Evaluation of the crossability between small grains

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification

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Abstract

The greatest concern associated with genetically engineered crops is the possibility of GM crops contaminating other non-GM crops as a result of cross-pollination. Pollen drift is the primary mechanism in which GM crops contaminate traditional crops. However, genes can only be transferred between species if a certain degree of crossability exists. The aim of this study was to determine the potential risks associated with the release of transgenic crops and how to minimize these risks. Therefore, in this study crossability between three small grains was evaluated as well as pollen mediated gene flow from blue aleurone wheat. The potential frequency of cross pollination as well as the distance at which gene flow takes place was determined.

When crossing triticale and rye the outcrossing was low in both directions. When rye was used as the female the F_1 hybrid emergence was equal to zero and when triticale was used as the female parent the F_1 hybrids were sterile. Therefore the potential for gene flow between triticale and rye is highly unlikely. The crossability between triticale and wheat was higher when triticale was used as the male parent, however these crosses did not produce many viable hybrid plants. When wheat was used as the male parent the F_1 hybrid emergence was much higher and the F_1 generation produced viable seed. In crosses between wheat and rye, gene flow is only possible when wheat acts as the female parent. The F_1 generation is also capable of producing seed.

In the pollen dispersal study an average OC of 0.4% was observed. A maximum OC of 2.4% was observed at a distance of 2.5 meters from the pollinator. There were samples with outcrossing percentages of 1% and 1.3% at distances of 50 meters and 60 meters respectively. Therefore, results indicated that prevailing wind direction is not necessarily associated with higher OC rates. Therefore, to reduce gene flow as much as possible an isolating distance of at least 65m should be used.

Uittreksel

Die grootste bekommernis geassosieer met geneties gemanipuleerde gewasse is die moonlikheid dat GM gewasse konvensionele gewasse kontamineer deur middel van kruisbestuiwing. Stuifmeel verspreiding is die primêre meganisme waardeur tradisionele gewasse met GM-gewasse gekontamineer word. Geenvloei tussen spesies kan egter net plaasvind as daar ‘n mate van kruisbaarheid bestaan tussen die gewasse. Die doel van hierdie studie was om die potensiële risiko’s verbonde aan die vrystelling van genetiese gewasse te bepaal asook hoe om hierdie risiko’s te verminder. Die kruisbaarheid tussen drie klein grane is bepaal asook die die stuifmeel bemiddelde geenvloei vanaf blou graan. Die potensiële frekwensie van kruisbestuiwing asook die afstand waarteen kruisbestuiwing kan plaasvind is ook bepaal.

Wanneer korrog met rog gekruis word is die kruisbaarheid laag in albei rigtings. Wanneer rog as die vroulike ouer gebruik word, is die opkoms van die F_1 generasie gelyk aan nul en wanneer korrog as die vroulike ouer gebruik word, is die F_1 generasie steriel. Die potensiaal van geenvloei tussen rog en korrog is dus hoogs onwaarskynlik. Daar is bevind dat kruisbaarheid tussen korrog en koring hoër is wanneer korrog as die manlike ouer gebruik is. Hierdie kombinasie het egter nie baie lewensvatbare plante geproduseer nie. Wanneer koring gebruik word as die manlike ouer is die opkoms van die F_1 generasie baie hoër en die F_1 generasie produseer lewensvatbare saad. In kruisings tussen koring en rog is geenvloei slegs moontlik wanneer koring as die vroulike ouer gebruik word. Die F_1 generasie is ook in staat om saad te produseer.

In die stuifmeelverspreiding-studie was ‘n gemiddelde kruisingsbaarheid persentasie van 0.4% waargeneem. ‘n Maximum kruisbaarheid persentasie van 2.4% was waargeneem teen ‘n afstand van 2.5m vanaf die stuifmeel donor. Daar was ook monsters wat 1% en 1.3% kruisbaarheid getoon het by afstande van 50 en 60 meter onderskeidelik. Resultate van die studie toon aan dat heersende winde nie noodwendig met hoër kruisbaarheid geassosieer word nie. Om geenvloei te verhoed, moet isolerende afstande van ten minste 65m gebruik word.

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Abbreviations

°	Degrees
°C	Degrees Celsius
%	Percentage
Ba	Blue aleurone
CIMMYT	International Maize and Wheat Improvement Center
cm/sec	Centimeters per Second
DNA	Deoxyribonucleic Acid
E	East
F_1	First Filial Generation
F_2	Second Filial Generation
GM	Genetically Modified
Ha	Hectares
Hg/Ha	Hectograms per Hectare
HMW	High Molecular Weight
km	Kilometers
kg/hl	Kilograms per Hectoliter
la	Liters of 100% alcohol per 100 kg of dry matter
m	Meters
m^2	Square Meters
MES	Mariendahl Experimental Station
N	North
NE	North East
NW	North West
OC	Outcrossing
PMGF	Pollen Mediated Gene Flow

QTL	Quantitative Trait Locus
RAPD	Random Amplifications of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
S	South
SE	South east
sp.	Species
SSR	Single Sequence Repeats
SSRs	microsatellite markers
SW	South west
W	West

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Language and style used in this thesis are in accordance with the requirements of the South African Journal of Plant and Soil. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters have, therefore, been unavoidable.

Chapter 1

Introduction

Chapter 1: Introduction

The improvement of wheat is of great importance since it is the third most produced cereal in the world after maize and rice. With 225 million hectare planted worldwide and when all uses are considered, it is clear why wheat is regarded as such an important crop. Between 1999 and 2020 the demand for cereal grains are projected to increase by 80%. By the year 2020 almost 70% of wheat consumption will take place in third world countries (Pinstrup-Anderson *et al*., 1997). In recent years the average wheat production has been between 590 and 600 million metric tons. By 2020 this will have to be increased to about 840 million metric tons to feed the growing global population. Approximately 2.5 tons per hectare is the current global average yield, therefore this will need to be increased to 4.2 tons per hectare to meet the demand (Rajaram, 2005).

In the modern word plant breeding has become increasingly important. However, to create new cultivars, the gene pool available for breeding needs to be enlarged as genetic variability is essential for crop improvement (Francki *et al*., 2002). Many superior wheat cultivars have been developed, but there are still traits of interest that do not exist in wheat. Conventional plant breeders have been introducing genes from tertiary gene pools due to a naturally occurring degree of crossability that exists between different grass species. Through inter-species hybridization we are therefore equipped with a continuing supply of novel genes, but it should be kept in mind that genes do break down with time, especially those associated with disease resistance (Fedak, 1999). Therefore a continuous enlargement of the gene pool is of great importance as we are always in need of genes that confer disease resistance, tolerance to abiotic stress as well as quality traits (Francki *et al.,* 2002).

With the advent of molecular biology, and improved biotechnology tools, genetically modified crops have become increasingly important. With genetic engineering it is possible to increase yield as well as the nutritional composition of a crop, by for instance inserting traits from further related species. One example is the altering of nutritional composition in order to increase the amount of starch and vitamins in certain crops (Gatford *et al.*, 2006). This is especially important for those people who lack the necessary amount of vitamins in their diet. Genetically modified (GM) crops can also reduce the amount of pesticides and

herbicides applied to crops. Consequently the amounts of greenhouse emissions are also reduced as the use of machines for the applications of these sprays are reduced (Gatford *et al.*, 2006). Not only is this benefitting the environment but also human health (Cox, 2008). South Africa was the first country on the African continent producing GM crops (Cloete *et al.*, 2006). Most GM crops produced in South Africa have been altered to be insect- and herbicide-resistant (Aerni, 2005).

Even though GM crops have many positive effects on the environment, there are concerns about the potential negative effect it may have on the environment (Messeguer, 2003). The greatest concern is the possibility of GM crops contaminating other non-GM crops as a result of cross-pollination (Belcher *et al.,* 2005), thereby increasing the risk of gene flow between GM crops and related wild species or weeds (Messeguer, 2003). The exchange of genes between crops and weeds have been taking place for hundreds of years, but genetic engineering introduces genes that confer novel-fitness related traits (Snow, 2002).

The primary mechanism in which transgenic crops contaminate conventional crops is through pollen drift. Pollen drift however requires wind or insects to distribute the pollen to non-GM crops. The popularity of organic foods have increased substantially (20% each year) over the last few years (Cox, 2008). As the production of organic food is highly regulated, GM crops make it difficult for producers to grow crops that are free from contamination. If any genetically engineered proteins are detected in their products, producers may lose their certification (Kuparinen *et al.*, 2007). Some consumers are also concerned about the possibility of allergies that can be associated with GM crops (Goodman *et al.,* 2008).

The aims of this study was to evaluate the crossability between wheat, triticale and rye as well as to determine the possibility of pollen mediated gene flow in wheat. The results of this study will be useful in making risk assessments as well as provide insight into the best way to avoid gene flow from transgenic wheat.

The study was therefore divided into two parts. In part one the crossability between the cereals was determined. Differences between genotypes were also determined.

This was achieved by:

1) Crossing of three cereals with one another in all possible cross combinations in order to calculate the outcrossing percentage.
2) Performing embryo rescue on all the F_1 seeds produced from the crosses. This data was used to determine the F_1 hybrid emergence of each cross.
3) Growing the F_1 plants to maturity in order to evaluate the fertility.

In the second part of the study the potential frequency of cross pollination as well as the distance at which gene flow takes place was determined. This was achieved by:

1) Planting a field trail that consisted of a central pollinator and recipient blocks in all eight mayor directions.
2) Evaluating F_2 seed for the blue aleurone trait indicating outcrossing.

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Chapter 2

Literature Review

Contents

1. Introduction to Wheat, Rye and Triticale

1.1 Wheat

Hexaploid bread wheat (*Triticum aestivum)* emerged as a crop over 10 000 years ago through the hybridization of *Triticum dicoccum*, which is tetraploid, with *Aegilops tauschii*. Great advances have been made since the 19th century when organized selection and breeding to improve wheat cultivars began. Breeding mainly focused on producing high yielding cultivars that are well adapted to localized environmental conditions. Wheat is now widely adapted and grown all over the world, and is one of the most important crops (Worland *et al.,* 2001). However domestication and modern plant breeding narrowed the genetic base of bread wheat. This is of major concern for future crop improvement (Reif *et al.,* 2005).

To achieve maximum yield, breeders introduced genes such as the semi-dwarfing genes that directly increases yield. A rye chromosome arm was also introduced in place of a homologous group 1 wheat chromosome. This segment introduced disease resistance to wheat and even now this segment is present in the top yielding varieties even though pathogens have overcome these resistance genes (Worland *et al.,* 2001).

1.1.1 Production and Uses

Wheat is the third major cereal crop after maize and rice with 225 million hectare planted worldwide. During 2009 2.5 t/ha was produced on the African continent and 3.0 t/ha (1.9 million tons) in South Africa (FAOSTAT, 2011). It's clear why wheat is such an important crop when all the uses are considered. Bread wheat is the most common wheat and is used to make bread as well as a variety of other baked goods. The second most common wheat is durum which is used to make pasta. Wheat alone contributes to 20% of the world's total plant derived edible dry matter (Stone *et al.,* 2000).

Apart from increasing yield, improvement of baking quality has also been one of the major targets when improving wheat and creating new cultivars. Some of the characteristics that are used to predict baking quality is: particle size index, flour protein content, water

absorption, flour paste viscosity, dough resistance and dough extensibility (Kuchel *et al.,* 2006).

1.1.2 Other uses

Wheat may be used as animal feed, and about 40% of the wheat marketed in developing countries consists of feed wheat. There is however some genetic differences between wheat for human consumption and those used for animal feed. A substantial amount of wheat is also used in distilling processes to produce alcohol such as beer. The milling characteristics of the grain may be important in the distilling process as it affects the way the granules are exposed to the enzymes, and softer wheats are preferred (Worland *et al.,* 2001).

Wheat is also used as bio-ethanol feedstock in Europe. One of the main objectives in the production of industrial bio-ethanol is to acquire the largest quantity of renewable energy per unit area. Therefore the use of cereals is ideal. The cultivation of cereals also does not require such large amounts of energy as in the case of sugar beets. According to many researchers wheat and triticale are the two cereals that produce the most bio-ethanol (Kučerová, 2007).

Current research also focuses on the production of bio-ethanol from wheat straw as it is one of the most abundant crop residues. In Europe 170 million tons are produced which make it the cheapest raw material for the production of bio-ethanol (Fang *et al.,* 2002).

1.2 Rye

1.2.1 History and Classification

The precise origin of rye is unknown, but it is thought to be south-western Asia (roughly the same area of origin as wheat). Rye is also not as old as wheat since there are no ancient writings suggesting the cultivation of rye. Rye was moved from its origin to northern Europe sometime during the first millennium and there it was cultivated for the first time (Deodikar, 1963).

Rye is a cross pollinating crop and for this reason it is difficult to keep rye lines genetically pure. This is also the reason why much less effort has been put into the improvement and development of new rye lines. Therefore there is much less cultivars of rye that are grown around the world (Bushuk, 2001). Mostly rye is planted in the fall (winter types) as it possesses superior winter hardiness. It can therefore be grown in areas with severe winter climate even when other cereals, such as wheat, cannot. There are also spring cultivars, but there end-use quality is inferior to that of winter types (Bushuk, 2001).

1.2.2 Production and uses

The worldwide production of rye has decreased considerably since the 1970's. During 1986 24 million hectares of rye was planted and this decreased with 29% by 1996. During this time the production also decreased from 30 to 22 metric tons (a 27% decrease). The decrease in planted area was mainly due to the increase in yield. From the 1960's to the 1990's the yield increased from 0.52 t/ha to about 0.82 t/ha. This 57% increase was due to improvement in agronomic practices and breeding. This includes: 1) the use of chemical fertilizers, 2) better crop rotation practices and 3) the development of higher yielding cultivars (Bushuk, 2001). Currently 66 million hectares of rye is planted worldwide, making rye the eighth major cereal crop. In Africa more than half of the rye is produced in South Africa (0.6 t/ha approximately 2000 tons) (FAOSTAT, 2011).

