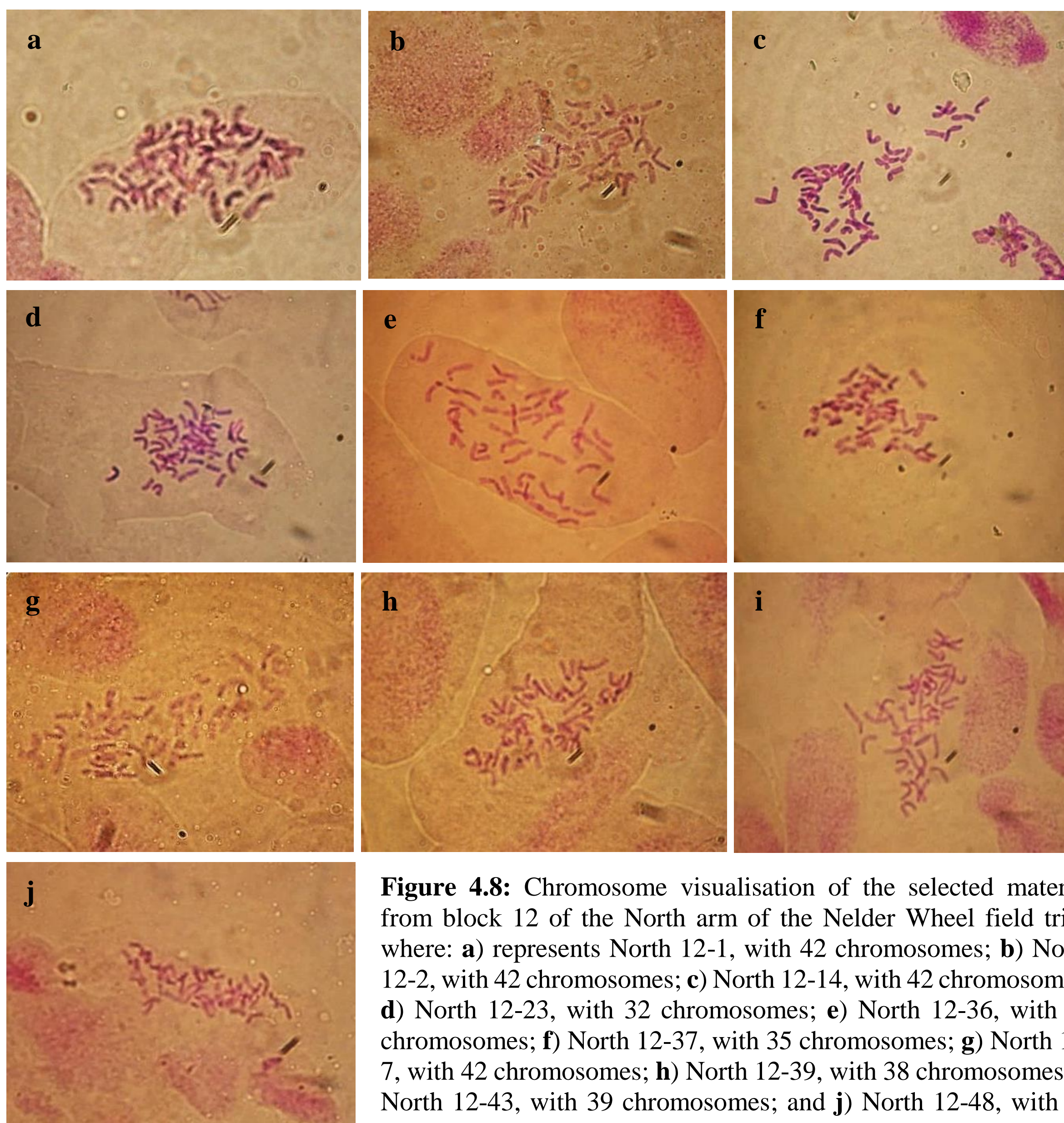

Figure 4.8: Chromosome visualisation of the selected material from block 12 of the North arm of the Nelder Wheel field trial, where: **a**) represents North 12-1, with 42 chromosomes; **b**) North 12-2, with 42 chromosomes; **c**) North 12-14, with 42 chromosomes; **d**) North 12-23, with 32 chromosomes; **e**) North 12-36, with 42 chromosomes; **f**) North 12-37, with 35 chromosomes; **g**) North 12-7, with 42 chromosomes; **h**) North 12-39, with 38 chromosomes; **i**) North 12-43, with 39 chromosomes; and **j**) North 12-48, with 35 chromosomes.

