The impact of pollen movement on Identity Preservation of maize (*Zea mays*)

By

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CONTENTS

ABBREVIATIONS AND ACRONYMS

AOSCA	Association of Seed Certification Agencies
ARC	Agricultural research council
Bt	*Bacillus thuriengensis*
CTAB	Cetryltrimethylammonium bromide
DNA	Deoxyribonucleic acid
E	East
EDTA	Ethylene diamine tetra acetic acid
ENE	East-north-east
ESE	East-south-east
g/l	grams per litre
GM	Genetically modified
GMO	Genetically modified organism
Ha	Hectares
Hg	Hectograms
HCl	Hydrochloric acid
i.e.	id est
IP	Identity preservation
k	Thousand
Km	Kilometre
L	Litre
M	Molar
m	metre

m^2	metre square
mg	milligram
ml	Millilitre
min	Minute
mM	millimolar
m/s	Metres per second
Mt	Metric ton
N	North
NaCl	Sodium chloride
NE	North-east
NNE	North-north-east
NNW	North-north-west
NW	North-west
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PCR	Polymerase Chain Reaction
pH	Percentage hydrogen
RH	Relative Humidity
rpm	Revolutions per minute
S	South
SE	South-east
sec	Second
SSE	South-south-east
SSW	South-south west

SW	South-west
V	volts
W	West
WNW	West-north-west
WSW	West-south-West
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TRIS	Tris (hydroymethyl) aminomethane
µg	Micro-gram
µl	Micro-litre
℃	Degree Celsius

PREFACE

The understanding of maize pollen, its movement and subsequent recombination potential is essential to managing gene flow. Identity preservation regulations are influenced by various mechanisms of gene flow. This study attempted to understand maize pollen movement under South African environmental conditions with the utilisation of advanced molecular techniques to detect maize pollen via genotype. A combination of phenotypic and genotypic markers was used to detect and quantify cross-hybridisation events.

Available research on Identity Preservation (IP) was scarce, especially for South African or even African environments. This reiterated the need for research into IP for South African conditions is necessary. During the course of this study, I have identified several areas that have not been researched sufficiently.

No scientific study is perfect in its original design and tends to perfection as the study progresses. In this regard, this study was no exception and throughout this dissertation I have attempted to identify weaknesses in the experimental design on which this study was based.

Each chapter in this dissertation was prepared as a separate article and therefore some recurrence may occur. As a result certain adaptations have been made to enhance readability as much as possible.

Chapter 1

A review of the importance of maize and Identity Preservation

INTRODUCTION

In the plant kingdom, the fundamental objective of pollen production is for species propagation. To achieve this, generous amounts of pollen are produced, transported to a receptive female so that fertilization can take place (Glover, 2002). Then, barring disease, predators and other environmental obstacles, the next generation of seed is produced. This natural practice of species propagation was exploited by early hunter gatherers using teosinte as a source of food (Falcon and Fowler, 2002). Early selections of seed with more desirable traits began the process now thousands of years old to alter the quality and agronomic traits of maize. Just as some qualities were selectively removed over time, certain qualities were maintained while others have been introduced.

In recent years, modern plant biotechnology has evolved to produce crops with specific quality and / or agronomic traits (Smyth *et al*., 2002). Despite this, pollen movement and longevity remain the most important factors of gene flow. The extent of fecundity of maize pollen with regards to the degree of movement, longevity, cooperative biotic conditions and regional environmental conditions has been neglected, in the light of modern biotechnology developments (Glover, 2002). The reasons for this are unknown but are perhaps due to the difficulty in working

with maize pollen, especially due to its limited longevity. The agronomic importance of maize as a food crop throughout the world is a compelling fact, motivating investigations into the contribution and extent of maize pollen to affect gene flow, contributing to ample, efficient maize production.

THE IMPORTANCE OF MAIZE AS A FOOD CROP

Maize forms part of the staple diet in many developing countries (Maredia *et al*., 2000; Pingali, 2001; Rohrbach *et al*., 2003) In Sub Saharan Africa, Central America and parts of South Asia, poverty and population growth have increased the demand for maize as food (Ortiz, 1998; Pingali, 2001). In the developing world, the demand for maize as livestock feed has increased due to the increased consumption of meat and poultry (Fig.1.1) (Pingali, 2001; Wisniewski *et al*., 2002).

By the year 2020, the global maize requirements are expected to increase to 504 million tons, from 282 million tons in 1995 (Pingali, 2001). The demand for maize in Sub-Saharan Africa is expected to increase from 27 million tons to 52 million tons, an increase of 93% (Altman, 1999; Pingali, 2001). In South Africa, maize is an important commodity crop with maize yields exceeding 29,000 Hg/Ha (Fig. 1.2) with the area harvested just above three million Ha in 2003 (Fig. 1.3) (FAO, 2004). Of the South African maize produced in the period from 1995 to 1997, 87% was for consumption, 48% of which was for human consumption and 39% for animal feed (Pingali, 2001).

High consumption levels, and growing population, have resulted in an intense pressure to meet production demands for maize. In Southern and Eastern Africa alone, maize accounts for over 50% of calories provided by starchy cereals (Maredia *et al*., 2000; Rohrbach *et al*., 2003). It has therefore become imperative to develop new maize varieties, to increase yield while lowering production costs (Phillips, 2002; Rohrbach *et. al*., 2003). The green revolution has provided developed countries with increased surplus crop production which continues to increase with successive generations (Wisniewski *et al*., 2002; Huffman, 2004). The advent of modern biotechnology has allowed the introduction of specific quality and agronomic traits through genetic engineering in the first world, all the while most third world countries are still contemplating the green revolution.

AGRICULTURAL BIOTECHNOLOGY

It is important to note that seed production in the developed world differs greatly to seed production in developing countries (Falcon and Fowler, 2002). In the first world seed production is commercially driven whereas in the third world, with the exception of small pockets of commercial farmers, seed production is informal and rural (Macliwain, 1999; Falcon and Fowler, 2002; Cohen and Paarlberg, 2004). It can be assumed that producers (irrespective of nationality or economic status) are interested in products that result in high yield at low cost i.e. properties of most first generation GM crops (Altman, 1999; Skerritt, 2000). However, most African rural or informal farmers for which these qualities are essential for sustainable food production may not fully understand the legal and economic complexity associated

with food production through modern biotechnology (Falcon and Fowler, 2002; Phillips, 2002).

The wide gap between the implementation of modern production management and traditional farming methods will have to be bridged in order for modern biotechnology to benefit rural farmers in developing countries. It is ironic that modern agriculture may actually hold several pitfalls for those it aims to help unless the necessary regulations can be put in place (Pingali and Traxler, 2002). Intellectual property rights and ownership is an alien concept to rural farmers and poverty and the need to feed families will always supersede the importance of implementing farm management. The concept of "owning seed" without a structure accommodating rural farmers, commercial farmers and seed companies is detrimental to the success of biotechnology, to achieve sustainable food development (Cohen and Paarlberg, 2004).

Genetic engineering (GM) holds great potential to introduce quality traits including improved nutritional value, longer shelf life and agronomic traits such as insect-resistance, herbicide-tolerance and disease-resistance from genetic sources such as bacteria, fungi or other plants without relying on sexual compatibility (Brookes, 2002; Auer, 2003). The introduction of quality traits through GM crops into commodity markets was slow to begin but global distribution of GM crops has increased from six countries in 1996 to 18 countries in 2003 (James, 2003) (Fig. 1.3). The total area of transgenic crops planted increased from 1.7 million Ha in 1997 to 67.7 million Ha in 2003, representing an almost 40 fold increase (Table

1.1), 30% off which was produced in developing countries. Complex health and safety, environmental, economic, political and consumer issues have resulted in the introduction of regulations to control the development, production and use of Genetically Modified Organisms (GMOs) (Sundstrom *et al.*, 2002). The requirement to regulate the development, production and use of GMOs forms part of other global initiatives such as the Biosafety Protocol and international labelling regulations (Table 1.2). Under article 18 of the Biosafety Protocol, to which South Africa has acceded, LMOs (living modified organisms) require safe handling, transport, and storage and use (CBD, 2004). Furthermore, other considerations such as international GM labelling regulations, breeder's rights, patent rights and intellectual property rights as well as the marketing importance of quality characteristics have made maintaining the genetic integrity and purity of GM and non-GM seed an imperative (Falcon and Fowler, 2002). To achieve this, a system of production, transport and handling known as Identity Preservation (IP) has been developed, that although not unique to the introduction of specific quality and agronomic traits through modern biotechnology has now become vital to its sustained use (Sundstrom *et al.*, 2002).

IDENTITY PRESERVATION

The concept of IP in agriculture has long existed to maintain the varietal genetic integrity and quality of agronomic crops. The introduction of genetically modified crops has created a new awareness of the importance of IP systems, due to the

demand for quality traits as well as the economic considerations of patent and breeder's rights (Brookes, 2002; Sundstrom *et al*., 2002).

IP can be defined as a system of production, handling transport and processing practice that maintains and verifies the integrity of agricultural commodities (Sundstrom *et al*., 2002; Smyth and Phillips, 2002). The point at which an IP system is maintained in the food chain also depends on the requirements of that specific product (Auer, 2003). The aim of IP is to limit adventitious co-mingling that can occur during any stage of production, handling, transportation, storage and processing (Glaudemans, 2001). This includes the use of isolation distances, proper field tillage to prevent volunteer plants, using clean equipment for harvesting, transport and storage practice (Glaudemans, 2001). However, one potential source of adventitious co-mingling in an IP system over which there is less control and uncertainty is through pollen movement (Glover, 2002). Therefore, gene flow is a primary factor in IP of maize (Glover, 2002; Snow, 2002).

All IP systems are designed to maintain the genetic integrity and purity of a crop, at a pre-determined threshold, as achieving 100% purity in a biological system would be near to impossible (Huffman, 2004). Seed tolerance levels refer to the maximum level of impurity allowed for seed production (Huffman, 2004). Seed companies use tolerance levels suggested by designated authorities such as the AOSCA (Association of Seed Certification Agencies) and OECD (Organisation for Economic Co-operation and Development). For example, AOSCA recommends a 98% tolerance level and the OECD requires 99%. Thus seed production in

developed countries is controlled by stringent regulations (Glover, 2002; OECD, 2001).

In addition to the need for IP, several countries have also put into place labelling regulations that govern the marketing and distribution of products produced through genetic engineering (Sundstrom *et al*., 2002; Brookes, 2002). Certain countries have implemented mandatory threshold labelling requiring genetically engineered products above a predetermined threshold (Table 1.2). For example Japan has a 5% threshold for food, South Korea has a 3%, Indonesia has a 5%, Australia and New Zealand have implemented a 1% while the European Union has reduced its threshold from 1.0% to 0.9% (Auer, 2003; Carter and Gruere, 2003; Agrifood Awareness Centre for Food Safety). In contrast to this, other countries apply voluntary labelling. Canada has a 5% threshold for voluntary labelling and the USA has not set any threshold levels (Auer, 2003; Carter and Gruere, 2003; Agrifood Awareness Centre for Food Safety) (Table 1.2). Thus the labelling requirements imposed by different countries have also become an additional IP consideration in order to comply with international regulations for export produce although it is argued that this has become a trade barrier (Smyth *et al*., 2002).

No policy no matter how well deliberated and regulated is flawless. The Achilles heel of an IP system is pollen-mediated gene flow (Ma *et al*., 2004). In maize, pollen is the primary vector of gene transfer and has the potential to create havoc in commercial seed production unless minimised (Treu and Emberlin, 2000; Eastham and Sweet, 2002).

MAIZE POLLEN MOVEMENT

Maize is an anemophilous (wind pollinated) monoecious species (Emberlin *et al*., 1999; Glover, 2002). It has been estimated that approximately 14 to 50 million pollen grains are produced per average-sized plant with some modern hybrids producing between four to six million pollen grains per plant during a single flowering period (Eastham and Sweet, 2002; Uribelarrea *et al*., 2002). Thus, a single average maize plant has the ability to pollinate an acre of maize plants, producing approximately 240 thousand seeds (Miller, 1985). This high capacity to produce large amounts of seed validates the ability of maize pollen to adequately effect pollination and thus enhance gene flow (Glover, 2002).

Maize pollen morphology

Maize pollen is one of the largest within the Graminae family at 90 to 125 x 85 µm with a volume of approximately 700 x 10^{-9} cm^3 and a weight of 247 x 10^{-9} g (Jones and Newell, 1948; Miller, 1985; Emberlin *et al*., 1999). Maize pollen grains are mono-porate and spheroidal to ovoid in shape with a slightly protruding aperture (Emberlin *et al*., 1999). A pollen grain consists of three layers the outermost layer is the multilayered exine, composed of a polymer called sporopollenin which is reported to be resistant to various chemicals such as sulphuric acid and phosphoric acid (Shaw, 1971). The second layer is the intine composed of cellulose and the third layer is the pollen coat composed of proteins, lipids and pigments (Edlund *et al*., 2004).

Gene Flow

Gene flow is the natural movement of genes between individual organisms through a process of sexual recombination or hybridization (Eastham and Sweet, 2002). In plants this occurs when pollen successfully cross-pollinates with another plant resulting in viable seed, known as out-crossing (Glover, 2002). A high level of gene flow results in homogenisation of population groups so that they become genetically similar, whereas a low level of gene flow together with selection pressure results in the maintenance of genetic variation (Lamkey, 2002).

Therefore, it is important to understand the nature of the factors influencing gene flow through pollen movement in order to achieve effective IP management and minimize adventitious co-mingling during production. Different levels of gene flow exist, crop to crop, crop to wild and crop to weed (Lamkey, 2002). Crop to wild or weed gene flow is generally regarded as a risk especially when GM crops have been introduced into an environment (Dale *et al*., 2002; Jarosz *et al*., 2003; Snow, 2002; Glover, 2002). The concern mainly surrounds the potential development of weeds resistant to herbicides (Dale *et al*., 2002; Snow, 2002). As well as the development of pest resistance to Bt toxin (Dale *et al*., 2002).

Gene flow in maize occurs only within members of the genus *Zea* (Eastham and Sweet, 2002). The ability of maize to cross-hybridise with other maize varieties such as sweet corn, is considered to be “medium to high level risk”, due to pollen dispersal (Eastham and Sweet, 2002; Glover, 2002). Gene flow via pollen movement can be studied via out-crossing (Raynor *et al*., 1972; Paterniani and

Stort, 1974; Jemison and Vayda, 2001; Luna *et al*., 2001; Henry *et al*., 2003; Ma *et al*., 2004; Garcia *et al*., 1998) while potential pollen-mediated gene flow (PMGF) (Levin and Kerster, 1974) is studies by measuring pollen concentrations (Jarosz *et al*., 2003) or computer modelling (Aylor *et al*., 2003; Fricke *et al*., 2004) to determine the extent of pollen dispersal.

Maize pollen longevity

Maize pollen is produced during the flowering period which lasts between 7 to 14 days (Treu and Emberlin, 2000). Pollen viability is enhanced by cool temperatures and high relative humidity (RH) levels (Schoper *et al*., 1987; Eastham and Sweet, 2002; Glover, 2002). In a study conducted by Luna *et al*. (2001) it was found that pollen longevity, under environmental conditions of 28 to 30°C and RH of > 53% was between one to two hours. However, the study was not able to conclusively determine the effect of temperature and RH on pollen longevity. Roy *et al*. (1995) found that maize pollen viability decreased with an increase in temperature above 50°C. A recent study by Aylor (2003) reported that maize pollen remains viable for 60 min. at 23°C and 50% RH. Viability was found to increase at higher levels of RH (Aylor, 2003).

Each of the pollen longevity studies has dealt with one or other variable that influences pollen viability in terms of either seed set or morphological characteristics. However, the simultaneous effect of different environmental conditions on pollen survival is still unclear. To date, no studies have attempted to

use genotypic identification to how far maize pollen of a specific genotype can travel and correlate this to actual pollination.

Maize pollen dispersal

Few studies on pollen dispersal have been published but all of these support the ability of pollen to affect gene flow (Snow, 2002; Henry *et al*., 2003; Jarosz *et al*., 2003). However, there does not appear to be a consensus of the degree to which this can occur. A study by Raynor *et al*. (1972) determined that 1% out-crossing can occur up to 60 m. Using a single plant as pollen source, Paterniani and Stort (1974) found that the furthest extent of out-crossing was 34 m at a level of 0.003%. Luna *et al*. (2001) reported that cross pollination in maize occurred at no more than 200 m. Similar results were also reported by Jemison and Vayda (2001) and Garcia *et al*. (1998). However, a study by Henry *et al*. (2003) across 55 sites in the United Kingdom found that recombination can occur at distances of up to 650 m. Furthermore, a recent study by Ma *et al*. (2004) reported an overall recombination of 2.2 to 62.3% between adjoining yellow and white maize rows with a decline to less than 1% at 28 m. Based on the analysis of these data the authors suggested that other factors, other than just distance, play an important role in pollen dispersal (Ma *et al*., 2004). However, Ma *et al*. (2004) made no further suggestion of what these additional factors could be.

The studies by Raynor *et al*. (1972), Paterniani and Stort (1974), Jemison and Vayda, (2001) and Luna *et al*. (2001) acknowledged the effect of wind on pollen movement but did not account for its effect on pollination potential. Henry *et al*.

(2003) considered the importance wind direction but not velocity, while Ma *et al*. (2004) considered wind velocity and direction but not relative humidity and temperature. From these studies it is clear that environmental conditions including Relative Humidity (RH), temperature, wind direction and speed play an important role in the distance viable pollen can spread. In a study by Roy *et al*. (1995) and Luna *et al*. (2001) it was determined that pollen can remain viable at higher levels of relative humidity and cool temperatures (Aylor, 2003).