Rye has many uses as it is a very versatile crop. As a green it can be used as livestock pasture, and as a grain rye is used as feed for livestock, distilling of alcohol and as flour. The flour can be used to make bread and other baked products. Rye is the second most commonly used grain for bread making (Bushuk, 2001). Although large amounts of rye flour is used for baking, rye is considered inferior to wheat as the dough isn't as elastic and the gas retention properties are lacking. It therefore cannot produce high volume breads, but it can be used to produce specialty products such as flat breads and rye crisps (Bushuk, 2001).

When using rye to produce bio-ethanol it is necessary to perform certain pretreatments, such as wet oxidation, to open the lignocellulosic structure to enable enzyme hydrolysis. This is because rye contains less starch and sugars than wheat and triticale and therefore

lignocellulosic materials will increase the amount of bio-ethanol produced by rye (Petersson *et al*., 2007).

Rye also has the potential for using it to produce bio-gas which consists mainly of methane and carbon dioxide. Biogas is the product of anaerobic digestion of the organic fraction of plants such as leaves and grass and therefore requires plants with a high carbohydrate contents and nitrogen content (Petersson *et al*., 2007).

Rye produces more bio-ethanol and biogas than both oilseed rape and faba beans. However, producing bio-ethanol from rye is not economical as these are other cereals such as triticale that produces much more bio-ethanol than rye. The income from producing bio-ethanol from rye is much less than the input cost of growing the crop (Petersson *et al*., 2007).

1.3 Triticale

1.3.1 History

The history and evolution of triticale is very unique in comparison to wheat and other allopolyploids. This is because the evolution of triticale only started 114 years ago and most of the dramatic evolutionary events (such as intergeneric hybridization and chromosome doubling) was almost all directed by humans. According to today's definition the German breeder, Rimpau, bred the first true triticale during 1888 (Ammar *et al*., 2004).

Triticale is a genetically amphiploid species consisting of the genomes of wheat (*Triticum aestivum*) and rye (*Secale cereale*) (Ammar *et al*., 2004). The first triticale was extensively studied during the first half of the twentieth century, but despite breeding efforts, triticale cultivars did not spread to a substantial extent (Ammar *et al*., 2004).

1.3.2 Production and uses

In 1964 the International Maize and Wheat Improvements Center (CIMMYT) started a Triticale breeding program under the leadership of the Nobel laureate Norman E. Borlaug

(Zillinsky and Borlaug, 1971). In the beginning, even though triticale had a vigorous growth habit, the plants were very tall, sterile, late, day-light sensitive and had shriveled seeds. Therefore there were many difficulties that had to be overcome to produce a viable crop (Mergoum *et al.,* 2004).

Today triticale is an accepted crop in many countries and nearly 43 million hectares are planted worldwide (FAOSTAT, 2011). CIMMYT has also become the leading supplier of triticale germplasm to agricultural research systems all around the world. Even though it is difficult to determine, data indicates that more than 200 cultivars (in more than 30 countries) have been released from direct CIMMYT germplasm introductions or through selection from segregating populations (Mergoum *et al.,* 2004).

Triticale is primarily used as animal feed or as forage. Generally winter triticale produces higher forage biomass and is therefore more suitable for grazing than spring types (Mergoum *et al*., 2004).

Since the amino acid composition of triticale fits the nutritional requirements of mono-gastrics as well as poultry it is perfect as an animal feed. Triticale can also substitute maize in poultry feed rations. Straw is also a major feed source in many countries and in dry areas it can have a higher value than grain. It has been found that triticale produces more straw than both wheat and barley (especially in arid and semi-arid regions) (Mergoum *et al.,* 2004).

Even though the use of triticale for human consumption is limited it has the potential to help feed the ever growing world population in especially resource limited environments (Curtis *et al*., 2002). This is because triticale is a good source of mineral nutrients as well as vitamins and the protein concentration is comparable to that of wheat. Therefore triticale cultivars with increased nutrients can have a large impact on communities with predominant cereal-based diets (Mergoum *et al*., 2004).

Over the years breeders have mostly focused on improving the agronomic and disease resistance of triticale. Consequently the improvement of traits associated with grain colour

and bread making quality have been neglected. However, today triticale can be used for making cookies and biscuits, replacing soft wheat.

Triticale is one of the cereals that can produce the most bio-ethanol. An important aspect in the production of bio-ethanol is the enzyme activity. The auto-amylolytic enzymes of triticale was found to have higher amylolytic activity than those present in cereals such as wheat and rye. Triticale also produces sufficient amounts of starch which is easily susceptible to the enzyme activity (Kučerová, 2007).

2. Crossability between small grains

Crossability refers to the ability of different species or cultivars to cross with each other, and in order to introduce a desired gene into a crop a certain degree of crossability must exist between crops (Fedak, 1999). This is the reason why it is important to assess the crossability between various cereals, and update this information as germplasm improves. There are however various barriers to crossability, with the large diversity of plants alone testifying to this fact (Solbrig, 1970). Most of these barriers are only partial and depends on physical separation (time, distance, barrier planting and wind). All of these can however be manipulated by plant breeders. Absolute barriers cannot be controlled by breeders as these include hybrid breakdown and incompatibility between gametes (Bates *et al.,* 1973).

There are two types of incompatibility between gametes. The first and most studied of the two is self-incompatibility that prevents inbreeding. The other is cross-incompatibility and is the opposite of self-incompatibility. It prevents hybridization and promotes specialization (Bates *et al.,* 1973).

2.1 Crossability genes

Research concerning the crossability between wheat and rye has been taking place since the start of the 20th century (Blackhouse, 1916; Alagu *et al.,* 2009). A breakthrough was made in 1942 when Lein and colleagues, using genetic studies, provided evidence for the existence of crossability genes. They proved that crossability is under the control of dominant alleles of two genes, *Kr1* and *Kr2*. These genes are located on chromosome 5B and 5A respectively

(on the long arm) and are responsible for the poor crossability between wheat and rye (Alfares *et al.,* 2009). The results from these early studies eventually lead to the discovery of other genes involved in crossability. These include: *Kr3* on chromosome 5D and *Kr4* on chromosome 1A (Zheng *et al.,* 1992).

Crossability is reduced by the Kr genes by inhibiting the entry of the pollen tube into the micropyle of the parent. Therefore it seems that the *Kr* genes are expressed in the floral tissue of the plant (Alagu *et al.,* 2009). There are two other genes, *Vrn1* and *Ph1*, present on chromosome 5B that play an important role in crossability (Griffiths *et al.,* 2006). The role of *Ph1* is important in the correct pairing of homologous chromosomes while *Vrn1*'s role is related to the vernalization requirement of wheat flowers (Alagu *et al.,* 2009). Only the basic molecular functioning of the *Kr* genes has been determined.

As in the case between wheat and rye, the dominant alleles drive incompatibility by inhibiting the production of intergeneric hybrids (Alfares *et al* 2009). In a study conducted during 1999, it was found that Chinese tetraploid wheat carries a recessive allele on chromosome 1A. This recessive allele causes an increased level of crossability between wheat and rye (Liu *et al.,* 1999). Further studies have also indicated that *Kr* genes have different effects on the crossability between wheat and rye. Six samples (different lines) were tested and the results indicated that *Kr1* has the strongest effect on crossability followed by *Kr2*, *Kr3* and *Kr4*. (Alagu *et al.,* 2009).

Results from crosses between wild barley and wheat indicated that *Kr1* and *Kr2*, which are involved in the crossability between wheat and rye, also control crossability between wheat and barley. The percentage of crossability was however much lower than with rye (Alfares *et al.,* 2009).

In 1998 the *SKr* gene was identified and was found to be another important gene controlling crossability. Using 187 doubled haploid lines; produced using anther culture of F_1 hybrids of 'Courtot' x 'Chinese Spring', the *SKr* gene was detected (Tixier *et al.,* 1998). Located on the short arm of chromosome 5B, *SKr* has been identified to be a major quantitative trait locus (QTL). The effect of *SKr* was found to be stronger (22.1% of heritability) than *Kr1* located on

chromosome 5B (5.5% of heritability). In the case of *Kr2*, no significant effect was detected (Lamoureux *et al*., 2002).

2.2 Embryo lethality genes

When two different species are crossed, incompatibility between the genomes can cause seedling or embryo lethality, death at later developmental stages or morphological abnormalities. It was suggested that these manifestations are under the control of specific genes. The genes responsible can have multiple alleles and certain combinations of these alleles cause lethality in F_1 hybrids. Two genes in rye inbred lines have been found to cause hybrid necrosis (Tikhenko *et al.,* 2005).

The number, effects and interaction patterns of these genes were studied by Tikhenko and colleagues during 2005. In the study they used two common wheat cultivars that contained two recessive alleles of the *kr1* and *kr2* gene. The genes conferred crossability with rye. The rye cultivars that were used were four inbred self fertile rye lines. Hybrid seeds obtained after crossing were classified according to their presence or absence of an embryo. Seeds that germinated were classified as "…grains with differentiated embryos…". Seeds that did not germinate were examined for the presence or absence of a differentiated embryo. The germinated seeds were planted and their hybrid status determined (Tikhenko *et al.,* 2005).

By determining the hybrid status they identified a rye gene that was involved in the formation of interspecific barriers at the post-gemetogenesis stage of fertilization. The development of a hybrid embryo is therefore under the genetic control of this gene. The gene was named *Eml* (Embryo lethality) and was represented by two alleles that differs in function. The *eml* allele was defined as normal and the *Eml* allele as abnormal according to the rate of appearance in rye lines and the expression thereof in wheat-rye F_1 hybrids. The appearance of the gene in wheat-rye F_1 hybrids, indicated a complementary interaction between the two parents. *Eml* has been found to be complementary to the corresponding gene in wheat. The abnormal *eml* allele however, was found to be non-complementary to wheat. Interaction between these complementary genes causes embryo lethality (Tikhenko *et al.,* 2005).

2.3 Wheat-rye crosses

In 1987 a crossability study was conducted in order to evaluate the crossability between wheat and rye. Zeven and colleagues (1987) summarized the crossability between wheat and rye of 1400 lines. Seed set was low for most of the crosses. Only the lines that were native to China, Japan and East Serbia had high crossability. This was attributed to landraces from China containing the crossability genes *kr1*, *kr2*, *kr3* and *kr4,* which is why many Chinese wheat landraces have high crossability with rye (Zheng-Song *et al.,* 1998).

During 1998 a similar but independent study was done by Zheng-Song. They also determined the crossability between wheat and rye, but used much fewer wheat lines. They only used 131 wheat landraces and one inbred line of rye as the male parent. They emasculated and pollinated the flowers by hand and the data was expressed as the total number of seeds with embryos over the total number of florets pollinated. In their study a landrace with a crossability of 5% or higher was regarded as a crossable landrace. They found that seed set was low when crossing wheat with rye. They also observed that only 14 – 16 days after pollination the embryo and endosperm started to deteriorate. The results seemed to be cultivar specific as 16 landraces had a crossability of 5 – 15% and three had a crossability of 40%. There were also three that did not cross with rye at all (Zheng-Song *et al.,* 1998).

2.4 Triticale-rye crosses

The dominant alleles, *Kr1* and *Kr2,* have been proven to reduce crossability between wheat and other species. In crosses between wheat and rye pollen tube growth can be retarded or even completely inhibited if the dominant *Kr* allele is present (Alagu *et al.,* 2009). Therefore the presence of *Kr1* and *Kr2* can also affect the crossability between triticale and rye (since the triticale genome includes the A and B genomes of wheat) (Guedes-Pinto *et al.,* 2001).

A crossability study was conducted during 2001 by Guedes-Pinto and colleagues (2001). They evaluated the crossability between triticale and rye as well as the genetic mechanisms involved. Their study consisted of two phases. In the one phase four rye lines was used as

the male parent and crossed with one triticale line. In the second phase four different triticale lines were crossed with only one rye. A wheat line was also used as a control since it has a high degree of crossability with rye.

The results from the first phase indicated that the crossability between rye and triticale is very cultivar specific with crossability between 5 and 21%. In the second phase one of the triticales showed a crossability of up to 58%. This was however still 30 – 50% lower than the control cross with wheat. This phase also indicated that crosses are cultivar specific, however, it would seem that early maturing cultivars show better crossability than late maturing cultivars. This might however be expected as the spring cultivars were from CIMMYT and their germplasm is known for containing crossability genes (Guedes-Pinto *et al.,* 2001).

The data from the crosses indicated that the trait of high crossability seems to be a recessive trait, confirming that the same *Kr1* and *Kr2* genes in wheat are also present in triticale. Some of the triticale genotypes were completely dominant for low crossability while other exhibited incomplete dominance. When crossing the F_1 generation with rye, the crossability was equal or slightly less than the parents that have the lowest crossability with rye. This is caused by the presence of complimentary inhibitory alleles in the F_1 generation that came from both the parents. Lower fertility of the F_1 generation may be caused by variations in meiotic irregularities. This could also affect the crossability between rye and triticale (Guedes-Pinto *et al.,* 2001).

In a recent study carried out by Hall and colleagues in 2007 they also found that outcrossing between triticale and rye seemed to be cultivar specific. The results from their study also indicated that outcrossing for reciprocal crosses were low. Therefore they concluded, based on the cultivars in used in their study, that the potential for rye crossing with triticale is very low. Even if all factors are favourable for crossing to take place, all the seeds that emerge will be infertile. Therefore if introgression should occur, the F_1 seeds are sterile and thus prevent gene flow (Hall *et al.,* 2007).