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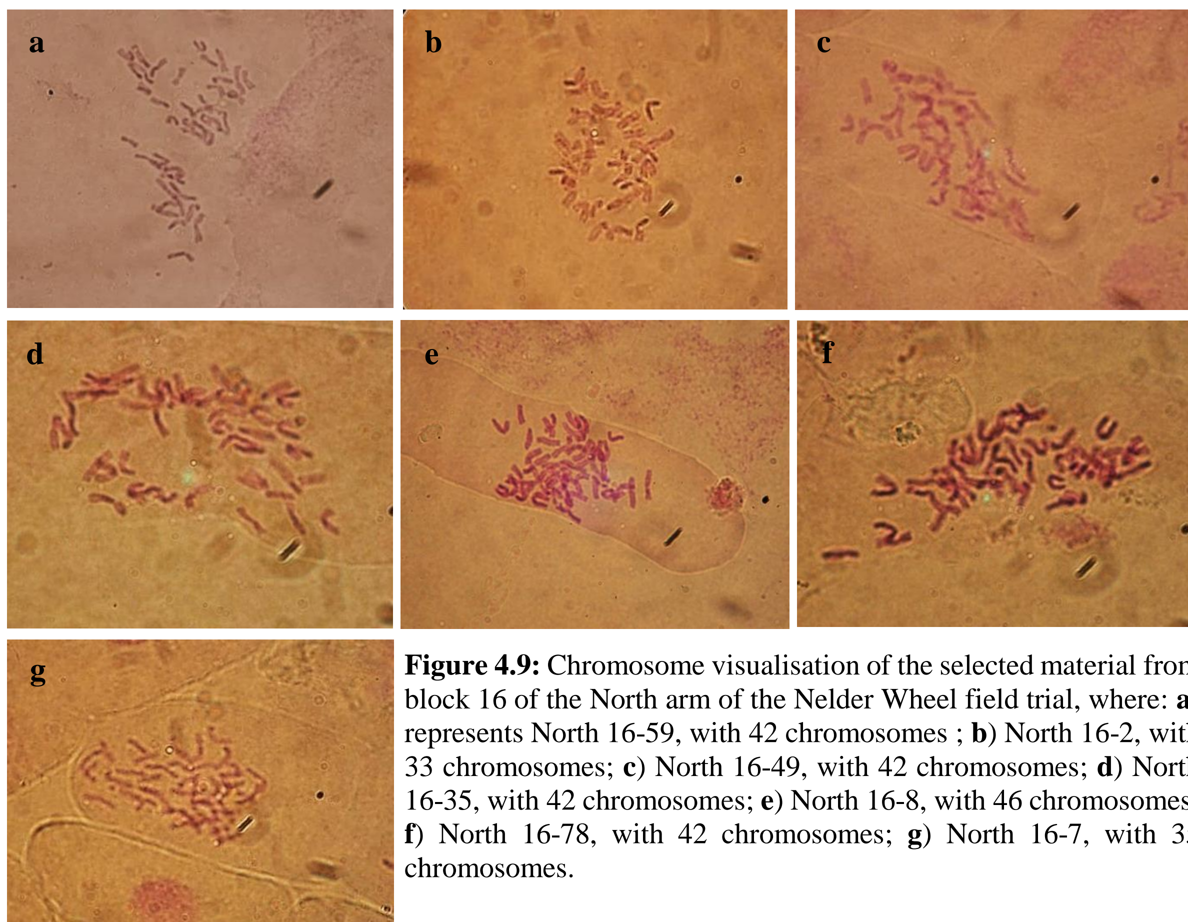

Figure 4.9: Chromosome visualisation of the selected material from block 16 of the North arm of the Nelder Wheel field trial, where: **a**) represents North 16-59, with 42 chromosomes ; **b**) North 16-2, with 33 chromosomes; **c**) North 16-49, with 42 chromosomes; **d**) North 16-35, with 42 chromosomes; **e**) North 16-8, with 46 chromosomes; **f**) North 16-78, with 42 chromosomes; **g**) North 16-7, with 35 chromosomes.