Markers to identify out-crossing

An important consideration to determine the impact of pollen on gene flow is the ability to identify pollination events. The studies of Raynor *et al*. (1972), Paterniani and Stort (1974), Garcia *et al*. (1998), Jemison and Vayda (2001), Luna *et al*. (2001) and Ma *et al*. (2004) used phenotypic markers to identify out-crossing. Raynor *et al*. (1972), Paterniani and Stort (1974), Luna *et al*. (2001) and Ma *et al*. (2004) made use of seed colour while Jemison and Vayda (2001) made use of herbicide (glyphosate) tolerance as a result of the EPSPS gene. Garcia *et al*. (1998) and Jemison and Vayda (2001) also made use of PCR (polymerase chain reaction) detection to confirm out-crossing. Henry *et al*. (2003) did not use phenotype but relied solely on genotypic evaluation using Real-time PCR to detect the presence of herbicide (glufosinate) tolerance mediated by the *pat* gene.

Genotypic markers

While phenotypic markers have successfully been used to identify out-crossing in experimental trials, these are not necessarily useful in production systems,

especially since the presence of a quality trait or gene cannot always be easily determine by phenotype. Modern molecular techniques such as PCR allow for the detection of genotypic markers in the absence of phenotype, on condition the trait or gene has a readily detectable marker (Gachon *et al*., 2004; Auer, 2003).

GENOTYPIC DETECTION

The advent of the polymerase chain reaction (PCR) has made it possible to detect specific DNA sequences (Saiki *et al*., 1987). The power of PCR based technology is that it allows the qualitative detection of a target sequence even if it is only present at low copy number (Auer, 2003). PCR has many applications in marker assisted selection (MAS), fingerprinting and population mapping (Kok *et al*., 2002; Mohan *et al*., 1997).

The development of the polymerase chain reaction has resulted in a new generation of DNA fingerprinting and detection techniques (Garcia-Canas *et al*., 2004; Liu and Cordes, 2004). PCR is used to synthesize or amplify target specific sequences using oligonucleotide primers to identify the target region. This results in copies of the target sequence known as amplicons. The PCR reaction consists of a reaction containing all the components necessary for DNA synthesis. The reaction conditions consist of repeated cycles of denaturing, resulting in single stranded DNA, annealing, during which primers bind to the target sequence, and extension or elongation, during which a complimentary strand of DNA is synthesized to the template (Saiki *et al*., 1985; Mullis and Faloona, 1987).

Peterson *et al*. (1996) reported on using PCR to amplify target sequences directly from barley pollen without DNA extraction. In another study using tomato pollen, Levin *et al*. (1997) were able to successfully PCR amplify target sequences from pollen suspensions using sequence specific amplification as well RAPD's (Random Amplified Polymorphic DNA) (Gachon *et al*., 2004). There is no published report on direct PCR amplification of maize pollen. However, this may prove challenging due to the short longevity of maize pollen and its effect on DNA integrity. Miller (1985), report that DNA topoisomerase may contribute to maize pollen DNA degradation. Furthermore, the complex of maize pollen components may also result in PCR inhibition.

In addition to the use of PCR to qualitatively detect specific target sequences, PCR based methods have also been developed to quantify the target sequence through the use of real-time PCR (Auer, 2003; Gachon *et al*., 2004). Real-time PCR combines PCR amplification and detection in a single assay due to the use of fluorescently labelled probes as opposed to normal PCR where post-PCR analysis uses gel electrophoresis for visualisation of PCR products (Saiki *et al*., 1987). Thus Real-time PCR allows for sensitive qualitative and quantitative sequence detection.

During Real-time PCR an increase in fluorescence is detected indicating an increase in the amount of PCR product present (Gachon *et al*., 2004). The point at which amplification is detected above a threshold level is known as the C_T (Threshold cycle) value (Spiegelhalter *et al*., 2001; Ahmed, 2002).

Real-time PCR has the same reaction components as traditional PCR but also includes hybridisation probes tagged with fluorescent dyes (Schweitzer and Kingsmore, 2001; Kok *et al*., 2002). Different methods of Real-time PCR have been developed, including QuantiProbes, Molecular Beacons, Hybridisation probes and Taqman probes (Kok *et al*., 2002; Ahmed, 2002). QuantiProbes are sequence-specific fluorescently labelled probes. A fluorophore is attached at the 3' end of the sequence and a quencher to the 5' end. In solution, the QuantiProbe forms a random structure, facilitating quenching. When the probe attaches to the target sequence, during PCR annealing separating the fluorophore and quencher a fluorescent signal is generated. QuantiProbes are displaced during the extension phase of the PCR reaction. Molecular beacons are probes labelled with a fluorphore attached to the 5' end and a quencher attached at the 3' end. The probes are designed such that the ends are complimentary. In solution the two ends of the probe hybridise and form a hair-pin loop structure, bringing the fluorophore and quencher in close proximity, preventing signal emission. When the probe attaches to the target sequence, during PCR annealing separating the quencher and fluorophore a signal is generated. Hybridisation or FRET (Fluorescence Resonance Energy Transfer) probes, the probe sequence is selected to hybridise to the amplified DNA fragment in a head to tail arrangement, so that when probe bindind occurs during PCR annealing the two fluorophores are brought in close proximity to each other and energy is emitted . In the use of Taqman probes, a fluorophore is attached to the 5' end and a quencher located at the 3' end of the probe. During the annealing/extension phase of PCR the probe is cleaved by the exonulcease activity of Taq DNA polymerase, thus separating the

fluorophore and quencher, allowing a signal to be emitted. In all of these systems the amount of emitted energy in the form light is proportional to the amount of amplified amplicon (Schweitzer and Kingsmore, 2001; Gachon *et al*., 2004).

The absolute quantification of a target sequence is determined using a standard curve of known concentration or copy number (Ahmed, 2002). The amplification of an endogenous sequence as reference is often used to normalise the amount of DNA present in the reaction. Endogenous genes such as the lectin gene for soybean and the HMG or zein gene for maize are used. External standards are used to determine the level of endogenous and target sequence. A standard curve is plotted with the C_T values of the standards against the log of the amount of known copy number or concentration. The C_T values of the sample are compared to the standard curve to determine the amount of target and endogenous sequence present. The percentage target sequence is calculated as a percentage of the amount of endogenous sequence present (Spiegelhalter *et al*., 2001; Ahmed, 2002).

CONCLUSION

Maize plays a significant role in commodity markets due to its national and global importance as a food crop. As a result, many varieties of maize currently exist, each with one or other specific quality or agronomic marketable trait.

New varieties, developed through modern biotechnology, have the potential of reducing production costs while increasing yield (Falcon and Fowler, 2002). Thus it would appear as if agriculture has achieved the “golden age” through the advance of technology, especially with increasing population growth and continued struggling developing economies.

First generation GM crops, with “input traits” such as those with agronomic qualities (Smyth *et al*., 2002; Sundstrom *et al*., 2002) carry an additional premium in terms the price of the seed, which is ultimately carried over to consumers. Second generation GMOs that carry traits that add value or perceived benefit directly to the consumer such as improved nutritional and health benefits. Third generation crops include the production of pharmaceuticals and other products with higher economic value (Smyth *et al*., 2002). As a consequence, IP will become increasingly important as the range of biotechnology crops becomes more specialised (Auer, 2003).

However, rural farmers, an undeniable component of African agricultural production, although not competitive, are in dire need of these first generation GM crops as high yields and low input costs will most definitely contribute to achieving sustainable food production, if not more so than for commercial farmers (Ortiz, 1998; Altman, 1999). Thus, IP in Africa is crucial if rural as well as commercial farmers are included in the new “doubly green” revolution (Wisniewski *et al*., 2002) and if intellectually property rights and breeder’s rights are going to be respected.

With biotechnology innovation also comes responsibility and management due to the requirement for protecting breeding rights, patent rights and intellectual property rights (Falcon and Fowler, 2002). The use of IP to preserve the integrity of a value added traits during production, handling, transport and storage has become important for consumer protection. The genetic integrity of a crop is directly affected by gene flow, the primary vector of which is maize pollen.

Although the potential of pollen with regard to gene flow has been studied, the complex impact of the environment on pollination potential remains largely unanswered, especially since the high potential for out-crossing does not appear to correlate to actual pollination in published data. In addition, different environmental conditions have to be assessed to determine whether they are significant variables contributing to pollen recombination (Treu and Emberlin, 2000).

There are different forces combining to determine pollination potential and its impact on gene flow. These include pollen load, viability determined by RH, temperature, wind speed and direction. The greater our understanding of the factors influencing gene flow the more effective the measures to achieve IP. There are no published studies available for South Africa in terms of the impact of pollen on out-crossing. However, in the final analysis, it is clear that geographic specific data is required. It is also notable that no published pollination data exists for any of the important maize growing areas in South Africa. Therefore it is necessary that such data be generated due to the importance of maize in agricultural production in South Africa.

Specific genotypic detection of maize pollen would provide more comprehensive data on the actual distance maize pollen can affect recombination. The use of PCR based detection would also allow the detection of co-mingling in the absence of phenotype.

The introduction of IP into existing seed production systems is clearly an imperative, especially if seed producers wish to produce seed with specific GM traits. The management and maintenance costs that will ensue are painfully obvious yet unavoidable. However, production systems that exclude GM seed, will incur higher costs as a result of low yield and greater input costs. Nonetheless, for IP to be beneficial, the costs involved are essential as the initial monetary investment in the development of varieties via biotechnology is far too great not to be secured. The alternative would result in repercussions that are financially suicidal for the agricultural industry.

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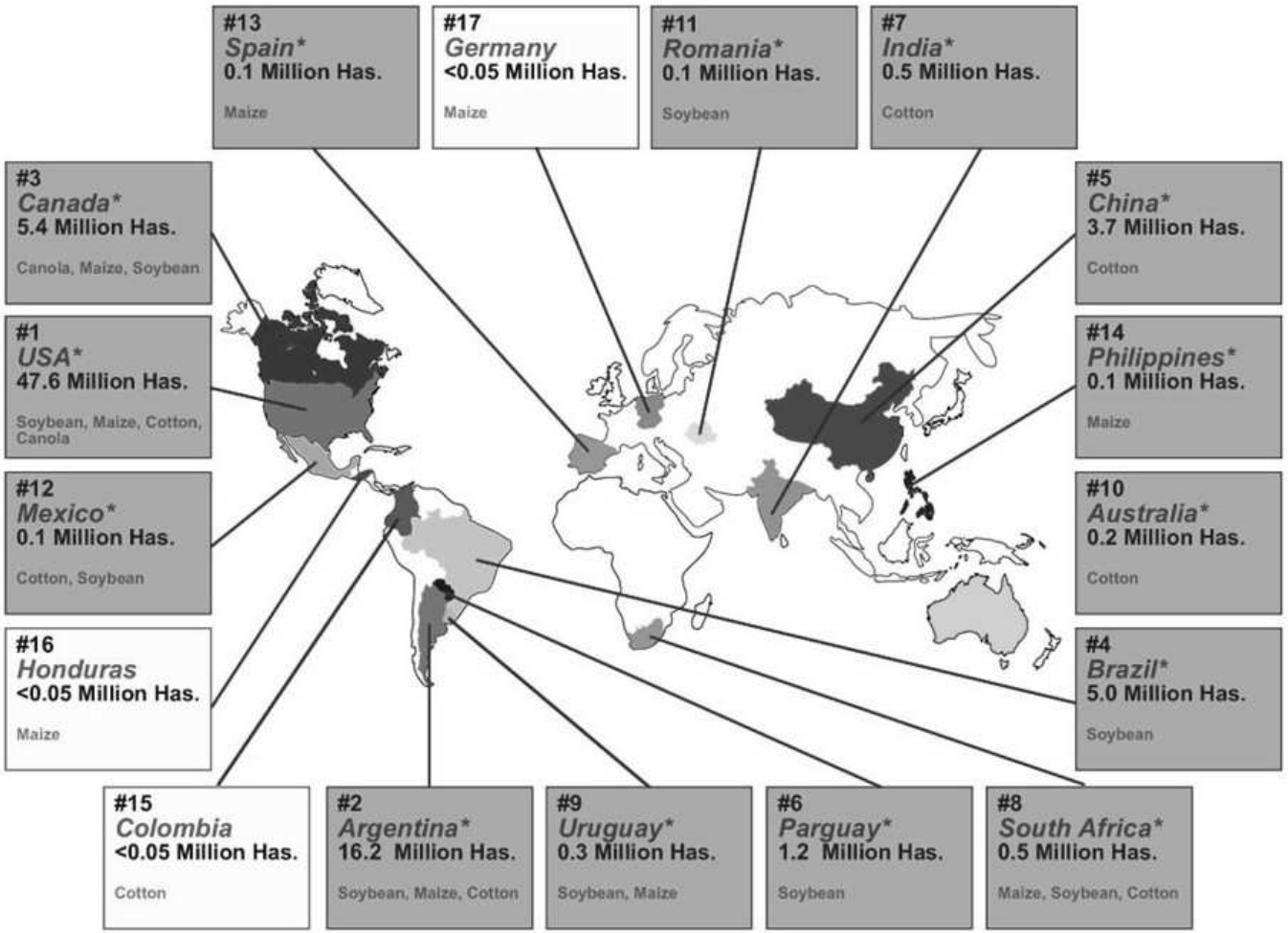

Figure 1.1 World distribution of GM crops planted during 2003 (James, 2003).

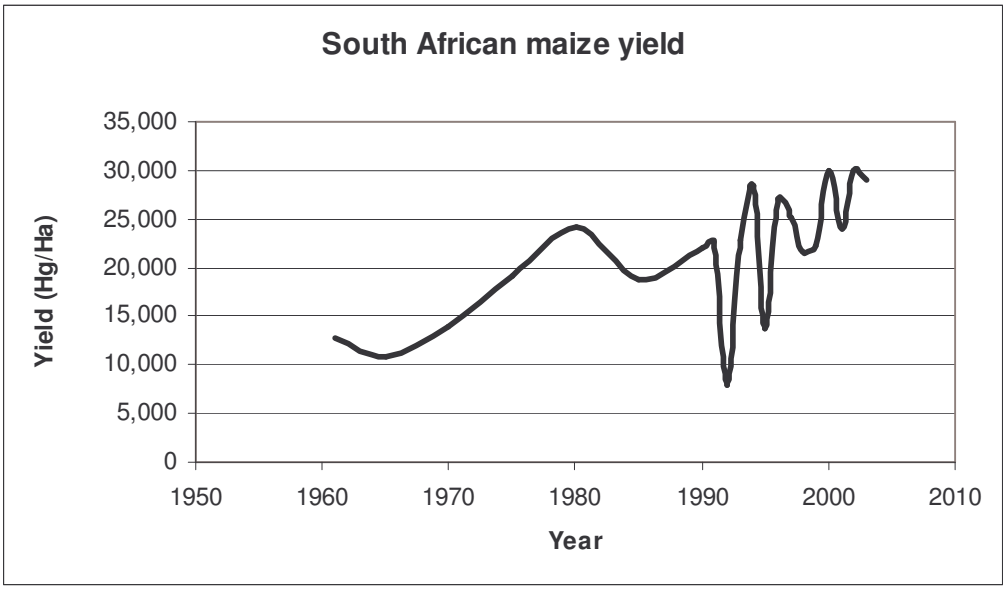

Figure 1.2 South African maize yields (Hg/Ha) from 1961 to 2003.

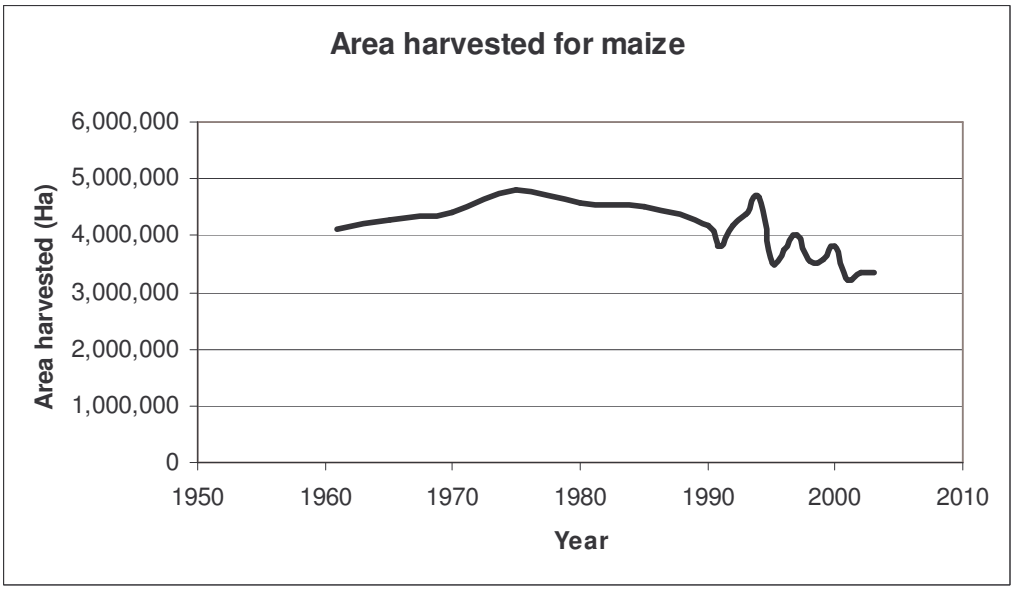

Figure 1.3 The area maize harvested for 1961 to 2003 in South Africa.

Table 1.1 Global GM crop production in hectares for different countries (James, 2003).