2.5 Wheat-triticale crosses

The crossability between triticale and wheat depends on which is used as the male and female parent. Earlier studies showed that the crossability was between 1.6 and 18.2% when triticale was used as the female parent (Vishwakarma *et al.,* 1985). The seed set was low, but the F_1 hybrid emergence was high (Khanna, 1990). However, when used as the male parent the complete opposite was observed. The seed set was high, but none of the seeds germinated (Khanna *et al.,* 1990; Jouve *et al.,* 1984). The low F_1 hybrid emergence was due to the fact that the embryos degenerated only a few days after pollination. The low crossability is due to poor germination of pollen and retarded growth of the pollen tube. In some cases pollen tube growth is completely inhibited (Khanna, 1990).

During a 2007 study it was determined that the crossability of triticale with wheat was higher when triticale was the male parent (>73%) and wheat the female parent. When triticale was used as the female parent in crosses with wheat the outcrossing was less than 23%. The emergence of hybrid F_1 seed from crosses between wheat (female) and triticale (male) was only 1%. Even though outcrossing between wheat and triticale was high, only a few seeds emerged and it was not viable. Viable seeds were however produced when triticale acted as the female parent (Hall *et al.,* 2007).

2.6 Molecular aids for the introduction of genes

2.6.1 Molecular markers

Although wheat-rye hybridization has been studied for over a hundred years the mechanism of wheat-rye hybridization is still not fully understood. To determine how the crossability genes in wheat have evolved, as well as how to overcome the reproduction barrier when making wide crosses, it is necessary to be able to clone the *Kr* genes (Mishina *et al* 2009). Therefore in 2009 two independent studies initiated 1) to develop a diagnostic marker for the *SKr* gene and 2) to localize the *SKr*-QTL on chromosome 5B using SSR markers (Alfares *et al.,* 2009).

Alfares and colleagues attempted to develop a diagnostic marker that can be used to introduce crossability into wheat germplasm by means of marker assisted selection. Introduction of these genes into advanced wheat breeding lines are usually done by backcrossing. Doing it in this way is however very time consuming, inefficient and laborious. The fact that rye is a cross pollinating species makes the process inefficient as a self-fertilized generation is required to determine whether the gene has been introduced. When assessing crossability without the use of markers an extra round of crosses is required (Alfares *et al.,* 2009).

Therefore the reliability and efficiency of the backcross process can be greatly improved by the use of markers. Identification will be possible without the need for testcrosses with rye or self-fertilization (Mishina *et al.,* 2009). Results from the study in 2009 revealed that cfb306 (a SSR marker) which is closely linked to *SKr* could be used as a marker for the introduction of crossability into the wheat germplasm. It has all the characteristics of an efficient marker and it has a high level of allelic polymorphisms, allowing it to be used with a large variety of wheat lines (Alfares *et al.,* 2009). Since cfb306 is a SSR marker it could also be used in high throughput genotyping automated techniques.

Integrating alien germplasm is possible if the donor represents primary or secondary gene pool species with one genome in common with the recipient. If these conditions are met recombination can take place, however the trait will only be successfully introduced after several rounds of backcrossing and selection. However the presence of the *Ph* locus on chromosome 5B will cause an insufficient degree of chromosome synapsis if the genomes of the donor and recipient are not homologous. In such cases substitution lines of the donor parents genome will need to be produced through backcrossing and screening of chromosomes (Fedak, 1999). The homoeology of these lines can then be identified by using molecular markers. These markers include: RAPD markers (randomly amplified polymorphic DNA) (Qi *et al.,* 1996), RFLP markers (restriction fragment length polymorphism) (Francki *et al.,* 1997), and microsatellite markers (also referred to as SSRs) (Peil *et al.,* 1998). The use of molecular markers decreases the selection time and increases the accuracy of selection.

2.6.2 Embryo rescue

Embryo rescue is a technique that can be used to recover embryos from interspecific or intergeneric hybrids. In these hybrids, the seeds that are produced may often not contain endosperm or an embryo at all. The seeds are then water filled sacs that may or may not contain a discernible embryo. If an embryo is present it is excised and it is placed on a culture media for further growth as it would be aborted if not rescued. In crosses made between closely related parents, embryos are well-differentiated, but in wide cross combinations embryos are usually very small with no further differentiation. Therefore the embryo formation frequency is cross-specific as well as the degree of the embryo differentiation (Fedak, 1999).

3. Pollen Distribution and Gene Flow

The difference between the development of GMO crops, also commonly referred to as transgenic plants, and traditional plant breeding is the fact that biotechnology allows for the transfer and introduction of genes from organisms that are naturally not compatible, whereas plant breeding only introduces genes from compatible related species. There is however potential negative effects that GM crops can have on the environment that researchers need to address. One of these challenges is gene flow from the GM crops to plants such as weeds, related wild species or even nearby non-GM cultivars of the same crop (Messeguer, 2003). The mechanism of gene flow differs between plant species. It is usually one of two mechanisms: 1) the movement of sporophytic seed and/or 2) distribution of haploid pollen followed by hybridization, also known as pollen mediated gene flow (PMGF) (Willenborg *et al.*, 2009). Even though crops and weeds have been exchanging genes for hundreds of years, GM crops have sparked new interest in understanding the implications of gene transfer as genetic engineering introduces genes that confer novel-fitness related traits (Snow, 2002).

Another important aspect to take into consideration is the ability of the hybrid seed to persist in the field and whether it may have any negative effects on the environment (Messeguer *et al.*, 2003). In the case of organic farming, producers lose their certification if

genetically engineered proteins are found in their products (Kuparinen *et al.*, 2007). European legislation stipulates that there may not be more than 0.9% contamination of conventional crop by GM crops. Therefore they recommend an isolation distance of 8m to avoid contamination of non-GM crops as well as the contamination of certified seed (<1%) (Loureiro *et al.*, 2007).

Interspecific and intergenic gene flow is commonly used by plant breeders to develop new or to improve cultivars. However gene flow as a result of outside pollen should be minimized in order to maintain genetic purity of breeding lines. In seed multiplication fields, gene flow is also unwanted (Hanson *et al.*, 2005).

3.1 Wheat pollen

Pollen mediated gene flow from wheat is expected to be low as it is a self-pollinating crop. In self-pollinating crops the onset of stigma receptivity and pollen shed occur at the same time within the floret (Gustafon *et al.*, 2005). During flowering the flowers open exposing the three anthers which releases the pollen. Therefore typically each floret is pollinated by anthers that are located within the same floret. Factors such as the duration of flower opening, anther extrusion as well as anther size are of vital importance for gene flow. All of these factors are controlled by genetic as well as environmental factors. Outcrossing rates increase with cultivars that exhibit a higher degree of floret opening and anther extrusion (Hucl, 1996; Hedge, *et al.,* 2004).

Previous research has lead to the following observations (Gustafon *et al.*, 2005):

1) Even among plants in close proximity (8-10 meters) the occurrence of gene flow is low (<1%);
2) An increase in distance between the pollinator and recipient decreases gene flow;
3) Gene flow is limited due to the fact that wheat pollen is viable for only a short period;
4) In comparison to related species, wheat produces only small amounts of pollen;
5) Wheat pollen settles quickly since it is relatively heavy if compared to other grasses;
6) Gene flow is also limited due to genotypic differences in flowering traits.

7) Insects do not play a role in the distribution of wheat pollen; and

8) Environmental factors such as humidity and temperature play significant roles in pollen dispersal.

As wheat is a self-pollinating crop it produces much less pollen than its outcrossing relatives. Wheat only produces one tenth of the pollen produced by rye; however possibility for gene flow does exist. Only 30% of wheat pollen is shed inside the flower leaving the rest available for outcrossing. Even though more than half of the pollen produced is shed outside the flower, wheat pollen is relatively heavy. Therefore the pollen mostly only travels short distances and wind is required to move it significant distances from the source (Hanson *et al.*, 2005). Studies have indicated that gene flow mostly occurs 0.5 – 1.5 meters from the pollinator (Hedge *et al.,* 2004). The average frequency of gene flow has been found to be 1%, but this value can increase to about 6.7% at distances less than 1m (Loureiro *et al.*, 2007). Other studies have however proven that gene flow can occur at distances greater than 6 meters. There have even been extreme cases reported where wheat pollen has been found as far away as 1000m from the pollinator (Hanson *et al.*, 2005). Table 1 contains the results from a few studies:

Table 2.1: Pollen mediated gene flow at increasing distances

	Year	Gene flow Frequency (%)	Distance (m)
Hucl and Matus-Cádis	2001	0.09	27
Lu *et al*.	2002	0.001	40
Matus-Cádiz	2004	0.005	300

Pollinating insects such as bees do not disperse pollen as wheat has small amounts of pollen and does not produce nectar (Treu *et al.*, 2000). High temperature and humidity also reduces gene flow as high humidity makes the pollen heavier and high temperatures cause a loss of pollen viability. High temperatures also reduce receptivity of the stigma and the duration pollen is shed from the wheat flower (Gustafon *et al.*, 2005).

3.2 Factors effecting pollen distribution

Environmental factors such as wind and humidity can directly affect gene flow by influencing pollen movement. However, environmental factors also affect floret opening, stigma receptivity, number and extent of anther extrusion, amount of pollen released, and pollen viability. These are affected by environmental factors such as rainfall, temperature, relative humidity, light intensity as well as various stress conditions (Hanson *et al.*, 2005).

3.2.1 Atmospheric conditions

For significant gene flow to take place pollen must be released on warm days with low relative humidity (Curtis *et al.*, 1995). These conditions cause the atmosphere to be moderately to highly unstable (Jackson *et al.*, 1999). The unstable and turbulent condition of the atmosphere is necessary for pollen to travel significant distances. There is however certain threshold wind speeds (<5cm/sec) needed for the movement of pollen (Jackson *et al.*, 1999). It is therefore necessary to monitor wind speeds as it has been proven that pollen movement is greater under unstable conditions than under neutral conditions. Simulations have also been used to illustrate the vast difference in pollen movement between stable and unstable atmospheric conditions. Unstable conditions cause an increase in vertical diffusion causing greater dispersal of pollen grains (Jackson *et al.*, 1999).

There is however other forces that affect the movement of pollen. Gravitational force causes pollen grains to resist movement. The size of the pollen as well as particle density, the roughness of the surface, and the degree of sphericity influences this resistance (Niklas, 1985). Increase in electrostatic force and surface adhesion can also cause pollen to resist movement in newly opened anthers (Jackson *et al.*, 1999).

3.2.2 Deposition parameters

Deposition velocity is responsible for the amount of pollen that is released into the air. Deposition velocity can be calculated by dividing the particle concentration in the air by the deposition rate. The velocity of sedimentation prevents pollen from being released into the air. Only if the deposition velocity is similar or higher than the sedimentation velocity can

pollen be released into the air. Sedimentation velocity is defined as the rate at which pollen descends to the ground as a result of gravity (in still air). The sedimentation velocity depends on genetic variation among grains from the same plant and different plants (Di-Giovanni *et al.*, 1995). If the only force responsible for the deposition of particles is sedimentation the pollen dispersal will only be influenced by gravity and horizontal wind speed (Jackson *et al.*, 1999). If the deposition velocity is halved the same level of pollen dispersal can be achieved by doubling the wind speed.

3.3 Effect of pollen viability on PMGF

Between 2000 and 2002 Loureiro and colleagues conducted a pollen viability study. They found that a hybridization rate of 86% was obtained from freshly collected pollen that was kept at 15°C. With increasing time and temperature the hybridization rate decreased. At 15°C the hybridization rate decreased by 14% and at 20°C it decreased with 23%. Zero seeds were set at 30°C and a hybridization rate of only 12% was obtained at 25°C. Time had an even more drastic effect on seed set with a hybridization rate of only 10% after 2 hours (15°C). No seeds were set at temperatures above 15°C after 2 hours. No seeds were set after 3 hours regardless of the temperature. Therefore it is clear that pollen viability decreases with time and exposure to the environment (Loureiro *et al.*, 2007).

Other studies have also been done to determine the survival of pollen when released into the atmosphere. It has been found that just two hours after the release of maize pollen into the atmosphere, a relative loss of 100% viability is observed. This is mainly due to a loss of moisture (Luna *et al.*, 2001).

3.4 How to minimize gene flow

3.4.1 Physical barriers

Gene flow can be minimized by the presence of vegetation barriers and border rows. Hedges and woodlands provide the most efficient barrier. These barriers allow air to flow through and this filters the air removing the pollen from the wind. These physical barriers can minimize this effect (Treu *et al.,* 2000).

3.4.2 Isolating distances

Isolating distances can also reduce gene flow. Isolating distances of 3 to 4 times the recommended distance should be used to try and eliminate gene flow (Treu *et al.*, 2000). It should be kept in mind that the isolating distance depends on pollen quality and viability, environmental conditions, flowering characteristics, compatibility with adjacent crops and the mode of pollen dissemination (Loureiro *et al.*, 2007). It has also been found that the size of the source can also influence gene flow. Larger source sizes increase gene flow at regional scale (Treu *et al.*, 2000).

Like crossability, the amount of PMGF that takes place, has been found to be cultivar specific. Hucl and Matus-Cádis conducted a field trail during 2001 to assess pollen mediated gene flow. The result from their study indicated that gene flow could take place at distance of up to 27 meters. In the study a wheat cultivar containing a blue aleurone was used as the pollinator, and surrounded by four different spring wheat cultivars. The recipient cultivars were planted in four directions, North, South, East and West, with a length of 35m. Using the results from their study they recommended that a minimum isolation distance of 30 meters should be used to avoid gene flow. However, the Canadian Seed Growers' Association recommends that only an isolation distance of 10 meters between crops of the same species should be used to avoid outcrossing (Hucl *et al.*, 2001).

Hanson and colleagues carried out a similar study. They studied gene flow by conducting field trails in five localities across three US states (Washington, Oregon and Idaho). The central pollinator block was 0.16ha in size and consisted of blue aleurone winter wheat. Surrounding the pollinator was 16 equally spaced strips consisting of two different cultivars ('Madsen' and 'Brundage 96'). The two cultivars were chosen to maximize the overlap of flowering. These strips were 46.3m in length starting at the edge of the pollinator. From the results from this study Hanson recommended that a 45m isolation distance should be used to avoid possible gene flow (Hanson *et al.*, 2005).