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4.3.2. Spring triticale

Cross-pollination events were identified by the expression of a light blue pigmentation within the spring triticale F2 seeds that were harvested from the greenhouse (Figure 3.6d). Out of the 150 631 seed screened, with an average of 1 183 seeds screened per block, 3 590 were blue - making the average outcrossing percentage 2.38% for this field trial. The outcrossing percentages ranged between 0.58 and 8.32% (Table 4.8), with the highest outcrossing percentages: 8.23%, 8.32% and 7.79% were at West 3, North-East 4 and North-East 5 respectively (Figure 4.7). Where, West 3 was 8.75m from the central pollen donor block (CPDB), North-East 4 at 10.25m, and North-East 5 at 13.75m from the central pollen donor block. The highest average pollen mediated gene flow (PMGF) at 3.75m from the CPDB occurred in the North-East direction (5.0%), and at 66.25m in the South-West direction (3.8%). The lowest average PMGF at 3.75m was in the North direction (1.0%) and at 66.25m in the North-West direction (0.8%).

When comparing the results that were obtained on PMGF of the central pollen donor block with pollen receptor blocks, with the wind data obtained for the ideal weather conditions for PMGF there tends to be a correlation as can be seen when comparing Figure 4.4 and 4.5 compared to Figure 4.13. For example: comparing the highest average PMGF at 3.75m and 66.25m from the CPDB in the North-East and South-West direction respectively with the wind data in Figure 4.5b, where wind speeds between 3 to 4ms^{-1} were recorded within the North-East and South-West directions.

Pairwise correlation coefficients was calculated as to determine whether the outcrossing percentages obtained for the eight arms differed significantly from one another. It was also conducted to determine whether the outcrossing percentages obtained for the different distances differed significantly from one another, see Table 4.8 and Table 4.9 for the results obtained.

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Table 4.8: The percent PMGF obtained for triticale in the eight major wind directions in the Nelder Wheel at distances 3.75 to 66.25m from the pollen source. The number of seeds screened and the number of blue seeds detected at each direction and distance are included.

Block	Mean Distance (m)	PMGF (%)									Seeds screened**	Blue seeds detected
		N*	NE	E	SE	S	SW	W	NW	Mean		
1	3.75	1.0	5.0	2.7	4.3	3.7	1.1	2.1	4.8	3.07	9092	284
2	6.25	0.8	4.8	3.5	3.5	1.9	1.6	3.9	1.6	2.70	8278	250
3	8.75	2.1	3.6	2.4	2.7	2.3	2.7	8.2	3.4	3.44	7807	214
4	11.25	0.7	8.3	4.8	3.3	2.3	1.8	4.4	3.7	3.65	7424	274
5	13.75	2.3	7.8	2.5	2.7	1.3	2.2	3.3	2.3	3.06	11540	390
6	16.25	2.7	1.8	1.9	1.9	1.6	2.7	2.2	1.7	2.07	10898	220
7	21.25	3.3	1.5	1.2	1.9	1.8	2.0	2.6	2.6	2.12	10773	216
8	26.25	3.0	1.0	2.4	3.5	3.4	1.7	2.4	1.5	2.37	9159	214
9	31.25	1.4	2.2	2.2	3.0	2.2	2.1	3.2	2.5	2.34	9633	227
10	36.25	2.0	1.3	1.7	3.2	2.5	1.8	3.4	3.5	2.42	8556	197
11	41.25	1.2	2.1	1.7	3.7	1.6	1.9	1.9	6.5	2.58	8167	165
12	46.25	2.9	1.6	5.6	4.2	1.0	1.0	1.5	3.0	2.58	7552	198
13	51.25	0.7	0.6	1.0	3.0	2.1	1.4	1.6	1.0	1.42	11015	166
14	56.25	1.9	1.0	1.3	2.2	1.2	2.7	1.7	1.5	1.68	10999	186
15	61.25	1.4	0.9	1.5	2.8	2.0	1.8	1.2	1.6	1.64	13660	219
16	66.25	1.2	3.1	2.5	3.0	2.8	3.8	1.8	0.8	2.37	6878	170
	Mean	1.79	2.91	2.43	3.06	2.10	2.02	2.84	2.63			

*N, North; NE, North-East; E, East; SE, South-East; S, South; SW, South-West; W, West; NW, North-West