Country	Million Hectares	
	2002	2003
USA	39.0	42.8
Argentina	13.5	13.9
Canada	3.5	4.4
Brazil	0	3
China	2.1	2.8
South Africa	0.3	0.4
Australia	0.1	0.1
India	<0.1	0.1
Romania	<0.1	<0.1
Uruguay	<0.1	<0.1
Spain	<0.1	<0.1
Mexico	<0.1	<0.1
Phillipines	0	<0.1
Columbia	<0.1	<0.1
Bulgaria	<0.1	<0.1
Honduras	<0.1	<0.1
Germany	<0.1	<0.1
Indonesia	<0.1	<0.1

Table 1.2 GMO labeling schemes and threshold levels in different countries.

Country	Labelling	Threshold	Scheme
Australia	Mandatory	1.0%	GM
Brazil	Mandatory	1.0%	GM
Canada	Voluntary	5.0%	non-GM
China	Mandatory	1.0%	GM
European Union	Mandatory	0.9%	GM
Indonesia	Mandatory	5.0%	GM
Israel	Mandatory	1.0%	GM
Japan	Mandatory	5.0%	GM
Russia	Mandatory	0.9%	GM
New Zealand	Mandatory	1.0%	GM
Saudi Arabia	Mandatory	1.0%	GM
South Africa	Proposed Voluntary	1.0%	non-GM
South Korea	Mandatory	3.0%	GM
Switzerland	Mandatory	1.0%	GM
Taiwan	Mandatory	5.0%	GM
Thailand	Mandatory	5.0%	GM
USA	Voluntary	5.0%	non-GM

CHAPTER 2

Genotypic detection of Bt maize pollen using PCR

INTRODUCTION

Due to a growing world population and the subsequent increase in maize demand for both food and fodder, several maize varieties have been developed to increase yield while reducing production costs (Maredia *et al*., 2000; Pingali, 2001). However, the use of conventional breeding is limited to sexually compatibly individuals and transfer of individual traits is tedious due to the need for back crossing to obtain true breeding lines (Altman, 1999; Pingali and Traxler, 2002). The advent of modern biotechnology has increased the speed with which new crop varieties are being developed (Nielsen *et al*., 2001). Furthermore, the use of genetic engineering has made it possible to transfer single genes across sexually compatible boundaries (Huffman, 2004). However, the products of modern biotechnology add a new dimension to crop management as a result of the effects of gene flow (Dale *et al*., 2002; Snow, 2002; Aylor *et al*., 2003).

Gene flow is the natural movement of genes between individuals through a process of sexual recombination or hybridization (Huffman, 2004). It is, therefore important to understand the nature of the factors affecting gene flow in order to achieve effective management and minimize adventitious co-mingling during seed production (Glover, 2002). In seed production it has also become imperative to conserve maize genetic resources (Garcia *et al*., 1998; Luna *et al*., 2001).

Adventitious co-mingling would result in legal and trade repercussions (Aylor *et al*., 2003; Jarosz *et al*., 2003).

Gene transfer via pollen flow or movement can be studied via out-crossing (Raynor *et al*., 1972; Paterniani *et al*., 1974; Garcia *et al*., 1998; Jemison and Vayda, 2001; Luna *et al*., 2001; Henry *et al*., 2003; Ma *et al*., 2004), measuring pollen concentrations (Jarosz *et al*., 2003) or developing computer models (Aylor *et al*., 2003; Fricke *et al*., 2004) to determine the extent of pollen dispersal. In practise, co-mingling can be kept to a minimum through the use of identity preservation (IP) (Ma *et al*., 2004).

IP refers to a system of crop production and handling to maintain the integrity and purity of a specific agricultural commodity (Sundstrom *et al*., 2002). One of the more important factors in IP is the potential for gene flow through cross-pollination (Eastham and Sweet, 2002). Gene flow refers to the movement of genes between genetic lines, cultivars or species, resulting in genetic exchange (Lamkey, 2002). Unlike self-pollinating crops, maize is an open pollinating cereal and thus at risk from cross-pollination (Miller, 1985). One of the suggested ways in which an IP system can limit cross-pollination is through the use of isolation distances (Sundstrom *et al*., 2002).

To contain the effect of pollen on co-mingling, different IP systems use different isolation distances. OECD (Organisation for Economic Co-operation and Development) guidelines require isolation distances to be 200 m as do most other

IP systems such as AOSCA (Association of Official Seed Certifying Agencies) in the United States that requires a 201 m isolation distance (OECD, 2001; Glover, 2002). Most studies on the movement of maize pollen and out-crossing indicate that 200 m is adequate to minimize co-mingling (Garcia *et al*., 1998; Jemison and Vayda, 2001; Luna *et al*., 2001; Ma *et al*., 2004). Despite this, some studies have reported on out-crossing occurring at distances up to 650 m from the source (Henry *et al*., 2003).

In theory, the potential of wind dispersal for maize pollen over distance is considerable. A wind speed of 1 m/s is sufficient to disperse a single pollen grain approximately 3600 m (3.6 km) in 1 hour (Treu and Emberlin, 2000). The potential for vertical pollen movement as reported by Brunet *et al*. (2004) found maize pollen captured at 800 to 2000 m above ground to be viable. These studies corroborate the fact that pollen has the potential to move over great distances, and would thus have quite an impact on IP regulations.

To date, pollen trapping studies have determined the overall movement of maize pollen but have not identified pollen from a specific source or the distance maize pollen can move from the source other than by using phenotypic markers. No published attempts have been made to determine potential maize pollen-mediated gene flow (PMGF) by Bt pollen genotypic detection. Thus to be able to trap and detect Bt pollen at a distance from a specific source, would provide a greater understanding into the actual distance maize pollen can disperse.

The advent of the polymerase chain reaction (PCR) has made it possible to detect specific sequences (Saiki *et al*., 1987, Garcia-Canas *et al*., 2004, Liu and Cordes, 2004).

Peterson *et al*. (1996) reported on using PCR to amplify target sequences directly from barley pollen. In a study on tomato pollen, Levin *et al*. (1997) were able to successfully fingerprint pollen directly and thus deduce its genotype. To date, direct PCR on maize pollen has not been reported. PCR amplification of maize pollen is potentially challenging due to short pollen longevity and the complexity of pollen structure. The complex molecular components of maize pollen, one of the largest pollen types, may result in inhibition of the PCR assay. Maize pollen survives only for a few hours, and this may affect the integrity of the DNA (Luna *et al*., 2001). Miller (1985) described an enzyme DNA Topoisomerase that may play a role in pollen DNA degradation affecting its viability. Therefore, the greatest potential obstacle in determining the genotype of trapped pollen would be maintaining the DNA integrity of the pollen as well as possible inhibition of PCR. Lui *et al*. (1985) reported that different organic solvents such as petroleum ether, acetone, benzene, chloroform and acetic acid could be used to maintain pollen viability for up to 40 days (Barnabas and Kovacs, 1997). The aim of this study was 1) to determine how to preserve the integrity of pollen DNA during pollen trapping and 2) to determine the distance at which pollen, of a specific genotype, can be trapped and detected under field conditions.

MATERIALS AND METHODS

Plant material

CRN 3505 white and CRN 4760 B yellow Bt maize seed (Monsanto, South Africa) was planted, one plant per pot in eight litre pots using soil typical to the local farming region, and maintained under glasshouse conditions at 25ºC for approximately 4 months. Fertilizer (6.5% Nitrogen, 2.7% Phosphorus, 13% Potassium, 7% Calcium, 2.2% Magnesium and 7.5% Sulphur) at 1 g/l was applied once a week. Pesticide (Metasytox R (Oxydemeton-methyl 5 ml/l), Wonder red spider spray (Amitraz 3 ml/l) and R.T Chemicals-AbamecPlus (Abamectin 0.6 ml/l) for aphid and red spider mite infestation was applied as required.

Trial layout

Maize fields were planted at two maize breeding regions in South Africa (Fig. 2.2), Delmas in Mpumalanga and Lichtenburg in the North-West Province. There was no significant slope at the two locations. The same varieties were used for both locations. A separate unrelated field trial consisting of a mixture of yellow and white maize, Bt and Roundup, was planted at Delmas, four weeks prior to the study trial. A CRN 3505 (white) and CRN 4760 B (yellow) maize varieties (Monsanto, South Africa) were planted at least 4 weeks after previous maize plantings at both locations (Table 2.2). The field positioning (Fig. 2.3 and 2.4) was based on prevailing wind patterns of that area (personal communication with plant breeders from each area).

Pollen Collection

Bt pollen was collected from individual maize plants, for up to two weeks during flowering by gently tapping the base of the anthers and allowing the pollen to fall onto a clean sheet of paper from which it was transferred to a micro-centrifuge tube containing silica gel crystals and stored at 4ºC (Fig. 2.1).

Pollen storage

The Bt pollen was subjected to different storage methods in aliquots of 1000 µg in duplicate, including dry storage on silica gel at 4°C, in 500 µl CTAB storage buffer (50 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8), dry after heat-treatment at 90°C for 15 min as well as different organic solvents including petroleum ether, acetone, benzene, chloroform and acetic acid (Lui *et al*., 1985) (Table 2.1). The efficacy of these methods was tested at different periods from zero, seven, 14, 21 and 42 days. Day zero served as a batch test to determine that the pollen was PCR amplifiable from which the other aliquots were taken for that series.

Pollen DNA Extraction

DNA was extracted from the 1000 µg aliquots by the addition of 10 µl CTAB extraction buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8) followed by homogenisation with a plastic micro-pestle in a 1.5 ml micro-centrifuge tube. For pollen stored in CTAB storage buffer, the 5% CTAB pollen mixture was centrifuged at 10k rpm for 5 min and the 5% CTAB storage buffer decanted and replaced with 10 µl 2% CTAB extraction buffer. After

homogenisation a further 490 µl of CTAB extraction buffer and 5 µl Proteinase K [20 mg/ml] was added to the crushed pollen and incubated at 60°C for 60 min. The mixture was incubated at 80°C for 5 min followed by the addition of 2 µl RNAse [10 mg/ml] and incubation at 60°C for 5 min. Chloroform extractions were performed by the addition of 500 µl of Chloroform-Isoamyl alcohol (24:1) followed by thorough mixing and centrifugation at 13k rpm for 5 min. The aqueous layer was retained and the chloroform extraction repeated. The nucleic acids were precipitated by the addition of 1 ml absolute ethanol on ice for at least three hours or overnight at 4°C. The precipitate was then centrifuged for 20 min at 13k rpm. The pellet was retained and washed twice by the addition of 500 µl 75% ethanol, followed by centrifugation at 13k rpm for 5 min. The pellet was re-dissolved in 50 µl sterile, double distilled water.

DNA purification

The extracted DNA was further purified using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Capture buffer (500 µl) was added to the dissolved DNA and mixed. The DNA capture buffer solution was applied to a micro-spin column and centrifuged in a 2 ml micro-centrifuge tube for 30 sec at 14k rpm. The flow-through was discarded and 500 µl Wash buffer applied to the column followed by centrifugation at 14k rpm for 30 sec. The collection tube was then discarded and the micro-spin column transferred to a 1.5 ml micro-centrifuge tube. Thereafter, sterile double distilled water (30 µl) was added to the column and incubated at room temperature for 1 min. followed by centrifugation at 14k

rpm for 30 sec to elute the DNA. The concentration of DNA was calculated at 260 nm wavelength in a spectrophotometer using the formula:

DNA concentration (µg/ml) = OD x dilution factor x 50

PCR amplification

PCR was performed on pollen and extracted pollen DNA using PCR and Real-time PCR. All negative PCR results were confirmed using Real-time PCR. PCR and Real-Time PCR were performed using mastermixes for 35S detection and quantification (GeneScan GmbH) on a Applied Biosystems 2700 and the Roche LightCycler.

For PCR analysis on extracted pollen DNA, the reaction contained, 19.9 µl 35S mastermix (GeneScan, GmbH), 0.16 µl Ampli-Taq Gold [5 U/µl] and 5 µl DNA. For the negative control, 5 µl 0.1 X T.E buffer [0.25 mM Tris, 2.5 mM EDTA] used and for the positive control, 5 µl of 1% Bt 176 maize control DNA was added. The PCR cycling conditions were 95^{o}C for 10 min (1 cycle), 95^{o}C for 25 sec, 62^{o}C for 30 sec, 72^{o}C for 45 sec (50 cycles), 72^{o}C for 7 min and 25^{o}C (1 cycle). PCR amplicons were visualized using 2% gel electrophoresis (Molecular Screening Agarose). Gels were run at 270 V for approximately 25 min, stained in ethidium bromide [100 µl/L] for 45 min, visualised under UV light and documented (BioRad Gel Doc 1000, Molecular analyst version 1.4.1).

For direct PCR, pollen stored in silica gel was placed onto a glass slide and, one to five, 10 and 20 pollen grains were transferred individually to a LightCycler™ capillary containing 5 µl sterile double distilled water, using an eyelash attached to a needle (Peterson *et al*., 1996). To each capillary, 15 µl of Reference Master Mix (containing dNTPs, primer for HMG detection, *Taq* polymerase, $MgCl_2$ and probes) or 15 µl of GM Master Mix (containing dNTPs, primer for 35S detection, *Taq* polymerase, $MgCl_2$ and probes) (GeneScan), was added, respectively, in duplicate. For the negative control 5 µl sterile double distilled water, was used and for the positive control, 5 µl of 1% Bt 176 maize control DNA was added. For Real-time reactions on extracted pollen DNA, 5 µl of the DNA extract was used.

The cycling conditions for Real-time PCR were, 1 cycle at 95ºC for 60 sec., 50 cycles at 95ºC for 5 sec. and 60ºC for 25 sec. and 1 cycle at 40ºC for 30 sec. For direct pollen amplification, the contents of the capillary were examined after Real-time PCR was completed to assess the amount of pollen breakage under light microscopy.

Adherent for pollen trapping

Different adherents including Tween 20 (Polyoxyethyllene (20) sorbitan monolaurate), glycerol and petroleum jelly were tested for applicability as an adherent in pollen capture by thinly coating it onto a glass slide using a glass slide. A pollen aliquot of 1000 µg was then applied onto the slide and the pollen recovered by rinsing the slide with 500 µl CTAB storage buffer into a plastic petri-dish and subsequent transfer to a 1.5 ml micro-centrifuge tube. The pollen mixture

was centrifuged for 5 min at 10k rpm and the pollen pellet re-suspended in 50 µl CTAB extraction buffer. Thereafter the DNA was extracted as described previously.

Pollen trapping

Pollen traps were constructed by the University of the Free State's Instrumentation Department. Each trap composed of two aluminium poles. One pole designed to fit firmly into the ground and the second designed to fit into the first pole. This allowed for variable height adjustment, a rivet set off-centre into the second pole ensured that when fitted into the first pole and turned slightly, it could be secured into position at the desired height. The top of the second pole was fitted with a clamp to hold a glass slide acting as pollen trapping unit. The pole height varied at the two locations was based on the approximate height of plants. Glass slides coated with Tween 20 were placed at distance intervals in two and four directions at Delmas and Lichtenburg, respectively. The distance to the pollen traps was measured from the edge of the Bt field for each specific direction (Table 2.3).

Glass slides were positioned at between 8 to 9 am and collected the following morning for Delmas and at 3 pm on the same day for Lichtenburg. Pollen was rinsed off collected slides using 1 ml of 5% CTAB storage buffer and stored in a 1.5 ml micro-centrifuge tube at 4°C. Pollen was maintained at 4°C until DNA extraction, purification and PCR analysis.

The 5% CTAB pollen mixture was centrifuged at 10k rpm for 5 min and the 5% CTAB storage buffer decanted and replaced with 50 µl 2% CTAB extraction buffer. Four microlitres of pollen was diluted 1:10 with 2% CTAB extraction buffer and used for pollen counting using a Neubauer Haemocytometre. The pollen was counted and calculated according to the manufacturer's instructions.

The average pollen count was calculated using the formula:

$$\text{Pollen/ml} = (\text{average pollen count}) \times (1 \times 10^4)$$

When pollen was only observed outside the haemocytometre squares, the pollen/ml was calculated using the formula:

$$\text{Pollen/ml} = \text{the number of pollen observed outside squares} \times \text{dilution factor} \times 1000 / (\text{Total volume of pollen suspension})$$

Pollen DNA extraction from traps

DNA extractions were performed on day one, two and four in Lichtenburg and day one and two in Delmas (due to rain). According to the method described previously.

Weather data and analysis

Weather data was obtained from the ARC, Agro-meteorology Department for Delmas (Monsanto-Petit) and Lichtenburg (Sheila Co-op). The weather data was

obtained hourly for nine days during the flowering period for temperature (°C), relative humidity (%), wind speed (m/s) and wind direction during flowering.

The weather data was categorised into the number of hours of high (>70%) and low (<70%), relative humidity and for temperature (high: >20°C and low: <20°C) as well as relevant (in the direction that would affect cross-pollination favouring the conventional field) and non-relevant wind directions. Chi-tests were performed on the weather data.

The relevant wind was further categorised into high (>2 m/s) and low (<2 m/s) speed. Chi-tests were performed on the weather data including relevant wind, non- relevant wind, high and low wind speed within the relevant wind directions.

RESULTS

Pollen storage

The pollen stored in diethyl ether, hexane, chloroform and 99.9% ethanol disintegrated totally after 5 min (Table 2.1). The pollen stored on silica gel at 4°C was PCR amplifiable at day 7, 14 and 21 but not 42 days. Pollen in CTAB storage buffer was PCR amplifiable at day 7, 14, 21, 42 and up to 9 months (Fig. 2.5). The heat treated pollen stored at 4°C was amplifiable at day 14 but not day 42.

For direct pollen amplification, pollen was amplifiable using a single pollen grain but not for batches of 20 pollen (Table 2.4). DNA from five and ten pollen grains

was amplifiable. Pollen counts after PCR amplification showed that 80 to 100% of pollen disintegrated during PCR cycling. The direct amplification from pollen was not reproducible.