3.4.3 Planting density

Planting density also has an influence on PMGF. During 2005 and 2006, Willenborg and Brûlé-Babel conducted a study to determine the effect of plant density and height on gene flow. They also determined the relationship between gene flow and flowering synchrony. Results from the study showed that planting density has a large effect on gene flow. A maximum gene flow of 0.31% was obtained at very low planting densities. This value decreased exponentially when the planting density was increased. The gene flow was as low as 0.0003% when the highest planting density of 600 plants per m^2 was used. The gene flow declined with increasing planting densities regardless of the genotype. There was a lack of variation among genotypes, but this could be due to the effects of the planting densities overriding the effects of the genotypes. The last objective of the study was to determine the association between flower synchrony and PMGF. The results indicated that PMGF cannot be predicted by flower synchrony (Willenborg *et al.*, 2010).

In Canada the recommended planting densities are between 250 and 300 plants m^{-2} (Manitoba Agriculture, Food, and Rural Initiatives, 2008). The study indicated that increasing the planting density reduces gene flow, however, increasing the density above the recommended rate of 300 wheat plants m^{-2} does not further reduce gene flow. Decreasing plant density below the critical plant density of 175 – 200 wheat plants m^{-2} causes an exponential rise in gene flow (Willenborg *et al.*, 2009). Therefore it is recommended that planting densities higher than 300 plants m^{-2} should not be used as it would be impractical (Willenborg *et al.*, 2010).

3.4.4 Reproductive isolation

Physical isolation of crops isn't the only form of isolation that can be used to reduce gene flow. A hybridization window has been identified for spring wheat cultivars and acts as reproductive isolation. Another study was conducted by Willenborg and Brûlé-Babel during 2005 and 2006. The aim of their study was to determine whether emergence timing could temporally isolate different crops and to identify the hybridization window for spring wheat crops (Willenborg *et al.*, 2010).

Flower synchrony is highest when the emergence coincides between two crops. The highest gene flow (0.396%) was observed when the emergence of the pollinator and recipient plants coincided. When the flowering became less synchronous, the result was a dramatic decrease in gene flow. However, the gene flow frequency undergoes a two- to four-fold increase when flowering of the pollinator and recipient coincide. From the data they determined that a hybridization window of 125 degree-days exist as a function of pollinator emergence. Therefore to minimize PMGF, the emergence of neighbouring plants must not occur within 125 growing degree-days of crop emergence. This will lower the PMGF regardless of the genotypes (Willenborg *et al.*, 2010).

The study provided evidence that gene flow can be reduced by temporal isolation. It is however recommended that temporal isolation should be used in conjunction with physical isolation (Willenborg *et al.*, 2010). In previous studies done with maize, it has been found that the isolating distance of 500m can be reduced to less than 62m when two weeks of temporal isolation was used (Halsey *et al.*, 2005).

3.5 Gene flow at commercial scale

Matus-Cádiz *et a.,* (2001) also conducted a study to determine if pollen mediated gene flow can take place at commercial scale. Blue aleuroned wheat was used as the pollinator and 33ha was planted at varying distances from neighboring wheat fields (0 – 11.8km). Results showed that outcrossing was detected up to 2.75km from the pollen source, however the frequency of outcrossing was only 0.00009% (approximately 100 times lower than 0.01%). Therefore oucrossing can take place over great distances, but with such low frequencies, gene flow seems to be a minor contributor to product admixture (Matus-Cádiz *et al.*, 2007).

From all the studies it is clear that gene flow cannot be entirely eliminated, but by combining physical barriers and good isolating distances gene flow can be greatly managed.

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Chapter 3

Evaluation of the crossability between small grains

Evaluation of the crossability between small grains

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Abstract

Enlargement of the gene pool can be achieved through hybridization. In order to introduce a desired gene into a desired crop a certain degree of crossability must exist between the crops. In this study crossability between three small grains was evaluated. Intra- and inter-specific crosses were carried out by manual emasculation and pollination of the flowers. Crosses were carried out under greenhouse conditions using the following triticale (2), wheat (2) and rye (2) cultivars: 'US2007', 'US2009', 'US1010', 'SST88', 'US3010' and 'Duiker'. The outcrossing percentage, F_1 hybrid emergence as well as the fertility of the F_1 generation was recorded. Outcrossing and F_1 hybrid emergence was highest between different genotypes from the same species as to be expected. However the inter-specific crosses were influenced by the species, genotype and the gender of the plant. When crossing triticale and rye the outcrossing was low in both directions. When rye was used as the female the OC was 8.7% with an F_1 hybrid emergence equal to zero. When rye was used as the male parent the OC was 10.6%, but in this case the F_1 hybrid emergence was between 0 – 33.5%. The crossability between triticale and wheat was higher when triticale was used as the male parent. The OC ranged between 48 – 66.4%, however these crosses did not produce many viable hybrid plants. When wheat was used, as the male parent the OC was 5.4 – 24.2%, however the F_1 hybrid emergence was much higher and ranged between 12.5 – 44%. In crosses between wheat and rye the highest OC was obtained when wheat was used as the female parent (22.1 – 39.1%). The F_1 hybrid emergence was between 12.4 and 48%. When rye was used as the female parent outcrossing was less than 5% and the F_1 hybrid emergence was zero for all crosses but one. When Duiker was crossed with SST88, all three of the F_1 plants emerged; indicating that even though outcrossing is low, there is a small possibility that gene flow can take place.

Keywords: Outcrossing, wheat, rye, triticale

Introduction

The improvement of wheat is of great importance since it is the third most produced cereal in the world (FAOSTAT, 2011). Between 1999 and 2020 the demand for cereals is projected to increase by 80% (Pinstrup-Anderson and Pandya-Lorch, 1997). In recent years the average wheat production was between 590 and 600 million metric tons. By 2020 this will have to be increased to about 840 million metric tons to feed the growing global population. Approximately 2.5 tons per hectare is the current global average yield, therefore this will need to be increased to 4.2 tons per hectare to reach this goal (Rajaram, 2005).

Improvement of wheat also requires enlargement of the gene pool that is available for breeding (Francki and Appels, 2002). Genetic variability is therefore of great importance in crop improvement. There are thousands of wheat cultivars available but there are certain traits that do not exist in wheat and therefore must be introduced through tertiary gene pools. These gene pools equip us with a continuing supply of new genes, but genes that have been introduced into a crop will also break down with time (Fedak, 1999). These genes can confer disease resistance, abiotic stress tolerance as well as quality traits. Enlargement of the gene pool will also allow the breeder to increase yield and other agronomic important traits. This can be obtained through hybridization (Francki and Appels, 2002). In order to introduce a desired gene into a specific crop a certain degree of crossability must exist between the crops (Fedak, 1999). There are however certain situations where outcrossing is not desirable. Outcrossing between GM- and non-GM crops should be avoided since this threatens the genetic purity of non-GM crop (Cox, 2008).

Genetically modified crops have been found to have many positive effects on the environment. The use of pesticides and herbicides are greatly reduced and the amounts of greenhouse emissions are also reduced as the use of machines for the application of sprays are less. There is also a need to feed the global population and GM crops have the potential to address this problem, as it is possible to increase yield as well as increase the nutritional composition of the crop (Gatford *et al.*, 2006).

One of the largest driving forces behind the development of GM crops is the pesticide industry, by 1990 the industry was facing several major issues as it had become increasingly difficult and expensive to develop new pesticides (Kuyek, 2002). Another key concern was the fact that blockbuster pesticides were soon to become off-patent (Wheeler, 2002). This will allow generic producers to reduce prices and therefore take an increasing share of the market. GM crops allow companies to circumvent this generic pesticide problem. Crops can be modified in such a way that they only grow optimally when sprayed with their own pesticides (Kuyek, 2002).

By 1999, approximately 78% of GM crops planted worldwide were engineered for herbicide resistance. Most of these crops were engineered to be Roundup resistant. Monsanto, the leading supplier of Roundup and the owner of most Roundup resistant crops, protected its sales revenue from Roundup, which was coming off-patent in 2000/2001, using GM crops (Kuyek, 2002).

There is however the potential for negative effects on the environment. The difference between the development of transgenic plants and traditional plant breeding is the fact that biotechnology allows for the introduction of genes from organisms that are not compatible, whereas plant breeding only introduces genes from compatible species (Messeguer *et al.*, 2003). When creating GM crops there are a few key challenges that researchers have to face. One of these challenges is gene flow from the GM crops to plants such as weeds or related/wild species (Messeguer, 2003). Crops and weeds have been exchanging genes for hundreds of years, but genetic engineering introduces genes that confer novel-fitness related traits (Snow, 2002). New phenotypic traits that can potentially be introduced include insect-, disease-, herbicide-resistance and even resistance to harsh growing conditions (Snow, 2002). Another important aspect to take into consideration is the ability of the hybrid seed to persist in the field and whether it may have negative effects on the environment (Messeguer *et al.*, 2003).

The existence of crossability genes

In the beginning of the 20th century researches experimented with the crossability between wheat as the female parent and rye as the male parent. In 1942 Lein reported the existence of crossability genes. Crossability is under the control of dominant alleles of two genes, *Kr1* and *Kr2*, which are located on chromosome 5B and 5A respectively (on the long arm). The poor crossability between wheat and rye is the result of the presence of these genes (Alfares *et al.,* 2009). Later more crossability genes were identified. These include: *Kr3* on chromosome 5D and *Kr4* on chromosome 1A (Zheng *et al.,* 1992).

Crossability is reduced by the presence of *Kr* genes. It inhibits the entry of the pollen tube into the micropyle of the female parent. Therefore it seems that the *Kr* genes are expressed in the floral tissue of the plant (Alagu *et al.,* 2009). There are two other genes, *Vrn1* and *Ph1*, present on chromosome 5B that play an important role in crossability (Griffiths *et al.,* 2006). The role of *Ph1* is important in the correct pairing of homologous chromosomes while *Vrn1* is associated with the vernalization requirement of wheat (Alagu *et al.,* 2009). To date only the basic molecular functioning of the *Kr* genes has been determined.

In 1998 another gene controlling crossability was found. The gene, *SKr*, is located on the short arm of chromosome 5B and has been identified to be a major QTL (quantitative trait locus) (Tixier *et al.,* 1998). The effect of *SKr* was found to be stronger than *Kr1* (Lamoureux *et al*., 2002).

Embryo lethality genes

Manifestations such as seedling or embryo lethality, death at later developmental stages or morphological abnormalities are due to incompatibility between genomes of different species. Karpechenko suggested that specific genes define these interactions. These genes can have multiple alleles, and certain combinations of these alleles can cause lethality in F_1 hybrids (Tikhenko *et al.,* 2005).

In 2005 Tikhenko and colleagues attempted to determine the number, effects and interaction patters of these genes. They identified a rye gene that was involved in the forming of interspecific barriers at the post-gametogenesis stage of fertilization. The development of a hybrid embryo is therefore under the genetic control of this gene. The gene was designated *Eml* (Embryo lethality) and is represented by two alleles that differ in function. The *eml* allele is defined as normal and the *Eml* allele as abnormal according to the rate of appearance in rye lines and expression in wheat-rye F_1 hybrids. The appearance of the gene in wheat-rye F_1 hybrids, indicates a complementary interaction between the two parents. *Eml* has been found to be complementary to the corresponding gene in wheat. Interaction between these complementary genes causes embryo lethality. The normal *eml* allele however, was found to be non-complementary to wheat (Tikhenko *et al.,* 2005).

Wheat-rye crosses

Zeven summarized the crossability between wheat and rye of 1400 lines in 1987. Most of the crosses had very low seed set, but those that had high crossability were native to China, Japan and East Serbia. It was subsequently postulated that the crossability genes *kr4* as well as *kr1*, *kr2* and *kr3* are present in landraces from China. Which is why many Chinese wheat landraces have high crossability with rye (Zheng-Song *et al.,* 1998).

In 1998 a study was done by Zheng-Song to determine the crossability between wheat and rye. An inbred line of rye was used as the male parent and crossed with 131 wheat landraces. The results from the study indicated that seed set was low when wheat was crossed with rye. It was also found that the embryo and endosperm started to deteriorate 14 – 16 days after pollination. Out of the 131 landraces, only 16 had a crossability of 5 – 15%. There were also three landraces that had crossability of 40% and three that did not cross with rye at all (Zheng-Song *et al.,* 1998).

Triticale-rye crosses

The dominant alleles of the *Kr1* and *Kr2* genes have been proven to reduce crossability between wheat and other species (Alfares *et al.,* 2009). In crosses between wheat and rye pollen tube growth can be retarded or even completely inhibited if the dominant *Kr* allele is

present (Alagu *et al.,* 2009). It has also been found that *kr1* has the strongest effect in a wheat background. Therefore the presence of the *kr1* and *kr2* genes can also affect the crossability between triticale and rye since the genome constitution of triticale consist of the A and B genomes of wheat (Guedes-Pinto *et al.,* 2001).

In 2001 Guedes-Pinto and colleagues evaluated the crossability between triticale and rye as well as the genetic mechanisms involved. The study indicated that the crossability when using rye as the male parent and triticale as the female is very cultivar specific with crossability between 5 – 21% (Guedes-Pinto *et al.,* 2001).

In a recent study carried out by Hall and colleagues in 2007 they also found that outcrossing between triticale and rye seemed to be cultivar specific. Outcrossing seemed to be low for crosses made in both directions. Even if all factors are favorable for crossing to take place, all the seeds that emerge will be infertile (Hall *et al.,* 2007).