** Total number of seeds screened from all blocks at the specific distance within the eight arms.

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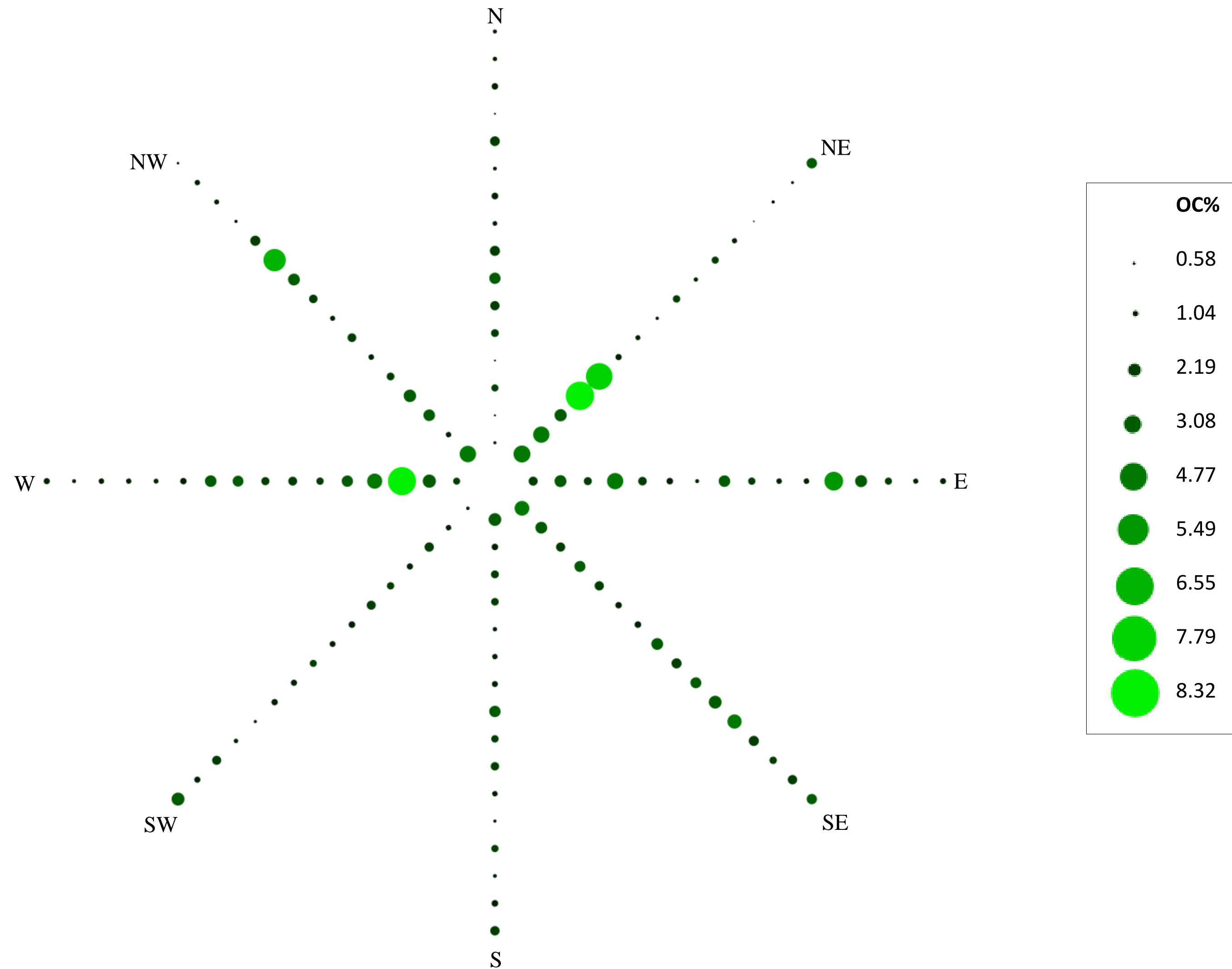

Figure 4.10: A Graph representing the outcrossing percentages (between 0.58 and 8.32%) obtained for each of the spring triticale blocks at 3.75m (i.e. N1) to 66.25m (i.e. N16) from the central pollen donor block in the eight arms within the Nelder Wheel field plot. Where, the OC% at each block is represented by the size and colour intensity of the circle.