Glycerol formed droplets when applied to a glass slide. Petroleum jelly could be applied evenly to the glass slide but posed a problem when recovering pollen from the slide. Tween 20 was easily applied to the glass slide and allowed easy pollen retrieval by washing with CTAB storage buffer. DNA extracted from pollen recovered from Tween 20 coated slides was PCR amplifiable.

Field trial phenology

The flowering period coincided with silk production at both trial locations for white and yellow maize, respectively (Table 2.2). Plants at both locations were uniform in growth pattern and size. The glass slide on pollen traps was set at the height corresponding to the average height of the maize plants, 1.7 m for Delmas and 1.9 m for Lichtenberg.

Pollen trapping

Trapped maize pollen counts for Delmas ranged from less than 1250 to 3750 per trap per day up to a distance of 400 m (Table 2.5). Pollen traps were only set for day one and two during flowering due to precipitation and in two directions due to access. The pollen counts for Lichtenburg ranged from less than 1250 to 101250 per trap per day, in four directions for seven days up to 350 m (Table 2.5).

Pollen counts higher than 1250 were observed at Delmas in a southerly and south west direction for day one and two, with relevant (to the position of pollen traps) wind on day one in a northerly (blowing south) direction and for day two in a north to north west direction as well as south-south east to north east (Fig. 2.6) (Table 2.5).

For Lichtenburg, pollen counts higher than 1250 were observed for day one for the south west and south east traps, day two for the south east, south and south west traps, day four for the south east traps, day five for the north west and south east traps, day six for the south east and south west traps and day seven for the south east and south west traps (Fig. 2.7) (Table 2.5). The 50 m south east trap was the only trap to be placed within the white maize field.

PCR pollen detection (Fig. 2.8) was achieved at a distance of 400 m in a westerly direction for Delmas on day two, with winds in a south-south east to east south east direction, and at Lichtenburg at a distance of 350 m in a southerly direction on day four, with winds in a north-north west and north east direction (Fig. 2.6 and 2.7) (Table 2.5).

Weather patterns

During the flowering period, the average temperature ranged from 16.1 to 19.6^{o}C for Delmas and 13.1 to 19.2^{o}C for Lichtenburg (Fig. 3.8). At Delmas there were 155 hours of less than 20^{o}C and 61 hours of greater than 20^{o}C, while at Lichtenburg there were 149 hours of less than 20^{o}C and 67 hours of greater than

20°C, average daily temperature. Average morning temperatures ranged from 14.2 to 21.4°C (overall average of 16.7°C) at Delmas and from 12.8 to 21.4°C at Lichtenburg with an overall average of 15.6°C.

The average relative humidity (RH) at Delmas ranged from 74.8 to 99.7%, while at Lichtenburg it ranged from 58.7 to 90.1% (Fig 3.9). At Delmas there were 166 hours of greater than 70% RH and 50 hours of less than 70% RH, while at Lichtenburg there were 154 hours of greater than 70% RH and 62 hours less than 70% RH. There was no statistical significant difference in average relative humidity ($P = 0.19$) and average temperature ($P = 0.53$) between Delmas and Lichtenburg.

The wind speed during flowering varied between sites. At Delmas the average wind speed per day ranged from 0.20 to 3.96 m/s and for Lichtenburg from 0.20 to 4.85 m/s (Table 2.6 and 2.7). The average wind speed in the relevant wind direction ranged from 0.32 to 1.90 m/s at Delmas and from 0.0 to 2.83 m/s at Lichtenburg. In Delmas there were 111 hours of wind in the relevant direction affect out-compared to 68 hours in Lichtenburg. There was a statistically significant difference in the relevant wind direction at Delmas and Lichtenburg ($P = 2.7 \times 10^{-5}$). Within the relevant wind direction there was also a significant difference between high wind speed (>2 m/s) and low wind speed (<2 m/s) at Delmas and Lichtenburg, respectively ($P = 0.02$).

The predominant frequency (direction and duration) of wind at Delmas was of a low wind speed in a south easterly direction (from which wind is blowing) while higher wind speeds were observed in a north, north-north east and easterly direction at a lesser frequency. At Lichtenburg, the predominant frequency of wind was of a low wind speed in a north-north east to easterly direction while higher wind speeds were observed in various directions.

DISCUSSION

Pollen storage at 4℃ using a desiccant was effective to maintain pollen DNA integrity for PCR amplification up to 21 days (Table 2.1). Storage in CTAB storage buffer proved effective in maintaining pollen DNA integrity for 42 days and as much as up to nine months (Table 2.1) (data not shown). Using organic solvents proved ineffective and resulted in pollen disintegration. Thus CTAB storage buffer was determined to be the most effective method to store pollen during pollen trapping experiments.

Direct pollen amplification from maize proved to be erratic and inconsistent (Table 2.4). Pollen breakage did not play a significant role in PCR failure. Levin *et al*. (1997) and Peterson *et al*. (1996) reported on PCR amplification from tomato and Barley, respectively. Both studies reported on a correlation between the ability to PCR amplify a target sequence from pollen with an increase in the number of grains of pollen. The study of Petersen *et al*. (1996) amplified target sequences in numerous copies in a single pollen grain but reported on unsuccessful results for

low copy sequences, even after increasing the number of pollen grains. Levin *et al*. (1997) report on the use of between 0.5 and 50 µg of pollen in PCR reactions with no evidence of PCR inhibition. In the present study, PCR inhibition is certainly a factor as PCR amplification was only obtained for 10 pollen grains or less. Furthermore, the target sequence was low copy number (one copy per haploid genome). The ability to detect PCR amplification in the present study was possibly due to the use of Real-time PCR which is considered to be more sensitive than normal PCR due to the use of fluorescent probes.

Tween 20 was determined to be a suitable pollen adherent for pollen trapping experiments. Glycerol could not be evenly coated onto the glass slide and petroleum jelly was water insoluble preventing pollen recovery. Tween 20 did not inhibit DNA extractions or PCR analysis, thus allowing for pollen to be captured in a field trial at various distances from a source, recovered with CTAB buffer and stored at 4°C until DNA extractions and genotypic identification using PCR.

In field trapping experiments, maize pollen was detected up to 400 m at Delmas and 350 m at Lichtenburg. Pollen movement may have exceeded this distance in both locations. The presence of Bt yellow pollen was not related to the amount of pollen from the traps at various distances (Table 2.3). Furthermore, the PCR detection of the Bt yellow pollen from pollen traps at consecutive distance intervals was not consistent indicating that swirling winds may have played an role in pollen distribution. The slope at both locations was such that this would not contribute significantly to pollen movement. Brunet *et al*. (2004) reported that maize pollen

could be carried up to 2000 m into the atmosphere. In the present study, pollen was detected up to 400 m from the source. The minimum recommended isolation distance by the OECD and the AOSCA is 200 m, which clearly may not be sufficient. This is the first report on potential PMGF by studying the movement of pollen of a specific detectable genotype. However, this study did not determine viability in terms of fecundity at these distances.

During the present study, the average daily temperature during pollination was below 20^{o}C, especially morning temperature and the overall relative humidity was above 70%. Thus, conditions at both locations were well suited for pollen survival.

Previous studies determined pollen longevity based on the ability to effect fertilisation (Luna *et al*., 2001) or on morphological appearance (Roy *et al*., 1995). Throughout this study, the ability to PCR amplify the correct target sequence was used as a measure of pollen DNA viability and hence DNA stability. DNA within the maize pollen remained stable for a few days after the onset anthesis.

A greater success with PCR was observed with DNA extracted from pollen than from the direct pollen amplification. Pollen storage at 4°C in silica gel successfully maintained DNA stability up to 21 days (Table 2.5), while contrary to previous studies on the effect of heat on pollen (Roy *et al*., 1995), heat-treated pollen remained viable for up to 14 days. CTAB storage buffer proved the most successful of all treatments and extracted DNA was PCR amplifiable for up to 42 days at 4°C and even up to 9 months. The use of organic solvents such as diethyl

ether, chloroform, hexan and ethanol to preserve the pollen resulted in the total disintegration of pollen grains, contrary to previous research by Lui *et al*. (1985) where these solvents was used to maintain pollen longevity for short periods.

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Table 2.1 Different pollen storage methods with corresponding PCR amplification. Successful PCR amplification is denoted as “viable” and unsuccessful PCR amplification as “non-viable”.

Storage method	Time (days)				
	0	7	14	21	42
Silica gel	viable	viable	viable	viable	non-viable
CTAB buffer	viable	viable	viable	viable	viable*
Heat treated pollen	viable	n.d	viable	n.d	non-viable
Organic solvents:	pollen disintergration				
Diethyl ether					
Hexan					
Chloroform					
Absolute Ethanol					

* **Viable for up to 9 months**

Table 2.2 A description of the trial layout for the different locations used in the study.

Field trial details	Delmas	Lichtenberg
Field dimensions	45 x 31.5 m	171 x 67 m
Field area	1417.5 m^2	11457 m^2
Distance between rows	0.75 m	0.80 m
Planting dates	08 December 2003	28 January 2004
Days to flowering	61	71
Flowering period	16 - 24 February 2004	4 - 12 April 2004
Number of white maize rows	30	96
Number of yellow maize rows	30	104
Pollen trapping period	2 days (21 - 22 February 2004)	7 days (6 - 12 April 2004)
Treatments	None	Fertlizer

Table 2.3 Direction and distance intervals for traps set at Delmas and Lichtenburg.

Delmas		Lichtenburg			
West	**South**	**North-west**	**South**	**South-west**	**South-east**
50 m	50 m	50 m	50 m	50 m	50 m
100 m	100 m	100 m	100 m	100 m	100 m
150 m	150 m	150 m	150 m	150 m	150 m
200 m	200 m	200 m	200 m	200 m	
250 m	300 m	300 m		300 m	
300 m	400 m	350 m		350 m	

Table 2.4 Direct PCR amplification on maize pollen to assess the limits of detection.

Pollen count	1st Replication		2nd Replication		3rd Replication	
	Amplification	**Intact pollen after PCR**	**Amplification**	**Intact pollen after PCR**	**Amplification**	**Intact pollen after PCR**
1	+	0	+	0	-	0
5	+	1	-	n.d.	-	0
10	+	0	-	n.d.	-	0
20	-	1	-	n.d.	-	0

\+ Amplification
\- No amplification

Table 2.5 The number of pollen/ml in each trap for Lichtenburg and Delmas as well as PCR amplification results, respectively.

Delmas			Lichtenburg							
WEST			NORTH WEST							
Distance (m)	Day 1	Day 2	Distance (m)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
50	<1250^{+}	<1250^{+}	50	<1250^{+}	<1250^{+}	<1250	<1250^{+}	1750 ^{1}	1250	1250 ^{1}
100	<1250 $^{-}$	<1250 $^{-}$	100	<1250^{+}	<1250^{+}	<1250	<1250 $^{-}$	1500 ^{1}	<1250	<1250
150	<1250 $^{-}$	<1250^{1} $^{-}$ (250)	150	<1250^{+}	<1250^{+}	<1250	<1250 $^{-}$	<1250	<1250	<1250
200	<1250^{+}	<1250 $^{-}$	200	<1250^{-}	<1250^{+}	<1250	<1250 $^{-}$	<1250	<1250	<1250
300	<1250^{-}	<1250 $^{-}$	300	<1250 $^{-}$	<1250^{+}	<1250	<1250 $^{-}$	<1250	<1250	<1250
400	<1250^{-}	<1250 $^{+}$	350	<1250^{+}	<1250 $^{-}$	<1250	<1250 $^{-}$	<1250	<1250	<1250
SOUTH			SOUTH WEST							
	Day 1	Day 2		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
50	<1250^{+}	1500 ^{1} $^{-}$	50	3750^{-}	3750 $^{+}$	<1250	<1250 $^{+}$	<1250	<1250^{1} (1000)	1500 ^{1}
100	3750^{+}	<1250^{1} $^{+}$(500)	100	<1250^{1-}	6250 $^{+}$	<1250	<1250 $^{-}$	<1250	1750 ^{1}	<1250^{1} (750)
150	<1250^{-}	<1250 $^{-}$	150	<1250^{-}	1250^{+}	<1250^{1}(250)	<1250 $^{+}$	<1250	<1250^{1} (250)	<1250
200	<1250^{+}	<1250 $^{-}$	200	<1250^{-}	1250 $^{+}$	<1250^{1}(500)	<1250 $^{-}$	<1250	<1250^{1} (250)	<1250
250	<1250 $^{-}$	<1250 -	SOUTH							
300	<1250 $^{-}$	<1250 $^{+}$		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
			50	<1250^{+}	8750 $^{+}$	<1250	<1250 $^{-}$	<1250	<1250	1250
			100	<1250^{1+}	18750^{+}	<1250^{1}(250)	<1250 $^{-}$	<1250	<1250	<1250
+ Amplification			150	<1250^{+}	2500^{+}	<1250	<1250 $^{-}$	<1250	<1250	<1250
- No Amplification			200	<1250 $^{-}$	1250^{+}	<1250	<1250^{1} $^{-}$(250)	<1250	<1250	<1250
1 Pollen observed outside haemocytometre squares			300	<1250 $^{+}$	1250^{+}	<1250	<1250 $^{-}$	<1250	<1250	<1250
			350	<1250 $^{-}$	<1250 $^{-}$	<1250	<1250 $^{+}$	<1250	<1250	<1250
			SOUTH EAST							
				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
			50	5000^{+}	85000 $^{+}$	<1250	101250 $^{-}$	6250	5000	7500
			100	<1250^{+}	1250 $^{-}$	<1250	2500 $^{+}$	<1250	<1250	<1250
			150	<1250^{-}	<1250^{1-}	<1250	<1250 $^{+}$	<1250	<1250	<1250

Table 2.6 Temperature and relative humidity data for Delmas during flowering.

Temperature (°C)																									Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	15.5	15.1	15.1	14.7	14.3	14.2	14.4	16.3	18.4	18.5	19.8	22.1	23.3	24	24.1	23.5	20.2	20.8	19.2	18.4	17.3	16.9	16.6	16.3	18.28
Day 2	16.4	16.4	16.2	16.2	15.5	14.8	15.1	17.7	19.1	20.1	21	21.7	22.7	23.4	24	24.2	23.9	23.1	21.6	19.8	19.5	18.4	18	18	19.44
Day 3	16.9	16.5	15.9	15.2	14.9	14.3	14.7	17.1	19.6	20.4	21.8	22.6	24	24.8	22.6	17.9	16.5	16	16.2	16	15.8	15.5	15.3	14.9	17.71
Day 4	14.4	13.9	13.7	13.2	12.9	12.9	12.7	14.7	17.2	19.7	21.1	22	22.5	23.1	23.8	23.9	24	23.5	21.2	18.7	19	17.9	17.2	16.1	18.30
Day 5	15.9	15.4	14.5	14.4	13.8	12.8	13.4	17.5	19.3	20.4	22	22.5	23.7	24.2	24.6	25.6	25.3	24.2	21.9	19.7	18.1	17.7	17.2	17.3	19.22
Day 6	16.1	15.5	15.1	14.8	14.7	14.7	15.5	16.8	18.7	19.9	21.1	22.6	23.8	24.4	25.1	24.9	25.3	24.9	22.3	19.9	18.9	18.6	18.2	17.6	19.56
Day 7	16.7	16.3	15.6	15	14.9	14.1	14.7	17.4	20.2	22.1	23.5	24.2	24.7	25.3	24.9	23.2	23.1	22.9	20.6	19	18.5	17.1	16.3	15.9	19.43
Day 8	15.5	15.3	15.4	15.6	15.4	15.3	15.1	15.1	15.6	16.3	17.4	18.3	19.1	19.6	19.4	19.3	19	17.8	16.8	16.8	16.8	16.5	15.7	15.4	16.78
Day 9	15.2	15.1	14.9	14.8	15	15	15.2	15.4	15.9	16.1	16.3	16.7	16.9	17.6	17.9	18.4	17.6	16.8	15.6	15.6	15.8	15.6	15.7	16.4	16.06
Ave.	15.83	15.50	15.15	14.9	14.60	14.22	14.53	16.43	18.23	19.29	20.44	21.42	22.31	22.93	22.93	22.32	21.66	21.12	19.49	18.21	17.74	17.12	16.69	16.42	
Max.	16.9	16.5	16.2	16.2	15.5	15.3	15.5	17.7	20.2	22.1	23.5	24.2	24.7	25.3	25.1	25.6	25.3	24.9	22.3	19.9	19.5	18.6	18.2	18	
Min.	14.4	13.9	13.7	13.2	12.9	12.8	12.7	14.7	15.6	16.1	16.3	16.7	16.9	17.6	17.9	17.9	16.5	16	15.6	15.6	15.8	15.5	15.3	14.9	
Ave. (am)	16.7																								
Overall ave.	18.3																								
Relative Humidity (%)																									
Day 1	95.7	96.5	97.5	98.3	98.7	99.1	99.4	96.2	93.8	91.8	79.2	74.6	61.5	61.6	67	77.8	68.3	78.5	87.8	93	95.4	94.9	96.9	97.5	87.54
Day 2	97.8	98.7	99.1	98.9	98.1	97.9	90.4	86.7	80.4	78.6	74.7	66.2	61.6	62.5	57.9	58.2	59.5	68.5	81.1	83	80.5	92	90	92.3	81.44
Day 3	95.1	95.9	92.7	98.2	98.9	99.2	98.1	89.3	80.6	70.2	71.7	59.2	52.6	51.7	85.8	87.7	92.6	93.2	94.7	97.5	98.3	98.9	99.4	99.7	87.55
Day 4	99.6	100	100	99.7	99.9	99.7	100	97.8	84.5	65.8	61.7	61.7	56.6	52.9	52.8	50.5	51.6	56.9	70.1	74.3	71.4	79.2	79.5	87.6	77.27
Day 5	88.9	92.7	95.8	96	97.5	97.8	97.4	87.1	76.2	67.1	60.1	60.4	57.8	52.1	47.2	47.4	46	59.7	67.6	75.6	78.9	76.9	83.1	86.4	74.82
Day 6	88.3	93.2	94.4	96.6	98.1	98.7	97.4	91.9	83.6	75.8	66.1	63.4	59.6	57.1	51.8	54	47.4	51.6	73	67.8	70.1	72.2	76.6	82.3	75.45
Day 7	82.8	87.5	91.1	92.6	95	96.8	92.3	85.5	72.9	67.4	56	55.7	54.6	48.8	54.7	69.8	57	74.4	78.2	75.6	82.7	91.1	93	96.3	77.16
Day 8	97.6	98	98.5	98.8	98.9	99.5	99.8	99.8	99.8	99	92.6	86.2	84	84.4	87.2	83	90.8	96.1	97.7	98.9	99.4	99.5	99.8	100	95.39
Day 9	100	100	99.9	100	99.6	100	99.8	99.8	99.4	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	99.7	99.67
Ave.	94.00	95.88	96.57	97.69	98.31	98.75	97.19	92.68	85.69	79.46	73.59	69.73	65.36	63.39	67.09	69.35	68.07	75.39	83.37	85.05	86.30	89.38	90.93	93.5	
Max.	100	100	100	100	99.9	100	100	99.8	99.8	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	100	
Min.	82.8	87.5	91.1	92.6	95	96.8	90.4	85.5	72.9	65.8	56	55.7	52.6	48.8	47.2	47.4	46	51.6	67.6	67.8	70.1	72.2	76.6	82.3	

Table 2.7 Temperature and relative humidity data for Lichtenburg during flowering.