Wheat-triticale crosses

In previous studies it was found that the crossability between wheat and triticale ranged from 1.6 – 18.2% when triticale was the female parent and wheat the male parent (Vishwakarma and Mani, 1985). It was found that seed set was low, but germination was good (Khanna, 1990). However, when triticale was used as the male parent and wheat as the female parent seed set was good but none of the seed germinated (Khanna, 1990, Jouve *et al.,* 1984). The low germination of the seeds was attributed to seeds lacking an embryo. The low crossability was attributed to poor germination of pollen, and retarded growth of the pollen tube. In some cases pollen tube growth was completely inhibited (Khanna, 1990).

In a more recent study in 2007 the previous findings were confirmed. When triticale was used as the male parent a very high outcrossing percentage was obtained (>73%). However, an OC of less than 23% was obtained when triticale was used as the female parent. Even though the OC was high when triticale was used as the male parent the F_1 hybrid emergence was only 1% and these plants were not viable. Viable seeds were however produced when triticale was used as the female parent (Hall *et al.,* 2007).

The aim of this study was to evaluate the crossability between three small grains produced in South Africa: wheat, triticale and rye. The possibility of gene transfer between species and the differences between the different genotypes will be taken into consideration. By determining the crossability in a controlled environment and performing embryo rescue it is possible to create the worst-case scenario for gene flow to take place. Therefore, gene flow in the field should still be less or similar to the outcrossing observed in this study, if all factors affecting outcrossing are at its most favorable

Materials and Methods

Plant material:

The following cultivars were used in this study: Triticale ('US2007', 'US2009'), rye ('US3010', 'Duiker') and wheat ('US1010', 'SST88'). All of these cultivars derive from the Stellenbosch University Plant Breeding Laboratory (SU-PBL) with the exception of 'SST88' (Sensako (Pty) Ltd, Randburg, RSA).

The plants were grown in sand filled pots (3 liter volume, height of 19cm and radius of 7cm) in a single water-cooled greenhouse. The temperature in the greenhouse was maintained at minus 5°C ambient. The photoperiod was provided by natural light as no electronic light source was used. Plants were watered daily with a fertilizer which consisted of 164 g Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, Milnerton, RSA), 2 g Microplex (Ocean Agriculture (Pty) Ltd, Muldersdrift, RSA) en 77 ml potassium nitrate in 100 liters of water. Plants were also treated with the appropriate insecticides when needed.

Hybridization

Emasculation and pollination of the intra- and inter-specific crosses was done by hand. The cultivars were used in all possible cross combinations. There were also six control crosses carried out in which the female parent was crossed with pollen from the same genotype as the female (Table 3.1). A minimum of five crosses was made per cross combination.

Table 3.1: Crosses made between wheat, triticale and rye.

		Female					
		US2007	**US2009**	**US3010**	**Duiker**	**US1010**	**SST88**
Male	**US2007**	Control	*	*	*	*	*
	US2009	*	Control	*	*	*	*
	US3010	*	*	n/a	Control	*	*
	Duiker	*	*	Control	n/a	*	*
	US1010	*	*	*	*	Control	*
	SST88	*	*	*	*	*	Control

Plants were manually emasculated using tweezers and scissors when the plants were 8 weeks old or just prior to anthesis. Ears were then covered with a crossing-bag until they were ready to be pollinated. Pollination took place 2-4 days after emasculation. This allows the flowers to mature and become receptive. Pollination was carried out with pollen from fresh yellow anthers. After pollination the spikes were again covered with the crossing-bag.

Embryo rescue

After 16-21 days embryo rescue commenced on all seeds produced from the crosses. Using tweezers the seeds were carefully removed from the ears and sterilized in 70% EtOH for 30 seconds and 8 – 10 minutes in 30% jik (3.5% sodium hypochlorite). The embryos were dissected under a stereomicroscope (Zeiss Stemi 1000 (Pty) Ltd) at 20x magnification and placed in 100ml glass bottles containing modified MS- (Murashige and Skoog, 1962) medium. The glass bottles were sealed with a plastic lid in order to prevent contamination. The medium contained only 10% (w/v) of ammonium nitrate (NH_4NO_3). Bottles containing embryos were then placed in the dark at 4°C for a week and then in a growth cabinet at 23-25°C with a 14 hour light / 10 hour dark cycle.

The following data were recorded from the crossing and embryo rescue: the number of flowers pollinated, number of set seed, seeds with embryos and number of embryos that formed viable plants.

Determination of F_1 fertility

When the plants reached the two-leaf stage (approximately 5cm tall), they were planted in small pots (filled with peat) and covered with a translucent plastic bag. The plants were then placed in a growth cabinet and hardened off over a period of five to seven days. When the plants reached the four-leaf stage they were planted in larger pots and moved to a greenhouse. The plants were then left in the greenhouse to flower in order to determine the fertility of the F_1 generation. Spikes were allowed to mature for about 6 weeks and then dry off for approximately 2 weeks. The spikes were then cut and threshed by hand. The number of seeds for each cross combination was counted and weighed to determine the fertility of the F_1 generation.

Data analysis

The percentage outcrossing (OC) was determined for each cross. The following equation was used: $OC = \left[\frac{\frac{HS}{F}}{P}\right] \times 100$

In this equation HS = number of F_1 hybrid seed produced, F = number of flowers emasculated and pollinated, and P represents the percentage outcrossing of the control crosses. P is determined using the following equation: $P = \frac{S}{F}$; where S is the number of seeds produced (Hall *et al.,* 2007).

Chi-square analysis was also done to determine whether the outcrossing between intra- and inter-specific crosses differed significantly compared to the control crosses. To determine the F_1 fertility the number of the F_2 seeds were recorded.

Results

Crossability

Approximately 230 ears were emasculated and pollinated by hand. The study included two triticale cultivars ('US2009' and 'US2007'), two wheat cultivars ('US1010' and 'SST88') and two rye cultivars ('US3010' and 'Duiker'). Each intra-and inter-specific cross, as well as its reciprocal cross, was carried out under greenhouse conditions over a 16 month period. Control crosses were also performed which consisted of crosses between plants of the same genotype. In the case of rye, the control crosses consisted of crossing the one rye genotype with the other rye genotype as it is a cross-pollinating, self-incompatable, species. These control crosses were carried out as a method to evaluate the success of the emasculation and pollination.

The outcrossing percentage was then determined for each cross combination (Table 3.2, 3.4 and 3.6). A chi-square analysis was also performed (Table 3.3, 3.5 and 3.7). The outcrossing percentage between triticale and rye was low in both directions. The seed set for all crosses differed significantly from each other ($p < 0.05$). When triticale was used as the female parent the OC ranged between 2.7 – 10.6%, The highest OC (10.6%) was obtained when 'US2007' (female) was crossed with 'Duiker' and this was also the only cross where the amount of embryo formation was not significantly different from the control. Therefore, gene transfer between 'US2007' and 'Duiker' seems to be the most likely. The F_1 hybrid emergence is also expected to be higher for this cross. For both triticale genotypes the highest OC was obtained when 'Duiker' was used as the male. When rye was used as the female the OC was even lower, ranging between 4.4 – 8.7%. In this case crosses between rye and 'US2007' showed the highest OC. The highest OC was obtained between 'US3010' and 'US2007' (8.7%).

Table 3.2: Outcrossing percentage between triticale and rye

Female	Male	OC (%)
US2007	Duiker	10.6
US2007	US3010	5.4
US2009	Duiker	5.4
US2009	US3010	2.7
Duiker	US2007	6.7
Duiker	US2009	5.7
US3010	US2007	8.7
US3010	US2009	4.4

Table 3.3: P-values associated with seed set from crosses between triticale and rye

Female	Male	Seed Set	Embryo Formation
US2007	**US3010**	2.424×10^{-7}	0.0052
US2007	**Duiker**	1.927×10^{-7}	0.1136
US2009	**US3010**	2.876×10^{-11}	0.0011
US2009	**Duiker**	1.305×10^{-10}	0.0305
Duiker	**US2007**	1.828×10^{-9}	3.462×10^{-6}
Duiker	**US2009**	7.889×10^{-9}	0.0014
US3010	**US2007**	7.403×10^{-12}	5.604×10^{-8}
US3010	**US2009**	9.400×10^{-14}	1.712×10^{-8}

Outcrossing between triticale and wheat was higher when triticale was used as the male parent, however the number of seeds set for all crosses were significantly different ($p < 0.05$). It should however be noted, that even though all the p-values were less than 0.05, when triticale was used as the female, the p-values were much lower. When triticale was used as the female parent the OC was lower. The OC was between 5.9 – 24.2% when triticale was used as the female parent. When 'US2007' was used as the female parent, the OC was higher (14.9 – 24.2%) than when 'US2009' was used as the female parent (5.9 – 16.4%). Also when 'US2007' was used as the female parent the highest OC was obtained when it was crossed with 'SST88'. Even though the OC was lower when triticale was used as the female parent the amount of embryo formation was not significantly different to the control crosses ($p > 0.05$). Therefore even though the OC is lower it is likely that the F_1 hybrid emergence will be higher when triticale is used as the female parent. When wheat was used as the female parent the OC ranged between 48.7 – 66.4%. When 'US1010' was

used as the female the OC was higher than for 'SST88'. The OC between 'US1010' and triticale was between 53.8 –66.4% and when 'SST88' was the female the OC was between 48.7 – 54.5%. However for each wheat genotype the OC was higher when crossed with 'US2009' as the male parent.

Table 3.4: Outcrossing percentage between triticale and wheat

Female	Male	OC (%)
US2007	US1010	14.9
US2007	SST88	24.2
US2009	US1010	16.4
US2009	SST88	5.9
US1010	US2007	53.8
US1010	US2009	66.4
SST88	US2007	48.7
SST88	US2009	54.5

Table 3.5: P-values associated with seed set from crosses between triticale and wheat

Female	Male	Seed Set	Embryo Formation
US2007	**US1010**	3.204×10^{-5}	0.2202
US2007	**SST88**	2.228×10^{-5}	0.1113
US2009	**US1010**	2.560×10^{-5}	0.3122
US2009	**SST88**	2.043×10^{-9}	0.0654
SST88	**US2007**	0.0013	6.480×10^{-10}
SST88	**US2009**	0.0002	1.257×10^{-6}
US1010	**US2007**	0.0053	4.599×10^{-7}
US1010	**US2009**	0.0046	0.0001

Outcrossing between wheat and rye was also low in both directions with the exception of crosses where 'US1010' was used as the female parent. However the seed set for all crosses was significantly different ($p < 0.05$). When wheat was used as the female parent, the OC ranged between 22.1 – 39.2% when 'US1010' was used as female and when 'SST88' was used as the female the OC ranged between 1.95 – 6.5%. When looking at the amount of embryo formation, there were two crosses that did not differ significantly from the control crosses. 'US1010' x 'US3010' and 'SST88' x 'Duiker' had p-values higher than 0.05. In both cases where 'Duiker' was used as the male parent the higher OC was obtained. When rye was used as the female parent, the OC ranged between 0 – 4.2%. When 'Duiker' was used as

the female genotype the OC was higher (0.6 – 4.2%). Using 'SST88' as the male parent produced the highest OC.

Table 3.6: Outcrossing percentage between wheat and rye

Female	Male	OC (%)
US1010	Duiker	39.2
US1010	US3010	22.1
SST88	Duiker	6.5
SST88	US3010	1.95
Duiker	SST88	4.2
Duiker	US1010	0.6
US3010	SST88	1
US3010	US1010	0

Table 3.7: P-values associated with seed set from crosses between wheat and rye

Female	Male	Seed Set	Embryo Formation
SST88	**US3010**	1.487×10^{-16}	0.0011
SST88	**Duiker**	2.042×10^{-16}	0.0521
US1010	**US3010**	0.001	0.0753
US1010	**Duiker**	0.0001	0.0398
Duiker	**US1010**	1.683×10^{-16}	3.237×10^{-9}
Duiker	**SST88**	4.583×10^{-15}	0.0409
US3010	**SST88**	2.528×10^{-18}	2.125×10^{-24}
US3010	**US1010**	2.763×10^{-17}	1.183×10^{-22}

F_1 Hybrid Emergence

In all cross combinations where rye was used as the female parent the F_1 hybrid emergence was equal to zero with the exception of 'Duiker' x 'SST88'. The F_1 hybrid emergence for this cross combination was 61.1%. For all other crosses only root growth was observed, but no plants were produced (see figure 3.1).

Figure 3.1: When rye was used as a female parent only minimal root growth was observed.

In crosses where rye was used as the male parent the F_1 hybrid emergence was higher when wheat was used as the female, however the F_1 hybrid emergence was significantly different for all crosses ($p < 0.05$). When 'US1010' was used as the female parent the F_1 hybrid emergence was 12.4% and 48% when 'US3010' and 'Duiker' were used as the male parents. When 'SST88' was used as the female parent the F_1 hybrid emergence was 11.1% and 27.6% when crossed with 'US3010' and 'Duiker'. The high F_1 hybrid emergence was expected when 'SST88' (female) was crossed with 'Duiker' (male) as the amount of embryo formation was not significantly different from the control cross. The highest F_1 hybrid emergence (48%) was obtained from crosses between 'US1010' (female) and 'Duiker' (male). From the results of the chi-square analysis, the F_1 hybrid emergence was however expected to be lower as the amount of embryo formation was significantly different from the control cross.