Table 4.9 indicates the pairwise correlation coefficients that was calculated for the outcrossing percentages obtained between the different arms. The pairwise correlation coefficients that were obtained for the eight arms of the Nelder Wheel indicated that there were significant differences (indicated in red) between the arms. The following arms were significantly different:

i) North from North-East - where the average PMGF recorded in the North arm was significantly lower than the average PMGF recorded for the North-East arm.

ii) North-East from East, South-East, South, South-West and North-West - where the average PMGF recorded for North-East was significantly lower than South-East, but higher than the average PMGF recorded for East, South, South-West, West, and North-West.

iii) East from South and South-West - where the average PMGF recorded for East was significantly higher than the average PMGF recorded for South and South-West.

iv) South-East from South-West - where the average PMGF recorded for South-East was significantly higher than the average PMGF recoded for South-West.

Table 4.9: The pairwise correlation coefficients that was calculated on the OC% that was observed within the different arms of the Nelder wheel field trial

		Direction							
		N	**NE**	**E**	**SE**	**S**	**SW**	**W**	**NW**
Direction	**N**	*	*	*	*	*	*	*	*
	NE	0.018	*	*	*	*	*	*	*
	E	0.459	0.043	*	*	*	*	*	*
	SE	0.428	0.025	0.988	*	*	*	*	*
	S	0.560	8.72E-07	0.014	0.219	*	*	*	*
	SW	0.824	1.77E-07	0.001	0.007	0.775	*	*	*
	W	0.418	0.133	0.154	0.250	0.711	0.663	*	*
	NW	0.473	0.001	0.117	0.228	0.383	0.081	0.223	*

A similar pairwise correlation coefficients was calculated for the different distances from the central pollen donor block (Table 4.10). From the 120 combinations of the different distances from the central pollen donor block, there were 57 combinations that were significantly different. The average PMGF at 3.75m from the CPDB was significantly higher than the average PMGF at 16.25m to 26.25m and 46.25m to 66.25m from the CPDB.

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The average PMGF at 66.25m from the CPDB was significantly lower than the average PMGF obtained at 3.75m, 8.75m, 10.25m, and 46.25m from the CPDB. The average PMGF at 66.25m from the CPDB was significantly higher than the average PMGF obtained at 51.25m, 56.25m, and 61.25m from the CPDB.

Within triticale there is a wide range of pollen production and distribution that occurs among different triticale cultivars (D'Souza 1970; Yeung & Larter 1972). It is expected that triticale cultivars will vary in PMGF (Kavanagh *et al*. 2012), due to the fact that triticale cultivars are heterogeneous in that they have a variable genetic background and the amount of retaining of the rye genome (Kavanagh *et al*. 2012).

In a study that was conducted by Kavanagh *et al*. (2012) with a blue aleurone pollen spring triticale donor and spring triticale "AC Alta" as pollen receptor, they found that the highest average PMGF, of 3.4%, occurred near the pollen source and rapidly declined to 0.09% at 50m from the source. The maximum outcrossing they observed at 0.2m from the source was 5.07% and the minimum outcrossing they observed at 50m from the pollen source was 0.04% (Kavanagh *et al*. 2012). Within their study they did observe directional differences with the highest PMGF corresponding with the prevailing wind direction at flowering. They found PMGF in triticale to be similar to that of spring wheat and concluded that it should not prevent the coexistence of GM and conventional triticale using the 0.9% threshold established by the European Union (Kavanagh *et al*. 2012).

In a study on pollen mediated gene flow in between herbicide tolerant (donor) and herbicide susceptible (receptor) wheat, Loureiro *et al*. (2012) observed average outcrossing to be higher near the pollen source – 0.029% and 0.337%. Where the outcrossing percentages rapidly declined as the distance increased, where they detected hybrids up to 100m from the pollen source. They also noted that drought environmental conditions may have influenced the extent of outcrossing. Pollen viability, hybridization as well as pollen dispersal is effected by the ambient conditions (Loureiro *et al*. 2007). Loureiro *et al*. (2007) emphasized the major role that environmental conditions play in outcrossing, and found that outcrossing data obtained in one area may not correspond with prevailing situations in different location and climates.