Temperature (°C)																									Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	14.2	14.2	13.9	13.5	13.6	13.8	13.2	15.7	18.5	20.6	21.9	23	23.3	23.8	24.2	24.4	23.9	22.5	21	20.3	19.5	17.8	16.8	16.1	18.73
Day 2	15.8	16.2	16.4	16.3	16.2	16.2	15.9	16.2	17	18.2	19.2	20.4	21.4	22.3	22.8	23	22.8	21.4	19.9	19	18.1	17.7	17.5	17.4	18.63
Day 3	17.3	17.3	17.5	17.3	16.9	16.6	16.5	17	19.3	20.6	21.5	22.3	22.5	23.1	21	19.4	18.2	17.4	17.5	17.4	17.3	17.2	17.1	17.2	18.55
Day 4	17.3	17.4	17	16.8	16.7	16.6	16.2	16.8	18.4	19.8	20.3	21.3	22.5	22.2	23.2	23.8	22.7	21.3	19.8	18.8	17.9	17.3	16.9	16.6	19.06
Day 5	15.7	15.1	15	14.6	14.3	14.1	13.6	15.6	19.2	21.6	22.8	23.9	24.6	25.2	25.2	25.4	24.8	23	20.8	18.9	17.3	16.3	16.5	16.5	19.16
Day 6	15.7	14.8	14.3	14.2	13.9	13.1	13.6	16.4	19	20.7	21.9	23.6	23.6	24.7	24.9	25.1	24.2	22.7	21	20.2	19	18.7	18.6	16.6	19.18
Day 7	13.4	12.9	11.8	10.3	8.79	8.09	7.42	8.93	10.6	12.5	14.6	16.3	17.8	19.1	19.8	19.8	19.5	18	14.3	12.2	11.1	9.87	9.11	9.25	13.15
Day 8	9.76	9.14	8.84	8.9	9.17	7.51	7.2	10.8	14.3	17.1	19.7	21.5	22.8	23.8	24.3	24.6	24.1	21.9	18.7	15.6	14.3	13.4	12	11.6	15.45
Day 9	11.4	11.1	10.7	10.6	10.8	11.2	12.1	13.9	15.5	17.2	19.1	20.5	21.7	22.1	23.2	23.3	22.7	20.9	19.7	17.8	15.9	14.7	12.9	11.6	16.28
Ave.	14.5	14.2	13.9	13.6	13.4	13	12.8	14.6	16.9	18.7	20.1	21.4	22.2	22.9	23.2	23.2	22.5	21	19.2	17.8	16.7	15.9	15.3	14.8	
Max.	17.3	17.4	17.5	17.3	16.9	16.6	16.5	17	19.3	21.6	22.8	23.9	24.6	25.2	25.2	25.4	24.8	23	21	20.3	19.5	18.7	18.6	17.4	
Min.	9.76	9.14	8.84	8.9	8.79	7.51	7.2	8.93	10.6	12.5	14.6	16.3	17.8	19.1	19.8	19.4	18.2	17.4	14.3	12.2	11.1	9.87	9.11	9.25	
Ave (am)	15.6																								
Overall ave.	17.58																								
Relative Humidity (%)																									
Day 1	96.8	96.9	98.9	99.4	99.6	99.9	100	91.3	76.2	69.5	64.1	61.4	63.4	60.4	55.6	57.1	60.4	67.5	74.4	76	95.3	98.2	100	99.8	81.76
Day 2	100	100	99.7	100	99.7	97.7	97.4	95.8	97.1	92.3	86.2	74	73	70.7	65.5	65.3	68.6	85.5	90.6	93.8	92.8	95.8	96.1	96.4	88.92
Day 3	97.2	94.3	94	97	98.2	97.9	99	98.3	84.4	76.4	74.7	70.4	67.7	71.5	86.2	91.9	93.4	94.9	97.3	96.8	99.2	99.7	99.7	99.5	90.82
Day 4	99.9	99.6	99.9	99.8	99.8	99.7	99.7	96	89	87.5	83.8	75.8	75.4	76.6	67.8	65	69.2	76.3	87.8	96.1	98.7	99.3	100	100	89.28
Day 5	100	100	99.8	100	99.7	99.9	100	98.8	80.1	74.2	63.4	64	52.9	47.9	45.9	49.6	58.2	70.2	80.3	83.9	83.2	80.1	75.9	74.5	78.45
Day 6	81.3	86.1	81.9	85.5	90.4	94.2	96.4	96.7	84.8	78.8	69.1	62.9	60.3	54.9	52.3	49.2	58.9	69.5	75.4	86.1	87.8	86.5	81.2	84.8	77.29
Day 7	83.9	79.5	66.2	72.9	83.3	86.4	85.5	73.3	71	58.9	48.6	42.7	40.2	32.7	32.5	35.8	34.9	44.3	57	64	70.9	80	79	73.5	62.37
Day 8	72.6	75.8	74.5	73.9	75.5	84.1	74.8	61.6	51	44	36.2	32.9	28.9	27.3	28.9	28.4	30.9	42.8	59.9	72.9	78.1	81.6	86.8	84.7	58.67
Day 9	88.7	87.7	93.8	92.1	89.7	97.6	99.5	98.4	85.6	75.3	66.4	57.2	56.8	48.2	44.7	43.2	48	57.7	66.5	74.2	80.5	83.9	90.4	92.8	75.78
Ave.	91.2	91.1	89.8	91.2	92.9	95.3	94.8	90	79.9	73	65.8	60.1	57.6	54.5	53.3	53.9	58	67.6	76.6	82.6	87.4	89.5	89.9	89.6	
Max.	100	100	99.9	100	99.8	99.9	100	98.8	97.1	92.3	86.2	75.8	75.4	76.6	86.2	91.9	93.4	94.9	97.3	96.8	99.2	99.7	100	100	
Min.	72.6	75.8	66.2	72.9	75.5	84.1	74.8	61.6	51	44	36.2	32.9	28.9	27.3	28.9	28.4	30.9	42.8	57	64	70.9	80	75.9	73.5	

Table 2.8 Wind speed and wind direction data for Delmas during flowering.

Wind speed (m/s)																									Daily average
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	0.39	0.3	0.2	0.2	0.2	0.22	0.2	0.28	2.63	2.3	1.92	2.04	1.48	1.55	1.78	2.74	3.53	1.78	0.82	0.24	0.36	0.25	0.39	0.29	1.09
Day 2	0.38	0.81	0.29	0.35	0.47	0.38	0.28	0.74	2.55	3.2	3.28	3.12	2.27	2.31	1.93	1.65	0.9	0.37	0.2	0.2	0.2	0.24	0.22	0.83	1.13
Day 3	0.5	0.33	0.31	0.72	0.3	0.23	0.32	0.91	1.42	1.01	1.18	1.86	2.08	1.28	2	2.2	3.96	2.93	2.42	1.29	0.74	0.2	0.2	0.2	1.19
Day 4	0.41	0.22	0.39	0.2	0.41	0.23	0.2	1.27	1.47	1.7	2.75	2.74	2.16	2.22	2.18	1.7	2.05	1.96	0.68	0.2	0.2	0.2	0.2	0.2	1.08
Day 5	0.21	0.2	0.2	0.29	0.2	0.2	0.2	0.4	2.03	2.53	2.17	1.1	1.84	2.27	1.54	1.96	2.01	1.45	0.31	0.2	0.2	0.21	0.26	1.04	0.96
Day 6	0.2	0.28	0.26	0.36	0.76	0.4	0.98	2.56	3	2.48	1.74	1.94	1.59	1.78	1.7	3.07	1.93	1.07	0.26	0.22	0.26	0.23	0.2	0.2	1.14
Day 7	0.2	0.27	0.21	0.2	0.2	0.2	0.2	0.69	2.21	1.94	2.15	1.78	1.65	1.36	1.21	0.92	1.11	0.5	2.16	1.55	1.6	1.44	1.61	0.32	1.07
Day 8	0.27	0.62	0.4	0.39	0.6	0.52	1.38	1.57	1.74	1.41	2.03	1.88	1.5	1.4	1.43	1.47	1.37	1.34	1.53	0.65	1.02	1.05	2.36	2.41	1.26
Day 9	2.3	1.86	1.73	1.46	1.48	1	1.74	1.96	2.21	2.06	1.53	0.91	0.95	1.03	0.76	0.54	0.2	0.33	0.2	0.2	0.2	0.22	0.22	0.62	1.07
Ave.	0.54	0.54	0.44	0.46	0.51	0.38	0.61	1.15	2.14	2.07	2.08	1.93	1.72	1.69	1.61	1.81	1.90	1.30	0.95	0.53	0.53	0.45	0.63	0.68	
Max.	2.3	1.86	1.73	1.46	1.48	1	1.74	2.56	3	3.2	3.28	3.12	2.27	2.31	2.18	3.07	3.96	2.93	2.42	1.55	1.6	1.44	2.36	2.41	
Min.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.28	1.42	1.01	1.18	0.91	0.95	1.03	0.76	0.54	0.2	0.33	0.2	0.2	0.2	0.2	0.2	0.2	
Wind Direction*																									
Day 1	ESE	SE	SE	SE	SE	SE	ESE	NNE	N	NE	NNE	NNE	NNE	NNE	NE	NNE	NE	NE	ENE	E	ESE	SE	SE	ESE	
Day 2	E	ESE	ESE	ESE	SE	ENE	E	NNE	NNE	NNE	NNE	NNE	NNE	NNE	N	N	NNW	WNW	SW	SSE	S	SE	SSE	SSE	
Day 3	NE	N	SW	SE	SSE	E	SE	E	ENE	N	NNE	NE	NNE	NE	SE	ESE	E	ENE	ENE	E	E	ESE	ESE	SE	
Day 4	SE	SE	ESE	SE	SE	ESE	SE	ESE	ENE	NE	NNE	NNE	NNE	NNE	N	NE	NE	NNE	NNE	E	NW	N	NW	N	
Day 5	ENE	E	N	N	SSE	WNW	W	NE	NE	N	NNE	NNE	ENE	NE	ENE	NNE	NE	NNE	ENE	NW	NW	NNE	N	N	
Day 6	N	NNW	NNW	NW	NNW	N	N	N	N	N	NNW	N	NNW	NNW	NW	N	NNW	NNW	NW	NW	W	W	WSW	WSW	
Day 7	WSW	WNW	N	NW	SSW	SE	ESE	N	NNW	NNW	NNW	NW	NW	NW	NW	N	NW	SSW	SSW	SSW	NE	ESE	SE	SSE	
Day 8	SSE	SSE	SSE	SSE	SE	SE	SE	ESE	ESE	E	ESE	E	NE	ENE	ENE	E	E	ENE	E	E	ESE	E	E	E	
Day 9	E	ESE	ESE	ESE	ESE	ESE	ESE	E	E	E	E	E	E	E	ESE	ESE	E	WSW	NE	ESE	ESE	ENE	ENE	ENE	

* Direction from which wind is blowing

Table 2.9 Wind speed and direction data for Lichtenburg during flowering.

Wind speed (m/s)																									Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	1.24	1.73	1.44	1.48	1.46	1.72	1.4	2.56	4.11	4.46	4.16	3.84	3.71	3.32	2.79	2.78	2.99	2.46	1.66	1.6	2.22	2.92	1.07	1.42	2.44
Day 2	1.41	0.46	0.88	1.2	0.98	1.16	0.56	1.93	2.2	2.19	1.61	1.13	0.95	1.49	1.44	1.13	1.03	1.78	1.44	1.67	1.3	0.74	0.66	0.7	1.25
Day 3	0.31	0.36	0.62	0.53	0.38	0.94	0.52	0.7	1	0.8	0.92	0.6	0.86	1.66	1.68	2.93	3.03	2.06	0.69	1.11	2.61	1.12	0.77	0.58	1.12
Day 4	0.35	0.28	0.34	0.51	1.22	2.09	2.38	2.79	4.1	3.71	2.88	2.63	3	2.24	2.43	1.59	1.41	0.67	0.35	0.22	0.46	0.39	0.48	0.2	1.53
Day 5	0.2	0.31	0.2	0.24	0.33	0.31	0.71	1.14	1.99	2.33	1.86	1.56	1.04	1.52	1.11	0.89	0.97	0.34	0.26	0.2	0.2	0.34	0.2	0.2	0.77
Day 6	0.21	0.2	0.2	0.2	0.2	0.2	0.2	0.21	0.97	2.02	3.08	2.61	2.6	2.6	2.58	2.62	2.04	0.87	0.3	0.29	0.2	0.51	1.59	4.32	1.28
Day 7	4.85	4.7	4.61	4.5	2.89	2.25	2.29	3.27	3.94	3.4	2.98	3.18	3.55	3.82	3.74	3.63	3.42	2.88	1.68	1.7	1.57	1.18	1.37	2.11	3.06
Day 8	2.93	2.98	3.14	3.26	3.12	2.04	1.56	2.59	2.98	3.5	3.58	3.12	3.05	2.45	1.7	1.48	1.49	1.54	1.2	0.33	0.2	0.2	0.2	0.2	2.04
Day 9	0.2	0.2	0.2	0.84	1.86	1.55	2.12	2.72	3.02	3.53	2.95	2.25	2.6	1.92	2.51	2.7	2.2	1	0.28	0.22	0.21	0.54	0.26	0.2	1.50
Ave.	1.3	1.25	1.29	1.42	1.38	1.36	1.30	1.99	2.70	2.88	2.67	2.32	2.37	2.34	2.22	2.19	2.06	1.51	0.87	0.82	1.00	0.88	0.73	1.10	
Max.	4.85	4.7	4.61	4.5	3.12	2.25	2.38	3.27	4.11	4.46	4.16	3.84	3.71	3.82	3.74	3.63	3.42	2.88	1.68	1.7	2.61	2.92	1.59	4.32	
Min.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.21	0.97	0.8	0.92	0.6	0.86	1.49	1.11	0.89	0.97	0.34	0.26	0.2	0.2	0.2	0.2	0.2	
Wind direction*																									
Day 1	NE	ENE	NE	NE	NE	NE	NE	NNE	NNE	NNE	N	N	N	N	N	N	NNE	NNE	NNE	NNE	WNW	W	NW	N	
Day 2	NNE	NNE	NE	NNE	NNE	NNE	NE	NE	NE	NNE	NE	NNE	N	NNE	NNE	N	NNE	E	NE	NE	NE	NE	NE	NE	
Day 3	NE	NE	NNE	SSE	SSW	ENE	SSW	ESE	ESE	S	SE	SSW	NE	E	ENE	ENE	NNE	NNE	NNE	SE	SSE	SE	E	NE	
Day 4	E	ENE	ENE	E	ESE	E	E	ENE	ENE	NE	NE	NNE	NNE	N	N	NNW	SSE	SSE	ENE	ENE	NE	NE	NE	NE	
Day 5	ENE	E	E	ESE	E	E	ENE	E	ENE	E	E	E	ESE	SE	ESE	SE	SE	ESE	ESE	ESE	ESE	N	NNW	N	
Day 6	NNE	NNE	NNE	NNE	NNE	NE	NE	NNW	WNW	WNW	WNW	WNW	NW	WNW	WNW	WNW	WNW	WNW	WNW	NW	WNW	W	W	SSW	
Day 7	SSW	S	S	S	S	SSE	SSE	SE	SSE	SSE	SSE	SSW	SSW	SSW	SSW	SSW	SSW	SSW	SSW	S	S	S	S	SSE	
Day 8	S	S	SSE	SSE	SSE	SE	SE	SE	ESE	ESE	ESE	E	E	E	SE	ENE	ENE	ENE	NE	NE	SE	E	E	E	
Day 9	E	E	E	ESE	ENE	NE	NNE	NNE	NNE	NNE	NE	NE	ENE	ESE	ENE	NE	ENE	ENE	NNE	NNE	NE	NE	ENE	E	

* Direction from which wind is blowing

Figure 2.1 Photograph of maize pollen under 100X magnification.