Table 3.8: F_1 hybrid emergence from crosses between wheat and rye

Female	Male	F_1 Hybrid Emergence (%)
US1010	**US3010**	12.4
US1010	**Duiker**	48
SST88	**US3010**	11.1
SST88	**Duiker**	27.6
Duiker	**US1010**	0
Duiker	**SST88**	61.1
US3010	**US1010**	0
US3010	**SST88**	0

Table 3.9: P-values associated with F_1 hybrid emergence from crosses between wheat and rye

Female	Male	Growth / Embryo	Growth / Seed
SST88	**US3010**	1.004×10^{-6}	1.266×10^{-6}
SST88	**Duiker**	0.0001	5.665×10^{-5}
US1010	**US3010**	2.685×10^{-5}	3.390×10^{-6}
US1010	**Duiker**	0.017	0.0111
Duiker	**US1010**	4.313×10^{-13}	4.975 x 10-8
Duiker	**SST88**	0.0172	0.0089
US3010	**SST88**	3.216×10^{-23}	1.674×10^{-21}
US3010	**US1010**	1.510×10^{-21}	6.134×10^{-20}

The F_1 hybrid emergence for all crosses between triticale and rye was significantly different ($p < 0.05$). When triticale was used as the female parent and crossed with rye, the highest F_1 hybrid emergence was observed when 'US2007' was used as the female. When 'US2007' (female) was crossed with 'US3010' and 'Duiker' the F_1 hybrid emergence was 12.5 and 33.5% respectively. The higher F_1 hybrid emergence between 'US2007' (female) and 'Duiker' (male) was expected, as the amount of embryo formation was not significantly different from the control cross. The F_1 hybrid emergence was 0 and 8.3% when 'US2009' was crossed with 'US3010' and 'Duiker'. The low F_1 hybrid emergence for crosses where 'US2009' was used as the female was also expected from the results from the chi-square analysis, as the amount of embryo formation for these crosses were significantly different from the controls.

Table 3.10: F_1 hybrid emergence from crosses between triticale and rye

Female	Male	F_1 Hybrid Emergence
US2007	**US3010**	12.5
US2007	**Duiker**	33.5
US2009	**US3010**	0
US2009	**Duiker**	8.3
Duiker	**US2007**	0
Duiker	**US2009**	0
US3010	**US2007**	0
US3010	**US2009**	0

Table 3.11: P-values associated with F_1 hybrid emergence from crosses between triticale and rye

Female	Male	Growth / Embryo	Growth / Seed
US2007	**US3010**	0.002	0.0003
US2007	**Duiker**	0.0056	0.006
US2009	**US3010**	6.105×10^{-5}	0.0016
US2009	**Duiker**	0.0087	0.0023
Duiker	**US2007**	2.239×10^{-14}	7.145×10^{-9}
Duiker	**US2009**	2.239×10^{-14}	7.145×10^{-9}
US3010	**US2007**	1.510×10^{-21}	6.134×10^{-20}
US3010	**US2009**	1.510×10^{-21}	6.134×10^{-20}

When wheat was used as a female parent and crossed with triticale the F_1 hybrid emergence was low for all cross combinations. When 'US1010' was crossed with 'US2007' and 'US2009' the F_1 hybrid emergence was 3.9 and 0% respectively and when using 'SST88' as the female parent the F_1 hybrid emergence was 3.1 and 0.8%. For both wheat genotypes, when crossed with 'US2007' the F_1 hybrid emergence was the highest. The wheat genotype had little effect on the F_1 hybrid emergence.

When using triticale as the female and wheat as the male parent varied results were observed. When 'US2007' was crossed with 'SST88' and 'US1010' the F_1 hybrid emergence was 41.9 and 33.3% respectively. Therefore crossing 'US2007' with 'SST88' yielded the highest F_1 hybrid emergence. However, when crossing 'US2009' with 'US1010' the highest F_1 hybrid emergence (44%) was obtained. This was also the only cross that did not differ significantly from the control crosses ($p < 0.05$). When crossing 'US2009' with 'SST88' the F_1 hybrid emergence was 12.5%. It would however seem that using 'US2007' as the female yields the overall highest F_1 hybrid emergence. The F_1 hybrid emergence observed when triticale was used as the female parent was also expected from the results from the chi-square analysis in Table 3.5.

Table 3.12: F_1 hybrid emergence from crosses between triticale and wheat

Female	Male	F_1 Hybrid Emergence
US2007	**SST88**	41.9
US2007	**US1010**	33.3
US2009	**SST88**	12.5
US2009	**US1010**	44
US1010	**US2007**	3.9
US1010	**US2009**	0
SST88	**US2007**	3.1
SST88	**US2009**	0.8

Table 3.13: P-values associated with F_1 hybrid emergence from crosses between triticale and wheat

Female	Male	Growth / Embryo	Growth / Seed
US2007	**US1010**	0.0145	0.0179
US2007	**SST88**	0.0097	0.0085
US2009	**US1010**	0.0909	0.193
US2009	**SST88**	0.0003	0.0031
SST88	**US2007**	2.087×10^{-6}	6.236×10^{-6}
SST88	**US2009**	4.054×10^{-15}	2.257×10^{-17}
US1010	**US2007**	0.0119	2.157×10^{-9}
US1010	**US2009**	/	1.840×10^{-16}

Discussion

The aim of this study was to determine the ability of gene transfer between wheat, triticale and rye. Therefore it was necessary to quantify the possibility as well as the frequency of the possible gene flow. This was observed by measuring the intra- and inter-specific outcrossing frequency between the three species. To perform the crosses ears were emasculated, as to prevent self-fertilization, and then manually pollinated. However, the formation of hybrid F_1 seed alone cannot be associated with potential gene flow. Only if the F_1 generation is fertile successful gene flow can take place. This is of particular importance when outcrossing with transgenic cultivars is to be considered. If the F_1 generation is sterile, there will be no seed formation and pollen mediated gene flow (PMGF) is prevented.

Significant outcrossing was observed between different genotypes from the same species, indicating that there is a large possibility for PMGF. The outcrossing frequency for all these

cross combinations was between 90 – 100%. The F_1 hybrid emergence of these crosses were all more than 80% and all the plants were fertile. This further emphasizes the possibility for PMGF.

When crossing triticale and rye the outcrossing was low in both directions. When rye was used as the female the highest OC was 8.7%, and when rye was used as the male parent the highest OC obtained was 10.6%. When considering the triticale genotypes, the crosses with 'US2007' as the male or female parent, yielded the highest OC. The rye that yielded the highest OC was 'Duiker' when it was used as the male parent. Therefore, because the outcrossing is so low, it is possible that the *Kr1* and *Kr2* loci present in wheat are also effective in triticale, causing low crossability (Guedes-Pinto *et al*., 2001).

When using rye as the female parent, PMGF were however prevented as the F_1 hybrid emergence was equal to zero. When rye was used as the male parent, the F_1 hybrid emergence was between 0 – 33.5%. The cross between 'US2007' and 'Duiker' yielded the highest F_1 hybrid emergence. Therefore, although the OC is low, this moderate F_1 hybrid emergence indicates that there is a possibility of successful hybrid production. However, gene flow is unlikely as the F_1 hybrids are infertile. These results are supported by the study done by Hall and colleagues in 2007. They also found crossability to be low for crosses made in both direction and that the outcrossing was cultivar specific (Hall *et al*., 2007). The risk of potential gene flow from a transgenic crop is therefore low.

The crossability between triticale and wheat was higher when triticale was used as the male parent. The OC ranged between 48.7 – 66.4%, however these crosses did not produce many viable hybrid plants as the F_1 hybrid emergence was low (<4%) when triticale was used as the male parent. The F_1 hybrid was also sterile producing no seed. These results are supported by previous studies that determined that seed set was good, but the F_1 hybrid emergence was very low when triticale was used as the male parent (Hall *et al*., 2007; Khanna, 1990). Therefore, geneflow is unlikely when triticale is the male parent, because the embryo will degenerate in the environment after only a few days following pollination.

When wheat was used as the male parent the OC was 5.9 – 24.2% which is significantly lower than when wheat was used as the female. However, the F_1 hybrid emergence was much higher and ranged between 12.5 – 44%. The high F_1 hybrid emergence was also validated by the chi-square analysis. Therefore, the possibility for gene flow between triticale and wheat is low, but viable seed can be produced when triticale is used as the female parent. These results were also supported by a previous study by Khanna. Khanna and colleagues found that seed set was low, but germination was good when triticale was used as the female parent (Khanna, 1990). Therefore geneflow is possible as the F_1 seeds are able to persist in the environment.

When considering crosses between wheat and triticale, 'US2007' was the triticale that yielded the highest OC when used as the female parent. The wheat line 'US1010' was responsible for the highest OC when used as the female parent.

When looking at crosses between wheat and rye the highest OC was obtained when wheat was used as the female parent. When 'US1010' was used as the female parent the OC was between 22.1% and 39.1%. However when 'SST88' was used as the female parent the OC was less than 7%. When both these lines were crossed with 'Duiker' as the male parent the highest OC was obtained. When rye was used as the female parent outcrossing was less than 5% and the F_1 hybrid emergence was zero for all crosses but one. When 'Duiker' was crossed with 'SST88' three viable F_1 hybrid plants was produced. Therefore there is little possibility for gene flow when rye was used as the female parent. However when wheat was used as the female parent the F_1 hybrid emergence was 12.4 and 48% respectively. Therefore outcrossing is possible only when wheat is used as the female parent as only then viable seed is produced. These plants also produced F_2 seed.

The low crossability between wheat and rye is due to the presence of the *Kr1* and *Kr2* genes present on the wheat genome (Guedes-Pinto *et al*., 2001). Also in this case the low F_1 hybrid emergence could be due to the *Eml* allele causing embryo lethality (Tikhenko *et al*., 2005). Therefore, PMGF seems unlikely when rye is the female as the outcrossing is very low and the F_1 hybrid emergence for almost all crosses are equal to zero. The results also show that

the crossability between wheat and rye is highly cultivar specific. This is validated by the study done by Guedes-Pinto in 2001.

The crossability between these two species is important in the production of primary triticales. The results indicate that successful gene flow can take place as a degree of crossability exists and also because F_2 seeds are produced when rye is used as the male parent.

When considering the results from this study it should be kept in mind that gene flow can also take place between a transgenic and a non-transgenic crop. This is not desirable and correct measures should be followed to avoid gene flow. Therefore species that exhibit a certain degree of crossability should be isolated from each other. This can be done using physical or temporal isolation (Messeguer, 2003).

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Chapter 4

Assessment of pollen-mediated gene flow from blue aleurone wheat

Assessment of pollen-mediated gene flow from blue aleurone wheat

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Abstract

The greatest concern associated with GM crops is the possibility of contaminating other non-GM crops as a result of pollen mediated gene flow (PMGF). Pollen drift is the primary mechanism in which GM crops contaminate traditional crops. Wind is therefore necessary to carry the pollen (containing transgene) from the GM crop to organic or conventional crops. In this study pollen mediated gene flow from blue aleurone wheat was evaluated. The potential frequency of cross pollination as well as the distance at which gene flow takes place was determined. During 2010 a 130 x 130 meter field trail was conducted. The trial consisted of a central pollinator block of blue aleurone wheat that was surrounded by blocks of 'SST047' up to a distance of 65 meters in all directions (N, S, W, E, NW, NE, SW, and SE). When the plants reached maturity the blocks were harvested after which the material was processed. A representative sample of each block was planted in a single greenhouse. The F_2 generation was harvested and each sample was evaluated visually for the presence of blue seeds. This data was used to calculate the OC for each sample. In this study an average OC of 0.4% was observed. A maximum OC of 2.4% was observed at a distance of 2.5 meters. High OC values were also observed at distances less than 15 meters from the pollinator. A few high OC values that were observed at distances higher than 35 meters, most of these however were in the direction of the prevailing winds. There is however evidence that indicates that the prevailing wind direction might not necessarily be associated with higher OC rates. Therefore, to reduce gene flow as much as possible a combination of isolating distance, temporal isolation and even physical barriers should be used.

Keywords: *Ba*, pollen, outcrossing

Introduction

The use of GM crops has increased substantially over the last few years. In the United States 70% of all corn, 80% of all cotton and 90% of all soybeans planted commercially are genetically modified. Approximately 60% of all food in supermarkets in the US contains some GM crop ingredient (Cox, 2008). The reason for this is the fact that GM crops have many benefits as well as positive effects on the environment (Gatford *et al.*, 2006). South Africa was the first country in Africa where genetically engineered crops were grown (Cloete *et al.*, 2006). Most GM crops produced in South Africa have been altered to be insect- and herbicide-resistant (Aerni, 2005). This allows for a decrease in the amount of chemicals applied to crops, benefiting both the environment and human health (Cox, 2008). GM crops have also been developed to include nutritional benefits such as higher concentrations of starch and vitamins (Gatford *et al.*, 2006).

There are, however risks associated with genetically engineered crops that needs to be considered. Possibly one of the greatest concerns of genetically engineered crops is the possibility of it contaminating other non-GM crops as a result of cross-pollination (Belcher *et al*., 2005). Some consumers are also concerned about the possibility of certain allergies that can be associated with GM crops (Goodman *et al.,* 2008). Pollen drift is the primary mechanism in which GM crops contaminate traditional crops. Wind or insects are therefore necessary to carry the pollen (containing transgene) from the GM crop to organic or conventional crops. This threatens the genetic purity of these crops. In recent years the popularity of organic foods has increased substantially (about 20% per year) (Cox, 2008). Organic foods are highly regulated, but GM crops make it more difficult for organic farmers to produce products that are free from contamination. Producers can lose their certification if genetically engineered proteins are found in their products (Kuparinen *et al.*, 2007).

The aim of this study was to evaluate the pollen mediated gene flow (PMGF) from wheat with blue aleurone. The blue pigmentation of the aleurone layer in wheat is controlled by a single dominant gene, *Ba* (blue aleurone), located on chromosome 4A of the wheat genome (Zheng *el al.,* 2006). Tschermak stated in 1938, that the trait was introgressed from *Thinopyrum ponticum* to wheat by the addition or substitution of chromosome 4Ag (Zheng

et al., 2006). The blue aleurone trait naturally only occurs naturally in one wheat species, the wild diploid, *Triticum boeoticum* (Morrison *et al.,* 2004).

In this study the potential frequency of cross pollination as well as the distance at which gene flow can take place was determined. Wheat carrying the blue aleurone trait acts as a marker, indicating gene transfer. The blue grained trait is a dominant single gene marker and has been successfully used in small scale studies determining gene flow (Hanson *et al.,* 2005). Successful cross-pollination is identifiable by the expression of a light blue pigment in the F_2 seed (Gustafon *et al.*, 2005).