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Table 4.10: Pairwise correlation coefficients on the OC% for the different distances from the central pollen donor block

Distance from CPDB (m)	Distance from CPDB[*] (m)															
	3.75	**6.25**	**8.75**	**10.25**	**13.75**	**16.25**	**21.25**	**26.25**	**31.25**	**36.25**	**41.25**	**46.25**	**51.25**	**56.25**	**61.25**	**66.25**
3.75	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
6.25	0.230	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
8.75	0.243	0.565	*	*	*	*	*	*	*	*	*	*	*	*	*	*
10.25	0.590	0.872	0.221	*	*	*	*	*	*	*	*	*	*	*	*	*
13.75	0.153	0.842	0.139	0.652	*	*	*	*	*	*	*	*	*	*	*	*
16.25	0.007	0.162	0.003	1.18E-05	0.004	*	*	*	*	*	*	*	*	*	*	*
21.25	0.009	0.030	0.015	1.00E-07	1.86E-04	0.989	*	*	*	*	*	*	*	*	*	*
26.25	0.001	0.017	0.001	1.44E-10	8.11E-08	0.897	0.883	*	*	*	*	*	*	*	*	*
31.25	0.299	0.677	0.204	0.003	0.033	0.919	0.812	0.819	*	*	*	*	*	*	*	*
36.25	0.063	0.073	0.115	2.30E-07	2.65E-05	0.872	0.949	0.919	0.990	*	*	*	*	*	*	*
41.25	0.334	0.126	0.001	1.40E-04	0.004	0.424	0.351	0.226	0.787	0.778	*	*	*	*	*	*
46.25	0.007	0.036	5.76E-07	1.38E-06	5.73E-05	0.318	0.390	0.188	0.293	0.241	0.203	*	*	*	*	*
51.25	3.94E-09	1.20E-06	1.56E-09	7.41E-25	1.47E-18	0.118	0.039	0.147	0.146	0.091	3.97E-06	1.33E-05	*	*	*	*
56.25	2.04E-05	0.002	3.86E-06	3.27E-13	1.44E-08	0.974	0.867	0.437	0.600	0.440	0.005	8.40E-03	0.097	*	*	*
61.25	1.26E-04	0.001	6.34E-09	8.05E-15	2.04E-10	0.774	0.560	0.675	0.542	0.454	0.015	1.99E-02	0.048	0.187	*	*
66.25	0.001	0.500	2.89E-05	3.40E-04	0.056	0.689	0.132	0.539	0.517	0.058	1.28E-07	1.76E-02	0.004	0.015	1.293E-13	*

[*]CPDB, Central pollen donor block.

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As PMGF was recorded at 66.25m from the central pollen donor block, this study finds similar to what was stated by Kavanagh *et al*. (2012) that it would be reasonable to expect that the pollen would have remained viable and that gene flow would have occurred past this point had the study extended past this distance from the central pollen donor block. This study also finds similar to what was found by Kavanagh *et al*. (2012) that the maximum impurity tolerance of 0.05% at 3m and 0.01% at 30m in Canada for certified and foundation seed respectively, and the 0.3% and 0.5% tolerance mixing with other seed in wheat and triticale at 5m for basic, pre-basic, and certified seed in South Africa will be exceeded within 66.25m from the pollen source. Isolation distances of more than 66.25m should thus be considered, or that as stated by Kavanagh *et al*. (2012) that the threshold may need to be increased, as the current thresholds are not realistic within 66.25m from the pollen source. The 5% labelling threshold of GM products in non-GM products of South Africa will be maintained by the 66.25m isolation distance, as the highest OC% obtained at 66.25m from the pollen source was 3.8% in the South-West arm. The effect of temporal isolation as well as the effect of other crops or bare ground (i.e. road) as physical barriers can also be further investigated to reduce PMGF. Isolation distances of further than 65.25m can also be further investigated in order to obtain an OC% of less than 1% that will make it possible for conventional farmers that are in close proximity to GM crops to be able to label their products as GMO free.

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CHAPTER 5 - CONCLUSION

The objectives that were set out to be conducted were completed and included: i) the optimization of screening methods for the blue aleurone and purple pericarp seed traits; ii) conducting the Nelder Wheel field trial that comprised of a central pollen donor block, containing blue aleurone triticale and purple pericarp wheat that was surrounded by eight pollen recipient arms, containing normal spring wheat and triticale, which were orientated to the cardinal and intercardinal wind directions; iv) planting the F1 spring wheat and spring triticale progeny that were harvested from the field trial in a single growth room and greenhouse respectively, under standard conditions; and v) the molecular screening of the growth room planted F1 wheat progeny for hybridization events through the use of molecular marker, *Cfe53*, and chromosome visualisations, as well as the morphological screening of the F1 spring triticale progeny that were harvested from the greenhouse planted F1 progeny for outcrossing events.