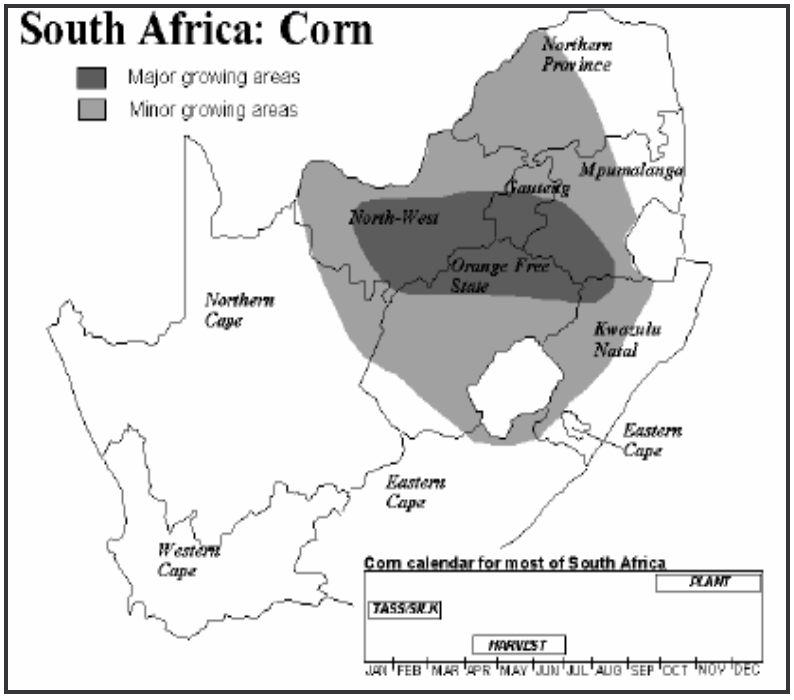


Figure 2.2 Map of South Africa showing the maize planting regions as well as a calendar for planting, flowering and harvesting (Rutter, 2003).

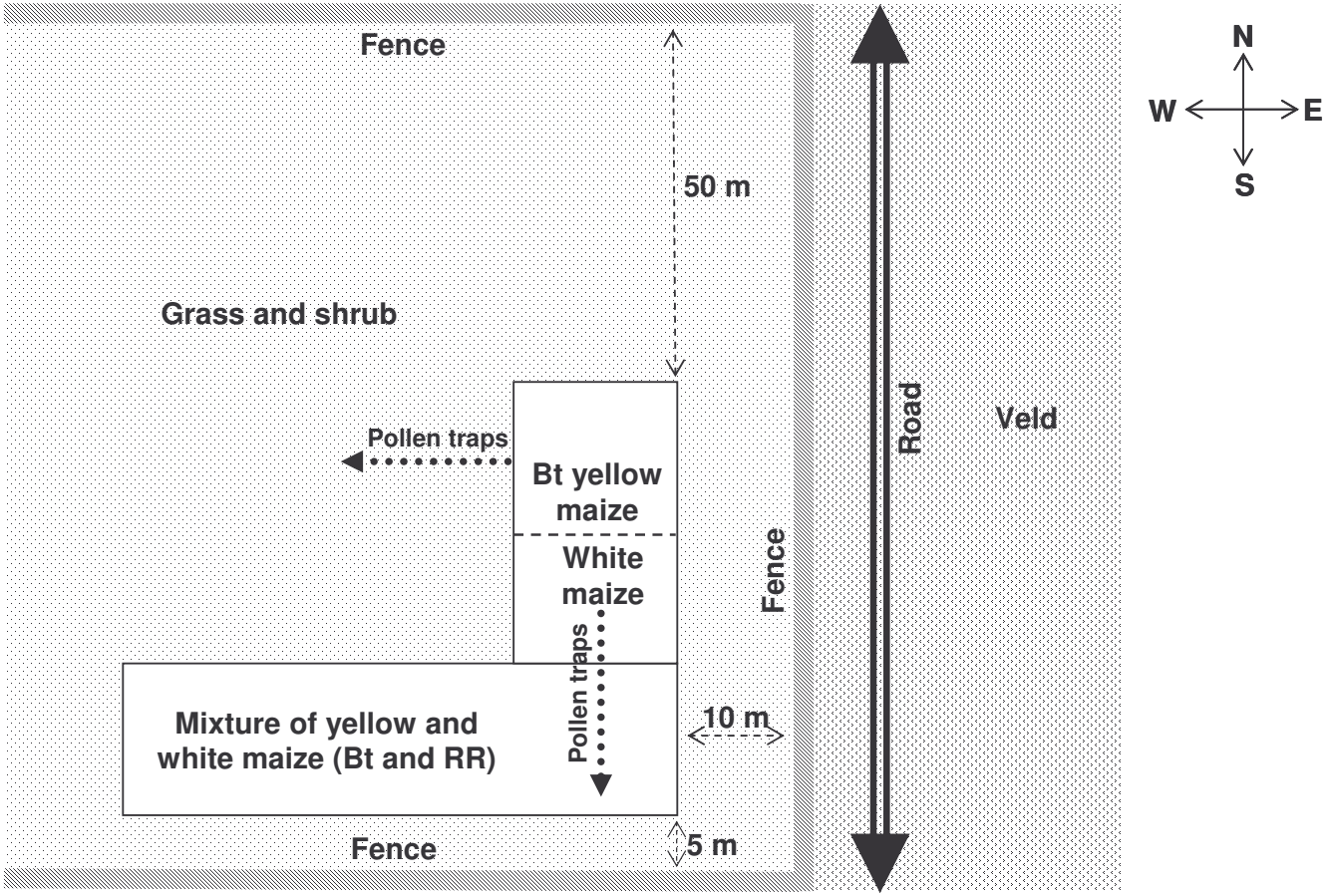


Figure 2.3 Schematic layout of field trial in Delmas; rows were planted parallel to the dotted line.

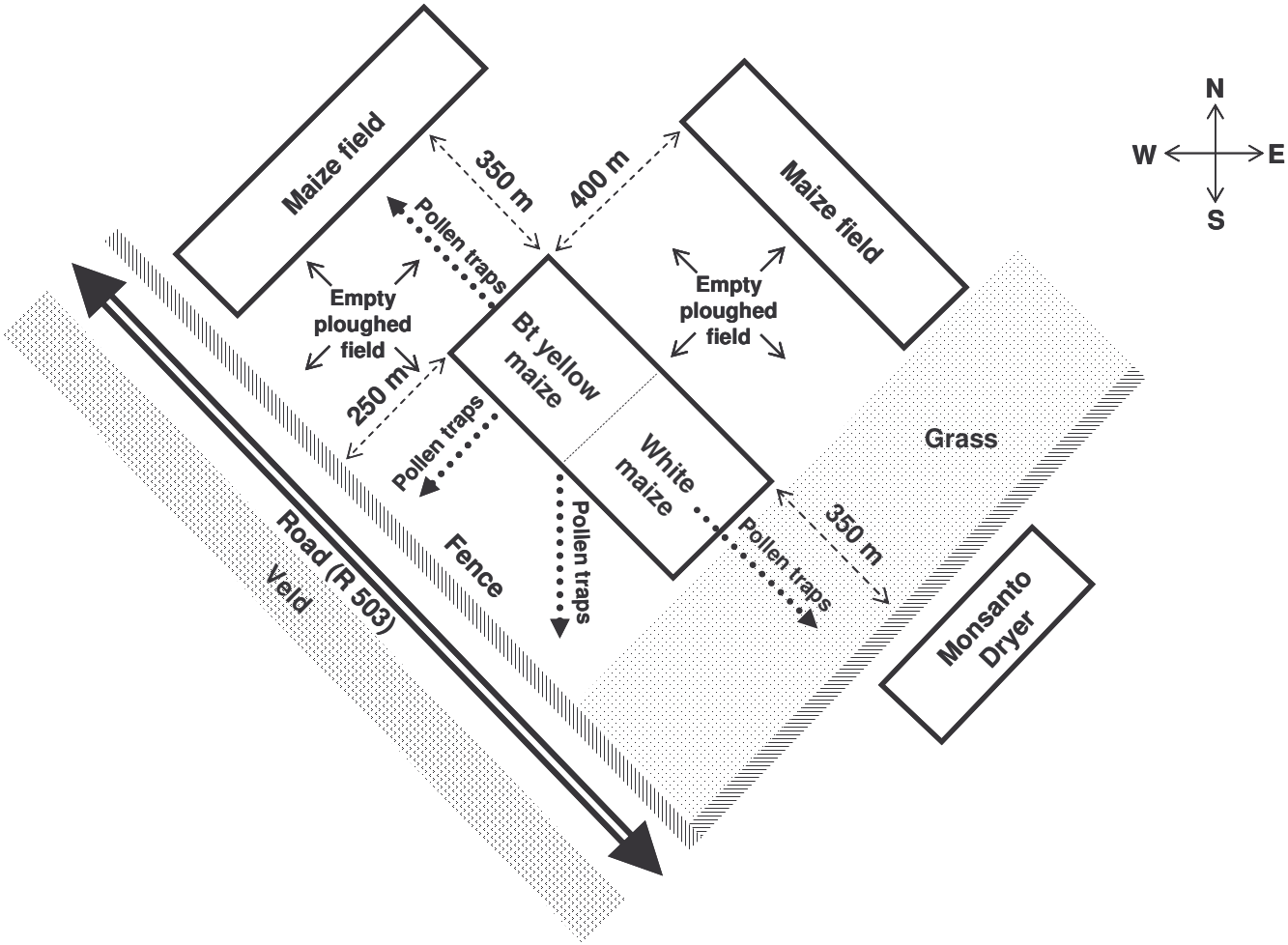


Figure 2.4 Schematic layout of field trial in Lichtenburg; rows were planted parallel to the dotted line.

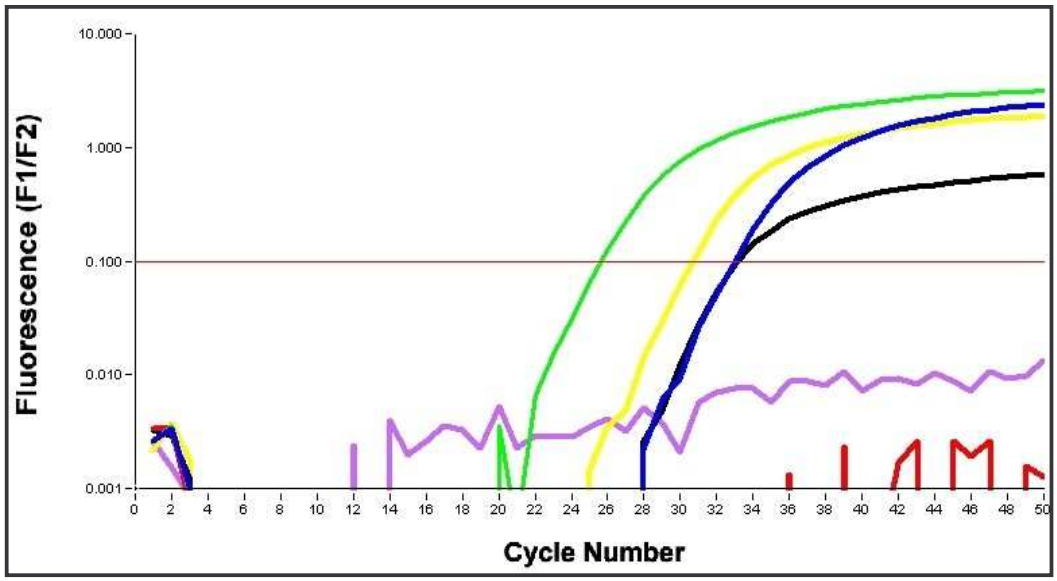
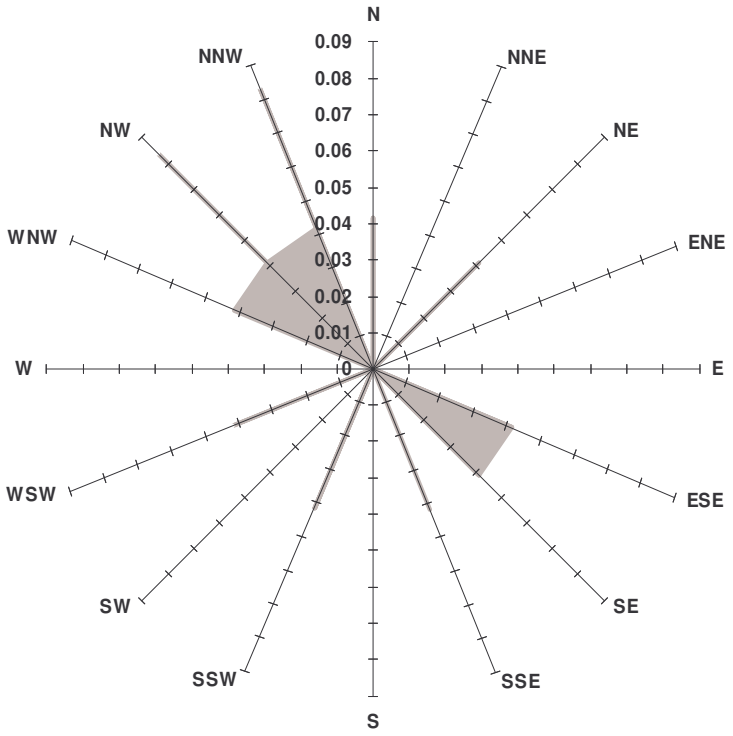


Figure 2.5 Real-Time PCR amplification of DNA extracted from Bt pollen stored in CTAB buffer for the maize species-specific (—) as well as positive (—) and negative control (—) and for the 35S (—) detection with the relevant positive (—) and negative (—) control.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ENE
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ESE
SE
SSE
S
SSW
SW
WSW
W
WNW
NW
NNW
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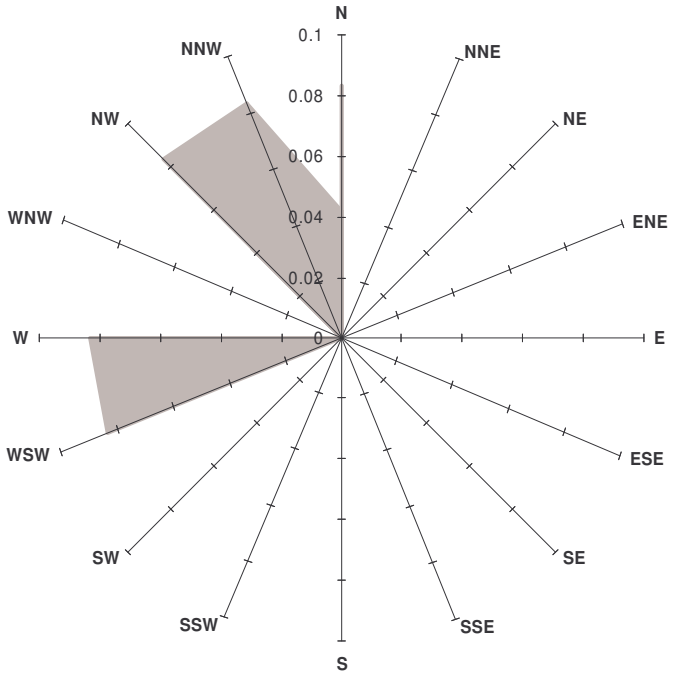
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0

Day 2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SSE
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NW
NNW
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0.08
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0.04
0.02
0