Wheat pollen and hybrid formation

Another aspect taken into consideration during this study is the possibility of hybrid formation. This depends on factors such as floret opening, stigma receptivity, number and extent of anther extrusion, amount of pollen released, and pollen viability (Hanson *et al.*, 2005). These factors are controlled by the environment as well as various genes.

Since wheat is a self-pollinating crop pollen mediated gene flow is expected to be low. This is because the stigma receptivity and pollen shed is in the same phase within the floret (Gustafon *et al.*, 2005). The duration of flower opening as well as anther extrusion is of great importance for gene flow (Hedge and Waines, 2004). This is controlled by genetic as well as environmental factors. Therefore, cultivars that exhibit a higher degree of floret opening and anther extrusion have higher outcrossing rates (Hucl, 1996). Anther size also plays a critical role in gene flow (Hedge and Waines, 2004).

Even though wheat only produces about one tenth of the pollen produced by rye, which is an outcrossing relative, possibility for outcrossing does exist (Hedge and Waines, 2004). This is because only 30% of the pollen is shed inside the flower leaving the rest available to outcross (Hedge and Waines, 2004). However, even though wheat pollen can be shed outside the flower the pollen travels only short distances (6m) as it is relatively heavy. Therefore wind is required to move the pollen significant distances from the source (Hanson *et al.*, 2005). Gene flow mostly occurs 0.5 to 1.5 meters from the pollinator (Hedge and

Waines, 2004). The average frequency of gene flow has been found to be 1%, but this value can increase to about 6.7% at distances less than 1m (Loureiro *et al.*, 2007).

Studies have been done to determine the survival of pollen when released into the atmosphere. High temperatures as well as high relative humidity reduce PMGF. High temperatures cause the loss of pollen viability (due to a loss of moisture) (Luna *et al*, 2001) and high humidity makes pollen heavier. High temperatures also reduce receptivity of the stigma and the duration pollen is shed from the wheat flower (Gustafon *et al*, 2005).

Factors affecting pollen distribution

There are multiple environmental factors that influence gene flow by affecting floret opening, stigma receptivity, number and extent of anther extrusion, amount of pollen released, and pollen viability. These environmental factors include: rainfall, temperature, relative humidity, light intensity and various stress factors (Hanson *et al.*, 2005).

Atmospheric conditions

It has been found that pollen release takes place on warm days (between midday and late afternoon) with low relative humidity (Curtis and Lersten, 1995). These conditions cause the atmosphere to be moderately to highly unstable (Jackson and Lyford, 1999). Thus gustiness and turbulent conditions are required for long distance dispersal of pollen. Pollen grains resist movement by means of gravitational force. This resistance is influenced by particle size, particle density, surface roughness and degree of sphericity (Niklas, 1985). Pollen grains in newly opened anthers are also subject to resistance of particle motion caused by an increase in electrostatic force and surface adhesion (Jackson and Lyford, 1999).

Deposition parameters

The amount of pollen that is released into the air depends on the deposition velocity. Deposition velocity is the deposition rate divided by the particle concentration in the air (Jackson and Lyford, 1999). Pollen can only be released into the air if the velocity of

deposition is similar or higher than the velocity of sedimentation (Jackson and Lyford, 1999). Sedimentation velocity is defined as the rate at which pollen descends to the ground as a result of gravity (in still air). If the only force responsible for the deposition of particles is sedimentation, the pollen dispersal will only be influenced by gravity and horizontal wind speed (Jackson and Lyford, 1999).

Minimizing pollen distribution

Studies have been done to find ways of reducing gene flow, but there is no way to completely eliminate it. In a Canadian field trail conducted by Hucl and Matus-Cádis during 2001 they found that gene flow can take place at distances of up to 27 meters. This was however found to be cultivar specific. From the results of their study they suggested that a minimum isolation area of 30 meters should be used to avoid outcrossing. The Canadian Seed Growers' Association however only recommends an isolation area of 10 meters between crops of the same species (Hucl and Matus-Cádiz, 2001).

Hanson and colleagues conducted another independent study in 2005. They assessed gene flow by conducting field trails in five localities across three US states. The results from this study indicate that both temperature and relative humidity play a key role in outcrossing as this affects the pollen viability. Hucl and Matus-Cádis (2001) recommended that 30m isolation distance should be used to avoid outcrossing, however Hanson recommends that 45m isolation distance should be used (Hanson *et al.*, 2005).

Another factor affecting gene flow is planting density. In a study conducted during 2005 and 2006, researchers attempted to determine the most practical plant density that results in the lowest possible gene flow (Willenborg *et al.,* 2009). Results from the study proved that planting density has a large effect on gene flow. At very low planting densities the maximum gene flow was predicted to be 0.31% and decreased exponentially at higher planting densities. At the highest planting density the gene flow was as low as 0.0003%. The gene flow also declined with increasing planting densities regardless of the genotype. In Canada the recommended planting densities are between 250 and 300 plants m^{-2} (Manitoba Agriculture, Food, and Rural Initiatives, 2008). The study also indicated that there was no

further reduction of gene flow above planting densities of 300 plants m^{-2}. Therefore it is recommended that planting densities higher than 300 plants m^{-2} should not be used as it would be impractical (Willenborg *et al.*, 2009).

Physical isolation of crops is not a feasible or effective manner to reduce gene flow. Temporal isolation can also be used efficiently to minimize outcrossing. Willenborg *et al* (2010) conducted another study during 2005 and 2006. Their main objective was to determine whether emergence timing could temporally isolate different crops and to identify the hybridization window for spring wheat crops. The highest pollen mediated gene flow was found to be only 0.396%, and was observed when the pollinator emerged just after the recipient plants. This frequency declined dramatically when the flowering became less synchronous. When pollinator and recipient plant flowering coincide the PMGF frequencies undergo a two- to four-fold increase. From the data they determined that a hybridization window of 125 growing degree-days exist as a function of pollinator emergence. The study provided evidence that gene flow can be reduced by temporal isolation. It is however recommended that temporal isolation should be used in conjunction with physical isolation (Willenborg *et al.*, 2010).

Materials and Methods

During 2010 a 130 x 130 meter field trial was sown in a field at Mariendahl Experimental Station (MES), nearby Elsenburg Agricultural Training College. The trial consisted of a central pollinator block of blue aleurone wheat ('US1010/4*Cltr1202STR(BA)·09US212') which was surrounded by blocks of 'SST047' up to a distance of 65 meters in all directions (N, S, W, E, NW, NE, SW, and SE). The pollinator had a radius of 2.5 meters, the recipient blocks were each 1 x 1 meter, and planted at a density of 300 plants m^{-2}. The first six recipient blocks of each row were planted 2.5 meters apart and the last ten blocks were planted 5 meters apart. The last block was up to 65 meters from the pollinator block. The pollinator as well the recipient blocks were planted at three different dates, each a week apart (3 June, 10 June and 20 June 2010). This was done to maximize the overlap in flowering between the pollinator and the recipient. The areas between the rays were planted with two triticale cultivars, US2007 and US2009. These cultivars were planted, at a density of 300 plants m^{-2},

three weeks before this trial was sown to avoid gene flow between the triticale and wheat. The trial was treated with appropriate herbicides, insecticides, fungicides and fertilizer when needed. Refer to Table 4.5 for the application of chemicals.

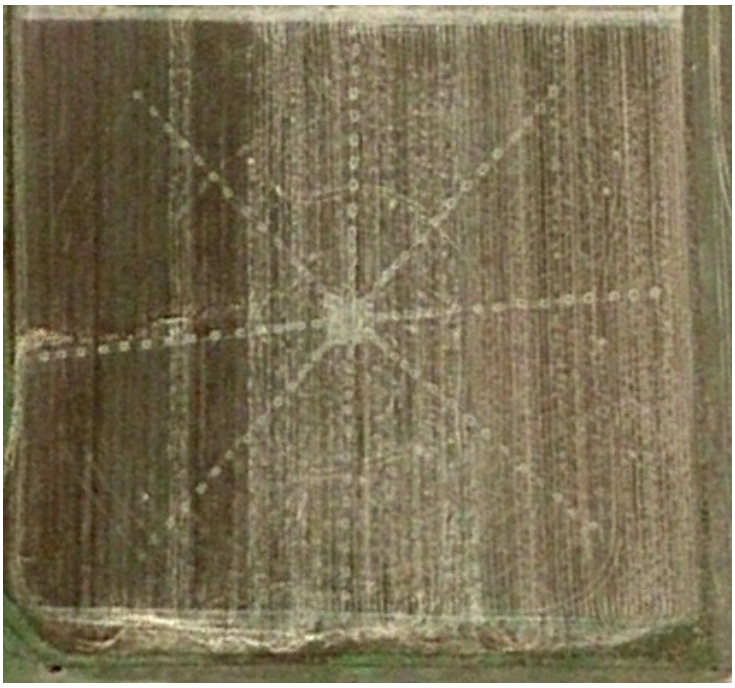

Figure 4.1: Aerial photo of field trail captured by Google Earth on November 24th 2010. (http://maps.google.co.za/maps?ie=UTF-8&hl=en&tab=wl)

The aerial photo was taken 24 November 2010 and acquired using Google Earth.

About 100 days after planting, the heading dates of the blocks as well as the pollinator were recorded. This is used to indicate whether there was good overlap in flowering between the pollinator and the recipient blocks.

When the plants reached maturity the entire 1 x 1m block was harvested (19 Nov 2010) and bagged by hand. Each block was given a number and each sample harvested was labeled with this number. In total 126 blocks were harvested in this manner. The ears were threshed with a stationary threshing machine (Wintersteiger (Pty) Ltd, LD 180) and stored until the weather conditions were suitable for the material to be planted. There were concerns that the extreme summer temperatures would cause sterility and therefore planting of the material was delayed to March 2011. A representative sample of each block

was planted in sand filled pots in a single greenhouse. Cross-pollination was prevented by covering one ear per plant with a crossing-bag before pollen shed. When the ears reached maturity the seeds were harvested, and samples in which cross-pollination occurred were identified based on the expression of the blue pigment. Each sample was evaluated visually in order to verify the 3:1 ratio expected for this single gene, dominant trait in the F_2 generation. The amount of blue seeds, indicating outcrossing, was counted and kept separate.

To determine the distance and rate of PMGF in all directions the outcrossing was expressed as a percentage blue seed in a sample. The following equation was used to calculate the percentage outcrossing:

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

(Hanson *et al.,* 2005).

Chi-square analysis was also done to determine whether the outcrossing between the eight directions differed significantly from each other. The same was done between the different distances from the pollinator.

The ARC supplied all wind data as well as the necessary meteorological data. Data was collected from a weather station located at Elsenburg (nearby MES).

Results

The average temperature and relative humidity during the pollination period ranged between 10.9 – 17.7 °C and 54 – 89% respectively. Rainfall was extremely low with an average of only 0.95mm per day and a total rainfall of 28mm for the entire pollination period. Wind during the pollination period was mainly from the South-East. There was also significant wind from the North, South, East and North-West. Of the 3150 samples analyzed 67 contained blue seed. The outcrossing ranged between 0.1 and 2.4% (table 4.1) with the highest outcossing percentage of 2.4% observed in a sample closest to the pollinator. Outcrossing occurred primarily in Southern, South-Western, Western and North-Western rays and the maximum distance at which outcrossing took place was 65m. Figure 2 indicates the percentage of outcrossing at each distance from the pollinator.

Table 4.1: Outcrossing percentage at each block

Distance	N	NE	E	SE	S	SW	W	NW
	% OC / Block							
2.5	0.1	0	0.1	0.3	0	2.4	0	0
5	0	0.1	0	0	0	0	0	0.6
7.5	0	0.9	0	0	0.4	1.3	0.1	0
10	0	0	0	0	0	0	0	0.1
12.5	0	0	0	0	0	0.2	0	0.7
15	0.1	0	0.1	0	0.1	0	0.4	0
20	0.1	0.2	0	0	0	0.2	0	0
25	0	0	0	0	0	0	0	0
30	0.1	0	0	0	0.2	0.2	0	0.1
35	0	0.1	0	0	0.1	0.1	1.3	0.3
40	0	0	0	0	0	0	0	0.4
45	0	0	0.8	0	0.1	0.5	0.4	0.1
50	0.2	0	0	0	0	1	0	0
55	0	0	0	0	0	0	0.5	0
60	0	0	0	1.1	0.7	0	0.2	0.2
65	0	0	0	0.1	0	0	0.1	0.4