Within this study the hybridization that was observed within the F1 wheat progeny (30% to 100%) from the North, South, West and East arms in open field conditions within the Western Cape was in range, with a few higher than with what has previously been reported by Hills *et al*. (2007) and Coetzee (2011), where outcrossing values of 86±4 to 89±5.7% and 5.4 to 24.2% were obtained respectively with triticale as the pollen donor, within greenhouse experimental conditions. The cytological confirmation of the results confirmed the outcrossing observed for North 12, but not North 16. Where the hybridization of wheat with triticale in some of the plant material in North 16 may have had the loss of chromosomes, but in those with the normal somatic chromosome number (42 chromosomes) single polymorphisms may have occurred, that led to the detection of the hybrids through molecular screening. As the samples were pooled samples of 10 plants per sample the heterozygotes that caused the heterozygous profile of the sample could not to be detected individually. It was then decided to molecularly screen North 12 (50%) and North 16 (80%) individually for hybridisation events, but the molecular marker encountered technical difficulties. The samples from North 12 and 16 could thus not be screened individually with the molecular marker, *Cfe53*.

With the spring triticale, outcrossing percentages was observed that ranged between 0.58% and 8.32%. A pairwise statistical test was performed, that indicated that the average PMGF recorded for North East arm was significantly lower than of the South-East arm, but was significantly higher than the average PMGF recorded for the East, South, South-West, West,

and North-West arms. A similar pairwise statistical test was also conducted for the different distances from the central pollen donor block (CPDB), where the PMGF obtained at 3.75m was significantly higher that the PMFG observed at 16.25m to 26.25m and 46.25m to 61.25m. The average PMGF at 66.25m was significantly lower than that observed at 3.75m, 8.75m, 10.25m, and 46.25m; and was significantly higher than PMGF at 51.25m, 56.25m, and 61.25m. Within this study there also seemed to be a correlation between the prevailing wind direction and wind speed frequencies with the PMGF that were observed.

The PMGF that was recorded in spring triticale at 66.25m from the central pollen donor block, indicates that it is reasonable to expect, as found by Kavanagh *et al*. (2012), that the pollen would have remained viable and that gene flow would have occurred past this point had the study extended past this distance from the central pollen donor block. This study also finds similar to what was found by Kavanagh *et al*. (2012) and Coetzee (2011) that the maximum impurity tolerance of 0.05% at 3m and 0.01% at 30m in Canada for certified and foundation seed respectively, and the South African impurity tolerance of 0.3% and 0.5% at 5m for both wheat and triticale for basic, pre-basic and certified seed will be exceeded within 66.25m from the pollen source. Isolation distances of more than 66.25m should thus be considered, or that as stated by Kavanagh *et al*. (2012) that the thresholds may need to be increased, as the current thresholds are not realistic within 66.25m from the pollen source. The 5% threshold stipulated in Regulation 293 (2011), under the South African Consumers Protections Acts of 2008 for labelling GM material in non-GM products will be maintained at 66.25m from the pollen source. Further risk assessment studies regarding the distance of pollen mediated gene flow between conventional triticale cultivars and possible GM triticale cultivars within South African ambient conditions will thus have to be conducted. Where the possibility of pollen mediated gene flow further than 66.25m from the pollen source will need to be tested in order to comply with the impurity tolerance of basic, pre-basic and certified seed in South Africa. Isolation distances further than 66.25m can also be further investigated to obtain OC% values lower than 1% in order for conventional farmers that are closely situated to GM farmers to be able to label the crops GM free.

The aim of this study which was to determine the crossability as well as the potential of pollen mediated gene flow between wheat and triticale under dry-land conditions within the Western Cape, was achieved. The data obtained within this study could be valuable in future research on biosafety in South Africa on wheat and triticale cultivars. Future research on hybridization and outcrossing between triticale and wheat can thus be conducted in the different areas in

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South Africa and on different wheat and triticale cultivars as there are also differences between cultivars concerning the amount of outcrossing, pollen weight, and pollen production. The quickest and most reliable approach to determine crossability and pollen mediated gene flow in small grains such as wheat and triticale, would be through the combined use of molecular markers and phenotypic observation, where as in this study cytological screening can be used as a confirmation of the results obtained. By using a wider set of molecular markers for the screening of hybridization events in future studies the effect that the molecular marker technical difficulties had on this study could be eliminated. The molecular markers could be linked to the seed colour trait, a certain herbicide resistance, or genome specific to the parental species involved. The effect of temporal isolation and different physical barriers (i.e. other crops or bare ground) can also be further investigated within South African conditions. This will help to develop isolation distances and strategies that will maintain the 5% GM labelling threshold that is stipulated in Regulation 293 (2011), under the South African Consumers Protections Acts of 2008 (Acts online 2013).