Day 1

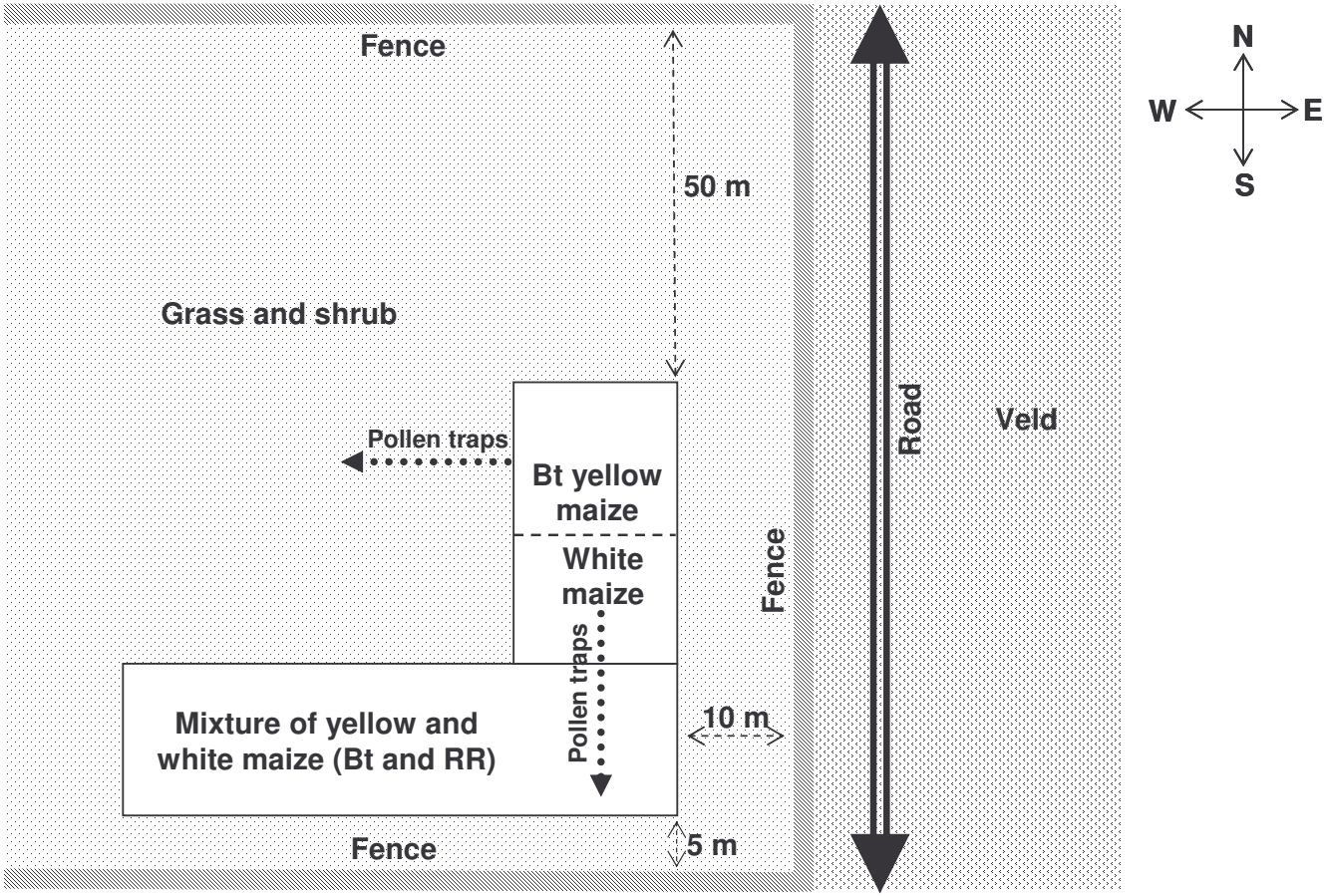
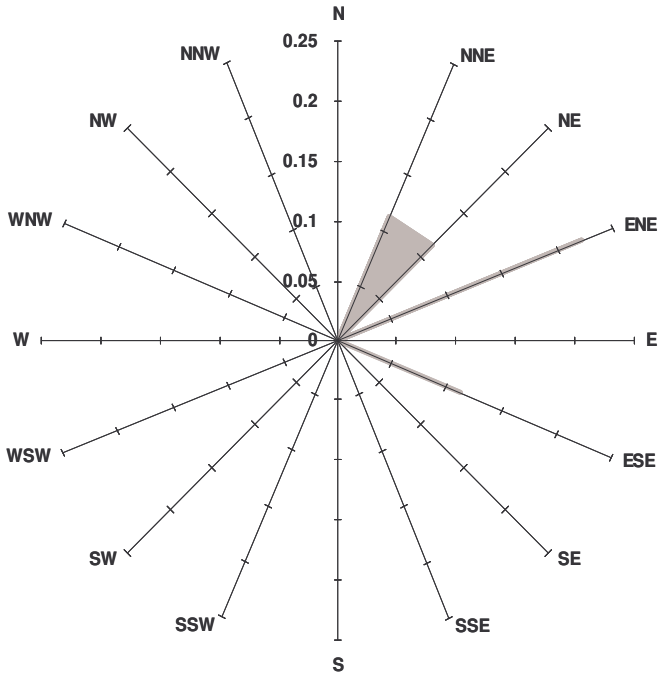


Figure 2.6 Wind rose for day 1 and 2 at Delmas.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0.15
0.1
0.05
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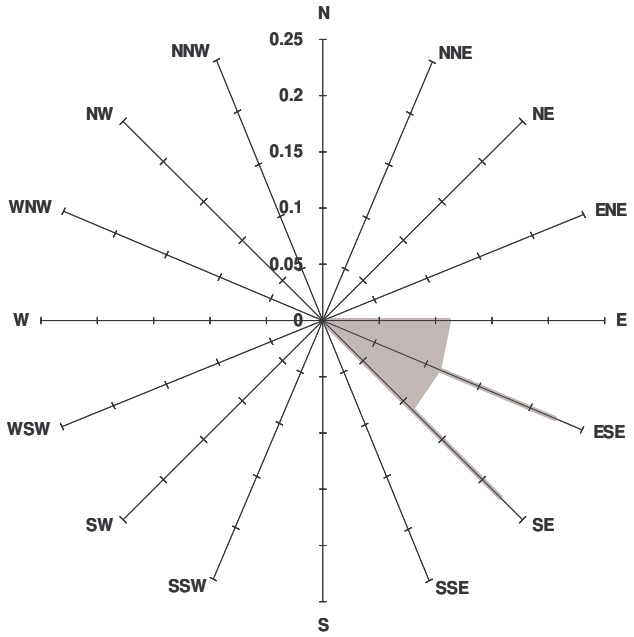
Day 7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ESE
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NW
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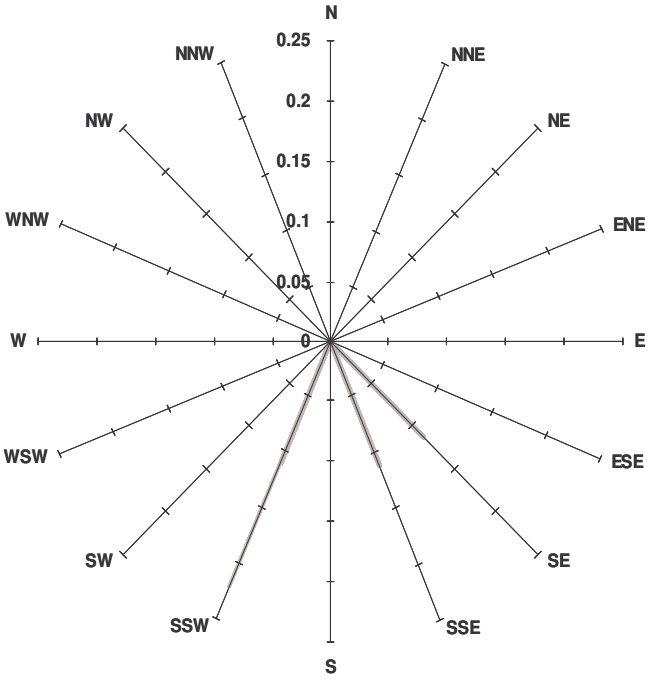
0.05
0

Day 6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ENE
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ESE
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SSW
SW
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W
WNW
NW
NNW
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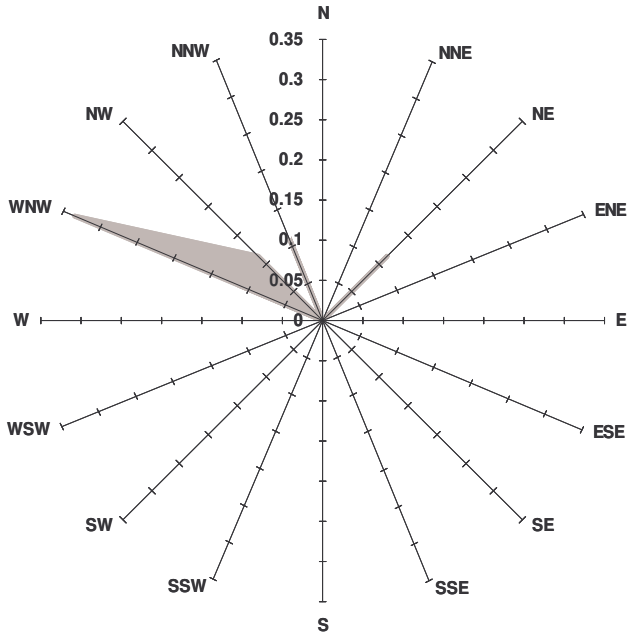
0.2
0.15
0.1
0.05
0

Day 5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SSW
SW
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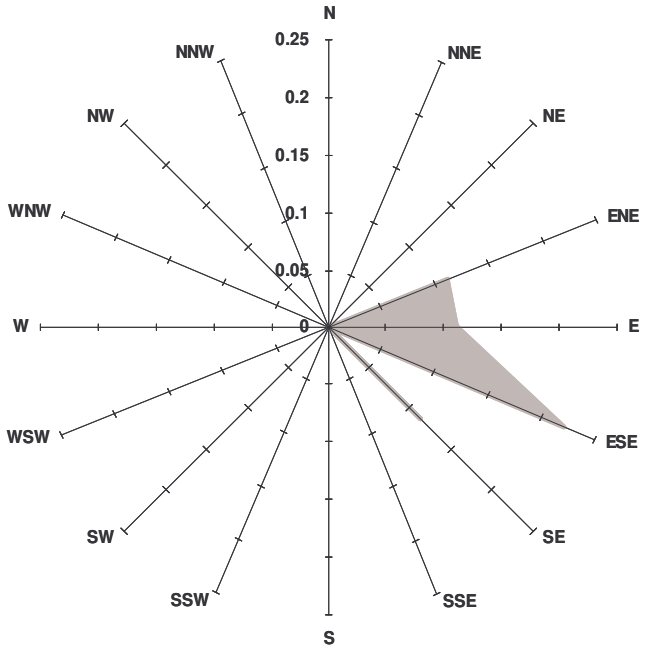
Day 4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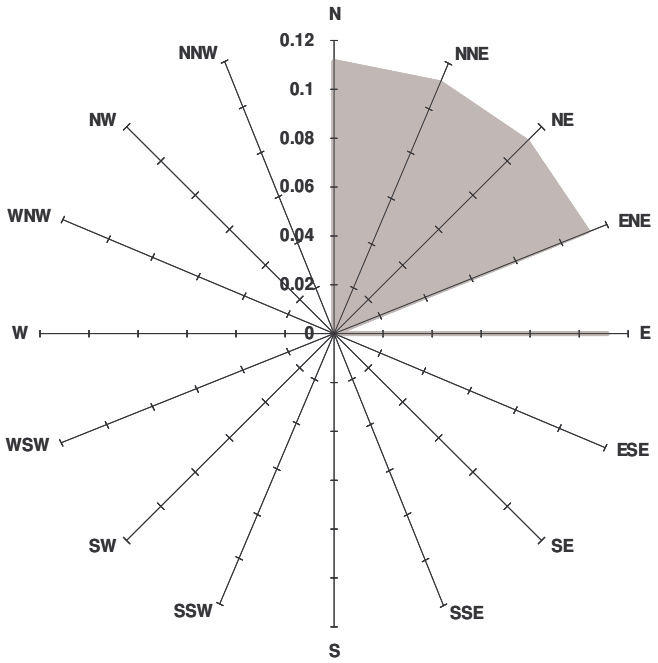
Day 3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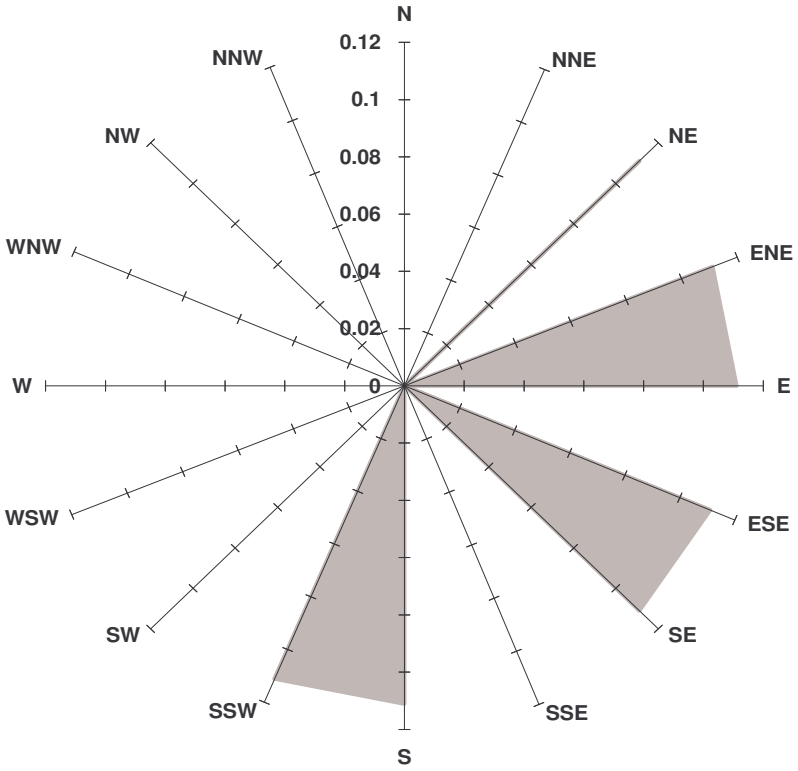
Day 2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Day 1

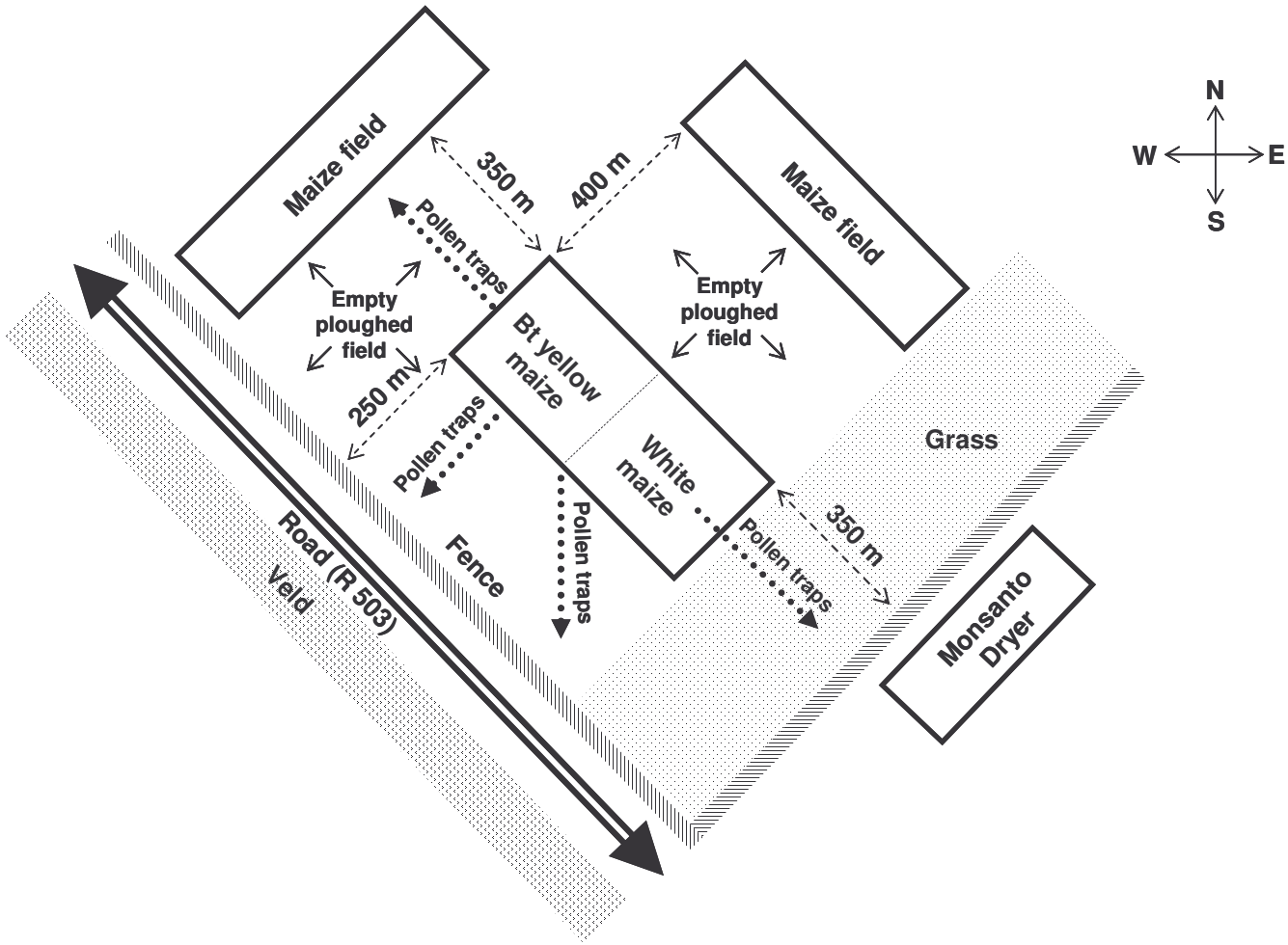
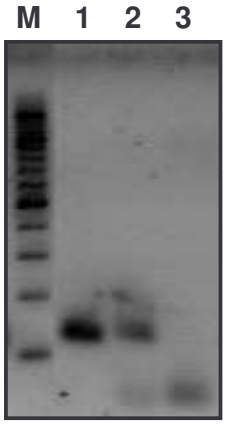


Figure 2.7 Wind rose for days 1 to 7 at Lichtenburg.



Lane: M Size standard molecular marker
1 Positive control
2 Sample DNA
3 Negative control

Figure 2.8 Gel electrophoresis indicating PCR amplification of pollen DNA (lane 2).

CHAPTER 3

The impact of pollen movement on Identity Preservation (IP) of maize

INTRODUCTION

Maize is one of the world's most important crops based on production volume and area cultivated (Pingali, 2001). In 1995, the global maize demand was 558 tons and is expected to increase to 837 tons by 2020. In the developing world, maize requirements are likely to increase from 282 million tons in 1995 to 558 million tons in 2020 (Pingali, 2001). Maize is a staple food in many third world countries (Maredia *et al*., 2000; Pingali, 2001). Population coupled with increasing poverty in Sub-Saharan Africa has increased the demand for maize as food. Thus sustainable maize production in Africa has become imperative and depends upon mass seed production (Altman, 1999). One of the most important aspects of using maize as food is the availability of adequate seed. Therefore, for maize production to succeed in Africa seed must be made available and affordable as well as meet the demands of a growing population (Macilwain, 1999).