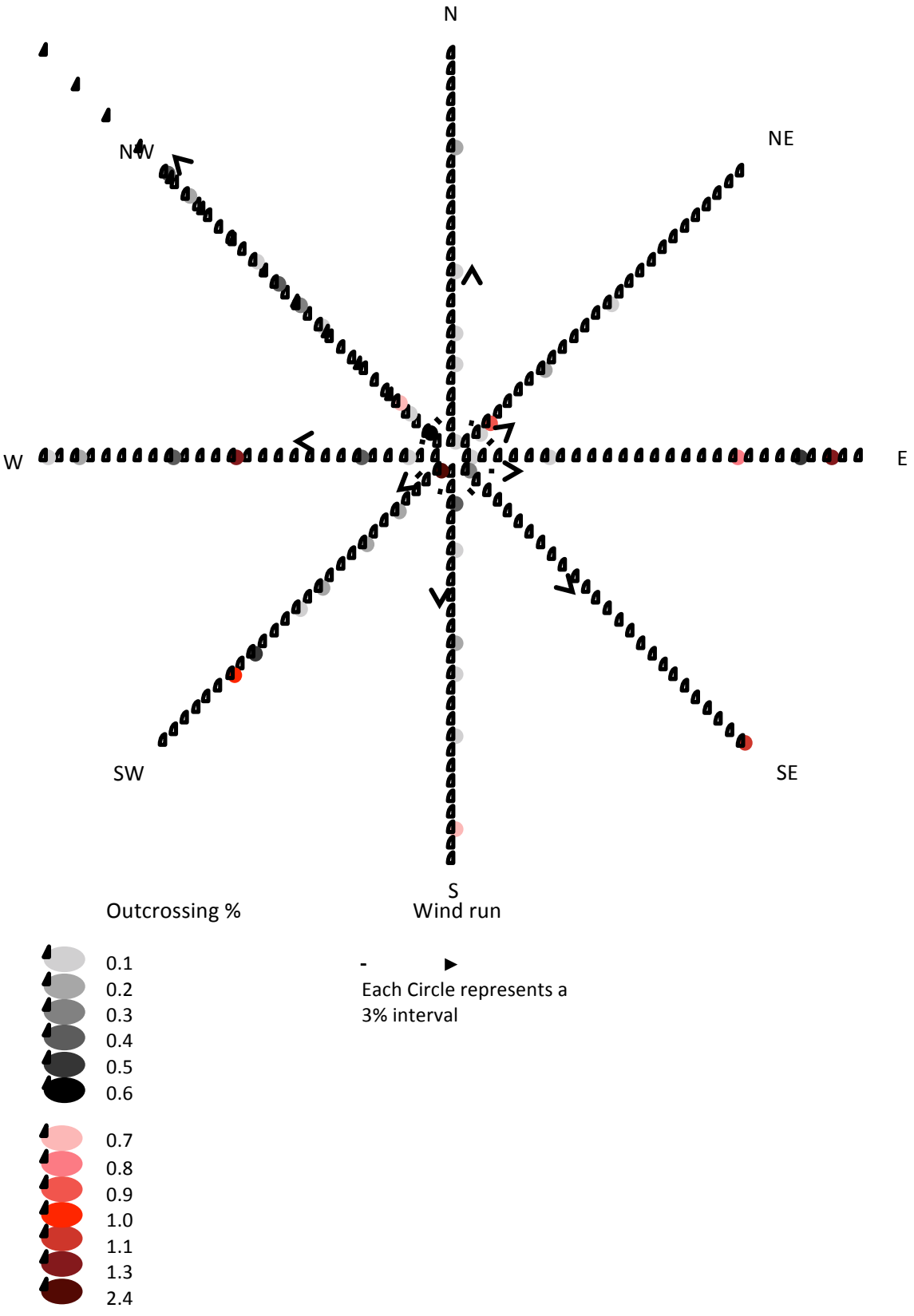

Figure 4.2: Outcrossing rate among winter wheat in 2010 gene flow experiment. Lines indicating percentage wind coming from each direction.

The chi-square analysis between the eight directions indicated that some of the directions do differ from one another. The following are statistically different: East from South-West, North from South-West, North from West and North from North-West (see table 4.2).

Table 4.2: Chi^2 analysis between wind directions

		Direction							
		N	NE	E	SE	S	SW	W	NW
Direction	N	*	*	*	*	*	*	*	*
	NE	0.230	*	*	*	*	*	*	*
	E	0.318	0.403	*	*	*	*	*	*
	SE	0.219	0.445	0.359	*	*	*	*	*
	S	0.114	0.401	0.297	0.471	*	*	*	*
	SW	0.028	0.056	0.044	0.069	0.066	*	*	*
	W	0.047	0.154	0.108	0.201	0.190	0.170	*	*
	NW	0.011	0.113	0.065	0.171	0.145	0.148	0.476	*

A similar analysis was done between the distances. From the 120 different combinations, there were only ten that were significantly different. The ten combinations are indicated in table 4.3 (for all combinations see table 4.4).

Table 4.3: P-value for distances that are significantly different

Distance		P-Value
7.5	10	0.04
7.5	20	0.04
10	30	0.04
10	45	0.03
10	60	0.05
15	25	0.04
20	25	0.04
25	30	0.02
25	45	0.02
25	60	0.04

Table 4.4: Chi^2 analysis between the different distances from the pollinator

Distance (m)	2.5	5	7.5	10	12.5	15	20	25	30	35	40	45	50	55	60	65
2.5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
5	0.19	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
7.5	0.47	0.11	*	*	*	*	*	*	*	*	*	*	*	*	*	*
10	0.13	0.17	0.04	*	*	*	*	*	*	*	*	*	*	*	*	*
12.5	0.21	0.42	0.14	0.14	*	*	*	*	*	*	*	*	*	*	*	*
15	0.19	0.50	0.10	0.08	0.40	*	*	*	*	*	*	*	*	*	*	*
20	0.16	0.38	0.07	0.09	0.30	0.34	*	*	*	*	*	*	*	*	*	*
25	0.12	0.13	0.04	0.17	0.11	0.04	0.04	*	*	*	*	*	*	*	*	*
30	0.17	0.44	0.08	0.04	0.35	0.42	0.39	0.02	*	*	*	*	*	*	*	*
35	0.36	0.20	0.34	0.09	0.25	0.19	0.14	0.07	0.16	*	*	*	*	*	*	*
40	0.16	0.34	0.07	0.24	0.27	0.30	0.42	0.17	0.34	0.14	*	*	*	*	*	*
45	0.35	0.13	0.32	0.03	0.19	0.11	0.07	0.02	0.08	0.50	0.06	*	*	*	*	*
50	0.26	0.34	0.20	0.14	0.40	0.32	0.25	0.12	0.28	0.33	0.23	0.30	*	*	*	*
55	0.17	0.40	0.08	0.22	0.32	0.38	0.50	0.17	0.43	0.16	0.44	0.09	0.27	*	*	*
60	0.40	0.13	0.39	0.05	0.18	0.12	0.09	0.04	0.10	0.43	0.08	0.42	0.26	0.10	*	*
65	0.18	0.45	0.09	0.12	0.36	0.43	0.42	0.07	0.50	0.17	0.36	0.09	0.29	0.44	0.11	*

Discussion

The aim of this study was to evaluate the pollen mediated gene transfer from blue aleurone wheat. It was therefore necessary to quantify the potential frequency of outcrossing as well as the maximum distance at which gene flow can take place. This was done by conducting a 130 x 130 meter field trail which consisted of a central pollinator block of blue aleurone wheat surrounded by a suitable winter wheat up to a distance of 65 meters in eight of the mayor wind directions.

According to previous studies the outcrossing percentage is expected to be less than 1%. There is however evidence that the OC can increase to almost 7%. In this study an average OC of 0.4% was observed. Even though the average is low there were a few samples that had an OC of more than 1%. A maximum OC of 2.4% was observed at a distance of 2.5 meters from the pollinator. This was one of the samples closest to the pollinator and therefore a higher OC was expected. Most of the higher OC values were observed at distances less than 15 meters from the pollinator, but there were a few high OC values that were observed at distances further than 35 meters from the pollinator. Most of these were however in the directions of the prevailing winds with the exception of two samples. There was no significant wind in the South-Western direction, but an OC of 1% was observed at 50 meters from the pollinator. The same was observed in the Eastern direction. An OC of 1.3% was observed 60 meters from the pollinator even though there was no significant wind.

According to Porter and Gawith (1999) the temperature was in range for anthesis and seed set to occur. Temperature also affects pollen viability. The temperature during this time was relatively low and would therefore not have significantly reduced pollen viability provided that pollination took place no longer than an hour after pollen release (Loureiro *et al.*, 2007).

From this study it appears that an isolating distance of more than 65 meters is required to adequately prevent gene flow. When considering the amount of outcrossing that took place in the South-Western direction it appears that prevailing wind direction is not always associated with higher OC distribution. The same results were observed by Hucl and Matus-

Cádis. The results of their study indicated that during the first year prevailing wind direction was associated with elevated OC rates. The following year, however there was no association between prevailing wind direction and elevated OC rates (Hucl and Matus-Cádiz, 2001).

Hanson recommended from the results of his study that an isolating distance of 45 meters should be used. Results from this study indicated that this might not be sufficient to prevent gene flow. Isolating distances of 65 meters or greater should therefore be considered. Results also indicated that prevailing wind direction is not necessarily associated with higher OC rates. Therefore, to reduce gene flow as much as possible a combination of isolating distance, temporal isolation and even physical barriers should be used. This may however not completely prevent gene flow, but will significantly lower the potential for cross contamination (Willenborg *et al.*, 2010).

Addendum

Table 4.5: Chemicals applied to field trial

Name	Concentration
Herbicides	
Hussar	200 g/ha
MCPA	500 ml/ha
Buctril	375 ml/ha
Ballista	500 ml/ha
Insecticides	
Mospilan	50 g/ha
Fungicides	
Duett	0.9 - 1 L/ha
Fertiliser	
With planting -	
4.1.1 (31)S 40N 10P 10K	193.2 kg/ha
First top application -	
TURBO 30 60N 6P 18K	280 kg/ha
Second top application -	
TURBO 30 60N 6P 18K	225 kg/ha

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Chapter 5

General discussion and conclusion

General discussion and conclusion

By performing a range of inter- and intra-specific crosses and embryo rescue of the resulting F_1 seeds, the crossability between three small grains represented by six contemporary cultivars were evaluated as a means of determining the possibility of gene flow from transgenic crops to conventional crops. The experimental procedure was designed to create the worst-case scenario for gene flow to take place. Therefore, if all factors affecting outcrossing are at its most favorable, gene flow in the field should still be less or similar to the outcrossing observed in this study. The results from this study compared well to that of previous studies. Differences can be attributed to the fact that crossability is highly cultivar specific. As expected, gene flow is highest between genotypes from the same species, with the OC and F_1 hybrid emergence all above 80%. The risk of gene flow from transgenic crops is therefore a concern that needs to be addressed.

The risk of gene flow between species is however considerably lower. The potential for gene flow between triticale and rye is highly unlikely as the F_1 hybrids are sterile when rye is used as the female parent. Therefore, as no F_2 seeds are produced further transfer of the transgene is prevented. When triticale is used as the female parent gene flow is even less likely as the F_1 hybrid emergence is zero.

When crossing triticale with wheat a large amount of F_1 seed was produced when triticale was used as the male parent. Gene flow is however restricted as the embryo starts to degenerate soon after fertilization. Gene flow is however possible when wheat was used as the male parent. Even though the outcrossing is low the maximum F_1 hybrid emergence is 44%. The F_1 generation also produced viable seed. Therefore, when planting transgenic wheat in close proximity to triticale, the necessary precautions should be taken to prevent gene flow.

When crossing wheat with rye, gene flow is more likely when using wheat as the female. Crossability was however considerably lower when rye was used as the female parent. The OC was less than 5% and the F_1 hybrid emergence was zero. Gene flow is therefore

completely restricted. Wheat as the female parent had an OC of up to 39% and a F_1 hybrid emergence of almost 50%. The F_1 generation is also capable of producing seed.

The primary mechanism of gene flow between species in the field is through the distribution of haploid pollen followed by hybridization. This is referred to as pollen mediated gene flow (Willenborg *et al*, 2009). In order to minimize gene flow it is necessary to determine the outcrossing frequency as a result of pollen mediated gene flow. It is also of great importance to know how far pollen can travel and still hybridize to produce viable seed. Therefore, a field trail was also conducted in conjunction with the crossability study.

In this study an average outcrossing percentage of 0.4% was observed. The maximum OC observed was 2.4%. This is however expected as the sample was located closest to the pollinator (2.5m). Most of the higher OC values were observed in the direction of the prevailing winds. There were however two samples with outcrossing percentages of 1% and 1.3% at distances of 50 meters and 60 meters respectively not associated with the prevailing winds. This may indicate that knowledge of the prevailing winds are not a completely reliable source of information to assist in managing PMGF restriction. The same was observed in a study conducted during 2001 (Hucl and Matus-Cádiz, 2001). Therefore, an isolating distance of at least 65 meters should be used to minimize gene flow.

Using the results from the two studies a few recommendations can be made in regards to minimizing gene flow. Previous studies have recommended that isolating distances of 45 meters should be used to prevent gene flow (Hanson *et al*, 2005). However, in this study outcrossing was observed at a distance of 65 meters. Therefore isolating distances of at least 65 should be used to prevent outcrossing between different wheat cultivars. Appropriate isolating distances should be used when transgenic wheat is planted in close proximity to triticale. As wheat is the pollen donor (as opposed to triticale) an isolating distance of at least 65 meters should be used in order to minimize gene flow. There is also a large possibility of gene flow from rye to wheat. Therefore, transgenic rye should also be physically isolated from wheat. However, as rye produces up to 90% more pollen than wheat (Hanson *et al*, 2005), it might be necessary to use an isolating distance greater than 65 meters.

In this study pollen mediated gene flow from wheat was evaluated. However, as there is a large possibility of rye crossing with wheat, it is necessary to evaluate rye pollen distribution. Apart from the fact that rye produces more pollen than wheat, its window of pollen release is also longer as rye is a cross-pollinating species. The pollen mediated gene flow from triticale should also be assessed, even though the outcrossing possibility with the other two grains is low. All risks associated with the release of transgenic triticale should be evaluated.

Another important aspect that should also be taken into consideration is the differences between locations brought about by differences in environmental conditions. The environment affects floret opening, stigma receptivity, the amount of pollen released and the viability of pollen. All of these factors influence PMGF. Environmental conditions that affect PMGF include: rainfall, temperature, relative humidity, light intensity as well as various stress conditions (Hanson *et al.,* 2005). The effect of environmental conditions on potential PMGF has been revised by Waines and Hegde (2003). Pollen mediated gene flow should therefore be studied over a range of different locations in order to determine the effect of the environmental conditions on gene flow.

Differences between specific cultivars should also be evaluated before a release is considered. In previous studies, pollen mediated gene flow was determined between a variety of cultivars. They found that that there can be considerable differences between cultivars and that the high/low OC stays consistent over different growing seasons (Hucl and Matus-Cádiz, 2001). This should therefore be done with cultivars that are commonly grown in South Africa.

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