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ADDENDUM A

Table 5.1: List of chemicals applied to the Nelder Wheel field trial. The concentration they were applied at as well as the dates they were applied on are also included.

Name	Concentration	Date applied
Herbicides		
Axial	6.97ml/L	24 June 2014
Roundup	28.57ml/L	24 June 2014
Axial	6.97ml/L	21 July 2014
Roundup	28.57ml/L	21 July 2014
Axial	6.97ml/L	30 July 2014
Roundup	28.57ml/L	30 July 2014
Axial	6.97ml/L	11 September 2014
Roundup	28.57ml/L	11 September 2014
Insecticides		
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	2.5ml/L	2 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	10 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	22 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	1 October 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	10 October 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	22 October 2014
Pesticides		
Storm (rat poison)	2 pellets/ arm	11 August 2014
Snailnail (Slug poison)	1 Cup/ block	29 August 2014
Snailnail (Slug poison)	1 Cup/ block	22 September 2014
Fungicides		
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	2.5ml/L	2 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	10 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	22 September 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	1 October 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	10 October 2014
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	22 October 2014
Fertilizer		
Nitrophosko	10:1:5(31)	25 June 2014
Nitrophosko	10:1:5(31)	2 July 2014
Nitrophosko	10:1:5(31)	30 July 2014
Nitrophosko	10:1:5(31)	10 September 2014
Nitrophosko	10:1:5(31)	22 September 2014

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ADDENDUM B

Table 5.2: List of chemicals applied to F1 plants replanted in the greenhouse at Welgevallen Experimental Farm. The concentration they were applied at as well as the date they were applied are also included.

Name	Concentration	Date applied
Insecticides		
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	30 January 2015
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	13 February 2015
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	16 March 2015
Fungicides		
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	30 January 2015
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	13 February 2015
Bifenthrin (synthetic pyrethroid)/Myclobutanil (triazole)	10ml/L	16 March 2015

ADDENDUM C

Table 5.3: Microsatellite marker used to screen for hybridization events

Genome	Marker	Forward/Reverse	T_A (°C)	Repeat	Expected Length (bp)	M.A.F*	Allele No.**	Gene diversity	PIC***	Source
A - genome	*cfe53*-2AL F	TGGACCGCAGAGACTTCG	60	$(TC)_{10}$	100-124	0.22	8	0.86	0.85	Botes & Bitalo 2013
	cfe53-2AL R	GTCCGCCCAAACCCTACC								
R-genome	*Xrems1266*-5R F	ACGACGGCAGTGAGAGAGAG	54	$(GA)_{8}$	168-271	0.3	7	0.82	0.80	Botes & Bitalo 2013
	Xrems1266-5R R	TCGGCTTCATCGTCTACTCC								
	SCM9-1RS F	TGACAACCCCCTTTCCCTCGT	60	$(GT)_{8}$	220	0.8	3	0.34	0.31	Botes & Bitalo 2013
	SCM9-1RS R	TCATCGACGCTAAGGAGGACCC								
	SCM40-7R F	CCCTTCAGCGGTCATTGTTG	60	$(GT)_{18}$	159	0.4	7	0.78	0.76	Botes & Bitalo 2013
	SCM40-7R R	CACATCTTGGGCCTGACACC								
	SCM152(F1)-5R	TAAAACGACGGCCAGTGACGA	68	$(AG)_{7}$	270-335	0.18	16	0.88	0.87	Ntladi 2011
	SCM152(F2)-5R	ACGGCCAGTGGAGCAGCAGCAG								
	SCM152-5R R	ATGTAGCCGAGGATGGTGAG								
	SCM159(F1)-4R	AAGAGCCAGTTTGGACTTGGAG	68	$(GAAA)_{5}$	114-167	0.28	26	0.88	0.87	Ntladi 2011
	SCM159(F2)- 4R	CGGCCAGTGGTTCCTTGGAT								
	SCM159(F2)- 4R R	CGGGAAGGAAAAACAGAAAACT								

*M.A.F. Major allele frequency, **No. Number, ***PIC, Polymorphism information content.