Maize seed production, as with most other crops, is different in developing and developed countries. Although there are notable exceptions, seed production in Africa is not commercially driven and informal/rural farmers retain part of their crop for subsequent planting seasons (Tripp and Rorhbach, 2001). In contrast to this,

most if not all first world seed production is commercially and technology intensive (Wisniewski *et al*., 2002). Due to competitive markets, seed companies are obliged to maintain certain agronomic and quality traits in commercial varieties to satisfy the needs of farmers. To do this, specific practises have to be implemented to ensure that the genotypic integrity of a particular line or variety is maintained (Lamkey, 2002). This is done through IP.

IP is defined as a system of production, handling and marketing practice that maintains the genetic purity and integrity of an agricultural commodity (Sundstrom *et al*., 2002). An IP system consists of different components of specific practise including crop production, handling, processing, transport and storage (Sundstrom *et al*., 2002). Although aspects of IP have long existed to maintain the varietal integrity and quality of agronomic crops, it is only since the commercialization of seed production that it has gained specific definition. Commercial seed production linked to specific agronomic traits or first generation GMOs such as insect-resistant and herbicide tolerant maize has increased the need for IP. Documentation authenticating the different steps of an IP system is necessary to ensure correct implementation and maintenance of IP (Brookes, 2002). However, one potential source of adventitious co-mingling in an IP system over which there is less control and uncertainty is through pollen movement. Therefore, gene flow is the principal factor in IP of maize.

IP regulatory stipulations depend upon factors that are under the control of an IP system and those that cannot be controlled but for which measures can be taken to

minimize its effect. Field tillage to prevent volunteer plants and the use of clean harvesting, transport and storage equipment are controllable factors. Pollen movement on the other hand is difficult to control due to the influence of environmental conditions. Spatial and temporal isolation are measures in IP systems used to minimise the potential cross-hybridisation via pollen movement.

One of the major challenges in maintaining an IP system is the potential cross-hybridisation between different maize varieties affecting quality, genetic integrity and more seriously providing consumers with a compromised product. (Smyth *et al*., 2002) Thus seed production is the most important stage in IP, as genetic purity is the backbone of an IP system and the maintenance thereof is essential. The introduction of adventitious co-mingling threatens the stability of IP systems and the reputation of seed companies. Hence pollination is the most important consideration for genetic purity in seed production.

Maize is a wind pollinated species and it has been estimated that approximately 14 to 50 million pollen grains per average-sized plant are produced during flowering (Miller, 1985). So, an average sized maize plant can produce enough pollen to produce approximately 240 thousand seeds (Miller, 1985).

To date, pollination studies have determined that maize pollen can effect pollination up to 650 m from its source (Henry *et al*., 2003). According to published data, the reported incidence of co-mingling decreases over distance (Luna *et al*., 2001; Burris, 2001; Jemison and Vayda, 2001; Henry *et al*., 2003). In a recent study by

Ma *et al*. (2004), it was found that factors other than distance also affect pollination, suggesting that site specific environmental conditions play an important role in pollination potential. Luna *et al.* (2001), found that environmental conditions play an important role in pollen longevity and hence its viability. They determined that maize pollen remained viable for between 1 to 2 hours at a temperature of 28℃ to 30℃ and a relative humidity > 53%. Aylor (2003) reported that maize pollen remained viable at 23℃ and 50% relative humidity for 60 min. and that viability increased with increasing levels of relative humidity. Furthermore, the potential wind dispersal for maize pollen over distance is considerable (Treu and Emberlin, 2000). A wind speed of 1 m/s is sufficient to disperse a single pollen grain approximately 3600 m (3.6 km) in 1 hour. The potential for vertical pollen movement is considerable and Brunet *et al*. (2004) captured maize pollen at 800 to 2000 m above ground. These studies corroborate that pollen has the potential to move over great distances, and would thus have a great impact on IP regulations. Although the potential for pollen to move over vast distances is great, it would appear that the actual pollination potential is much lower than expected. Luna *et al*. (2001) determined that pollen recombination could occur up to 200 m. Furthermore, Henry *et al*. (2003) found that maize pollen can be wind dispersed up to 650 m.

Various authoritative bodies such as the OECD (Organisation for the Economic Co-operation and Development) and the AOSCA (Association of Official Seed Certifying Agencies) have recommended regulations in IP systems to minimise pollen movement. The OECD, comprised of 30 member countries, recommends

an isolation distance of 200 m for maize. The AOSCA which is comprised of various seed certification bodies recommends a 201 m isolation distance for maize (Glover, 2002). The isolation distances recommended by seed certification authorities such as the OECD (OECD, 2001) and the AOSCA (Glover, 2002) are in the range of 200 m. From previous studies it is obvious that the potential for gene flow though pollen dissemination is much greater than the minimum average distance required by most seed certification schemes.

In conclusion, the production of maize will become increasingly important in future to maintain quality, agronomic and other traits in a modern biotechnology environment through the use of identity preservation. An IP system is only as effective as the understanding of the different factors it aims to control. The pollination potential relies on pollen movement and pollen longevity, both of which are dependant on the environment. To date no studies on pollen movement under South African conditions have been published. The aim of this study was to use a combination of phenotypic and genotypic markers to detect pollen recombination at varying distances from a pollen source The varieties used in this study were CRN 3505 (white maize) and CRN 4760 B (yellow insect resistant maize) (Monsanto, South Africa). PCR analysis was used to confirm phenotypic recombination and its absence. Trials were planted at two maize breeding regions in South Africa viz., Delmas (Mpumalanga) and Lichtenburg (North West Province).

MATERIALS AND METHODS

Trial layout

Maize fields were planted at two maize breeding regions in South Africa, Delmas in Mpumalanga and Lichtenburg in the North-West Province. There was no significant slope at the two locations. The same varieties were used for both locations. A separate unrelated field trial consisting of a mixture of yellow and white maize, Bt and Roundup, was planted at Delmas, four weeks prior to the study trial. CRN 3505 (white) and CRN 4760 B (yellow) maize varieties (Monsanto, South Africa) were planted at least 4 weeks after previous maize plantings at both locations. The field positioning was based on prevailing wind patterns for that area (personal communication with plant breeders from each area (Table 3.1) (Fig. 3.1 and 3.2).

Trial evaluations

The trials were evaluated for phenotypic hybridization events after the cobs were approximately 80 to 90% dry. The cobs in the white maize plot were checked for visible out-crossing, indicated by yellow seed. Due to the difference in plot size, the trials at Delmas and Lichtenburg were assessed differently. For the Delmas trial, the top most cob of every plant in the trial was assessed. At Lichtenburg the white maize plot was divided, into 12 transects, starting 4 m from the outer row with approximately 4.9 m between transects, with the first of every alternate row along each transect being evaluated. For Delmas, all cob losses due to disease, theft or damage by birds, as well as plants that did not produce cobs were noted as

missing data. For Lichtenburg, if cob loss or damage was observed, the cob from an adjacent plant in each row was assessed.

Phenotypic data analysis

Statistical analysis was performed on phenotypic data for both areas. Yellow seed counts were statistically analyzed using Excel (Windows XP). The minimum, maximum, standard deviation, variance and the means were determined. Each yellow seed count, mean and standard deviation was transformed to proportion values using the formula (Table 3.2 and 3.3):

Proportion value = (Number of yellow seeds)/Average number of seeds per cob

Where,

Average number of seeds per cob = $\sum$ (total no. of seeds for 10 randomly selected cobs)/10.

These data were plotted with distance as the dependent variable (x-axis) against the percentage recombination as the independent variable (y-axis). F-tests (to test for differences in variances) and T-tests (to test whether the different means were significantly different) were performed to compare the two areas. Selected distances in each region were selected for comparison (Table 3.4). The α was set at 0.05 for all the tests performed. Regression analysis was performed on the transformed data using Excel (Windows XP). The regression analysis was performed using linear and non-linear functions.

Random sampling for PCR analysis

The fields were divided into distance intervals (Table 3.5), 10 cobs were randomly selected per distance interval. Two cobs from each distance interval were de-seeded and yellow and white seeds carefully separated and homogenized using a Waring Blender for DNA extraction and PCR analysis.

Weather data and analysis

Weather data was obtained from the ARC, Agro-meteorology Department for Delmas (Monsanto-Petit) and Lichtenburg (Sheila Co-op). The weather data was obtained hourly for nine days during the flowering period for temperature (°C), relative humidity (%), wind speed (m/s) and wind direction during flowering.

The weather data was categorised into the number of hours of high (>70%) and low (<70%), relative humidity and for temperature (high: >20°C and low: <20°C) as well as relevant (in the direction that would effect cross-pollination favouring the conventional field) and non-relevant wind directions. Chi-tests were performed on the weather data. Chi-tests were also performed on the hours of relevant wind directions categorised into high (>2 m/s) and low (<2 m/s) speed.

DNA Extraction and Purification

DNA extractions were performed in duplicate on 2 g of the parental lines, as well as interval A for Delmas, and interval A and B for Lichtenburg. CTAB extraction buffer 10 ml was added to each 2 g sample. Proteinase K, 30 µl [20 mg/ml] was added to the sample and incubated at 60°C for 2 hours, the centrifuge tubes were

periodically inverted. The samples were centrifuged at 4.5k rpm for 5 min. The supernatant was decanted and incubated at 80°C for 5 min. RNAse, 5 µl [10 mg/ml] was added and the samples and incubated at 60°C for 5 min. Chloroform-isoamyl alcohol (24:1) was added in a 1:1 ratio and centrifuged for 5 min at 13k rpm, the aqueous layer was retained and this step repeated. Absolute ethanol was added to the final aqueous layer in a 2:1 ratio and the sample precipitated for 1 hour on ice or overnight at 4°C. After centrifugation at 13k rpm for 10 min., the DNA pellet was washed with 5 ml 75% ethanol by centrifugation at 13k rpm for 5 min. (repeated twice). Thereafter the pellet was re-dissolved in 100 µl 0.1 X TE buffer [0.25 mM Tris, 2.5 mM EDTA] with intermittent periods in a 60°C water-bath and mixing with an automatic pipette.

A GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), was used to purify the extracted DNA. Capture buffer (500 µl) was added to the dissolved DNA and mixed. A micro-spin was placed in a 2 ml collection tube. The DNA and Capture buffer mixture was added to the column. The tube was centrifuged at 14k rpm for 30 sec. The flow-through was discarded and Wash buffer (500 µl) was added to the column, the tube was again centrifuged at 14k rpm for 30 sec. The collection tube was discarded and the column transferred to a 1.5 ml micro-centrifuge tube. 0.1X TE buffer (30 µl for pollen DNA and 100 µl for seed DNA) was added to the column and allowed to incubate at room temperature for approximately 1 min. then centrifuged at full speed for 1 min.

Qualitative PCR and Qualitative Real-Time PCR analysis

PCR was performed on extracted pollen and seed DNA. Real-time PCR was used to confirm negative results and quantify the presence of transgene. For PCR analysis, an ABI 2700 was used. A master mix of 19.9 µl 35S PCR buffer (GeneScan, GmbH) and 0.16 µl Ampli-Taq Gold per PCR reaction including negative and positive control was made. Each reaction contained 5 µl of sample DNA or 5 µl 0.1X T.E buffer for the negative control and 5 ul 1% Bt 176 DNA for the positive control. The PCR parameters were 95^{o}C for 10 min (1 cycle), 95^{o}C for 25 sec, 62^{o}C for 30 sec, 72^{o}C for 45 sec (50 cycles), 72^{o}C for 7 min and 25^{o}C (1 cycle). PCR amplification of the 35S promoter sequence was confirmed using a 2% Agarose (Molecular Screening) gel run at 270 V for 20 to 25 min. and then visualised under UV light.

Real-Time PCR was performed using the 35S Screen Corn DNA Quantification System (GeneScan GmbH) on the LightCycler™ (Roche). To each capillary, 15 µl of Reference Master Mix (containing dNTPs, primer for HMG detection, *Taq* polymerase, $MgCl_2$ and probes) or 15 µl of GM Mastermix (containing dNTPs, primer for 35S detection, *Taq* polymerase, $MgCl_2$ and probes) (GeneScan, GmbH), was added to each capillary, respectively, in duplicate. An additional 5 µl sterile double distilled water was added to the negative control and 5 µl 1% Bt 176 Corn Positive Control was added, respectively. For Real-time reactions on extracted DNA, 5 µl of the DNA extract (1:50 dilution) was added. Cycling conditions for the Real-time PCR were, 1 cycle at 95ºC for 60 sec., 50 cycles at 95ºC for 5 sec. and 60ºC for 25 sec. and 1 cycle at 40ºC for 30 sec.

Quantitative Real-time PCR

For the quantification of the transgene present, the 35S Screen Corn DNA Quantification System (GeneScan, GmbH) was used as described above except for the inclusion of calibration standards for an endogenous reference gene and the 35S promoter sequence. A standard curve was plotted using the C_T values of the calibration standards against the log of the amount of the copy number standards. The amount of transgene present in the sample was determined using the standard curve.

RESULTS

Field trial phenology

The flowering period coincided with silk production at both trial locations for white and yellow maize, respectively (Table 3.1). Plants at both locations were uniform in growth pattern and size. The average height of plants was 1.7 m and 1.9 m at Delmas and Lichtenburg, respectively. Cob sizes differed, with smaller cobs observed in Delmas (378 seeds per cob) than at Lichtenburg (544 seed per cob) (Table 3.6).

Field trial evaluation

The level of cross fertilization varied between the different geographic locations (Fig 3.3, 3.4, 3.5 and 3.6). The mean percentage of hybridisation in the first adjoining row of white maize was 15.2% for Delmas and 39.1% for Lichtenburg, with a decline up to 22.5 m (maximum distance of white maize at Delmas) and 20.4

m, respectively. The mean out-crossing per row after 25 m at Lichtenburg averaged 0.36%, with 0.14% at 81.6 m (Table 3.2 and 3.3). The mean percentage out-crossing over distance for Delmas and Lichtenburg, using an exponential function was R^2 = 0.91 and 0.93 (Fig 3.3 and 3.4) (Table 3.4) and for the linear function was R^2 = 0.72 and 0.36, respectively.

According to the exponential equation, the estimated zero (0.0001%) mean percentage out-crossing would have occurred at 128 m for Delmas and at 110 m for Lichtenburg. The variance in percentage means between Delmas and Lichtenburg were statistically similar up to a distance of 18.70 m and 18.75 m, respectively, with the exception of the first row (Table 3.4).

The regression analysis of the mean percentage out-crossing over distance was R^2 = 0.73 ($P = 4.9 \times 10^{-13}$) for Delmas and R^2 = 0.37 ($P = 9.39 \times 10^{-9}$) for Lichtenburg. There was a distinct lack of randomness in the residual and normal probability plots for percentage mean out-crossing versus distance (Fig. 3.5 and 3.6) (Table 3.7). Transformation of distance data, using the square root of distance and the log of distance, in the regression analysis did not influence the lack of randomness in residual and normal probability plots.

The Bt gene was absent in the CRN 3505 and was quantified at 37.93% (SD = 3.7) in the CRN4760 B parent line. The Bt genotype was detected in yellow seed taken from white cobs for distance interval A in Delmas and Lichtenburg, respectively. The amount of Bt gene was quantified for Delmas and Lichtenburg at 17.55 (SD =

1.70) and 15.03% (SD = 1.50), respectively. The Bt gene was also detected in white seed from interval A at Delmas and Lichtenburg. The presence of Bt gene was quantified in white seeds from interval A at Delmas and Lichtenburg as 0.12% (SD = 0.06) and 0.9% (SD = 0.18), respectively. For interval B at Lichtenburg, the presence of the Bt gene in yellow seed from white cobs was quantified at 17.60% (SD = 1.80) while the amount of Bt gene in white seed was below the limit of quantification (0.05%) (Fig. 3.7).

Weather patterns

During the flowering period, the average temperature ranged from 16.1 to 19.6^{o}C for Delmas and 13.1 to 19.2^{o}C for Lichtenburg (Fig. 3.8). At Delmas there were 155 hours of less than 20^{o}C and 61 hours of greater than 20^{o}C, while at Lichtenburg there were 149 hours of less than 20^{o}C and 67 hours of greater than 20^{o}C, average daily temperature. Average morning temperatures ranged from 14.2 to 21.4^{o}C (overall average of 16.7^{o}C) at Delmas and from 12.8 to 21.4^{o}C at Lichtenburg with an overall average of 15.6^{o}C.

The average relative humidity (RH) at Delmas ranged from 74.8 to 99.7%, while at Lichtenburg it ranged from 58.7 to 90.1% (Fig 3.9). At Delmas there were 166 hours of greater than 70% RH and 50 hours of less than 70% RH, while at Lichtenburg there were 154 hours of greater than 70% RH and 62 hours less than 70% RH. There was no statistical significant difference in average relative humidity (P = 0.19) and average temperature (P = 0.53) between Delmas and Lichtenburg.

The wind speed varied between sites. At Delmas the average wind speed per day ranged from 0.20 to 3.96 m/s and for Lichtenburg from 0.20 to 4.85 m/s. The average wind speed in the relevant wind direction to affect out-crossing ranged from 0.32 to 1.90 m/s at Delmas and from 0.0 to 2.83 m/s at Lichtenburg. In Delmas there were 111 hours of wind in the relevant direction to affect out-crossing compared to 68 hours in Lichtenburg. There was a statistically significant difference in the relevant wind direction affecting the out-crossing at Delmas and Lichtenburg ($P = 2.7 \times 10^{-5}$). Within the relevant wind direction there was also a significant difference between high wind speed (> 2 m/s) and low wind speed (<2 m/s) at Delmas and Lichtenburg, respectively ($P = 0.02$).

The predominant frequency (direction and duration) of wind at Delmas was of a low wind speed in a south easterly direction (from which wind is blowing) while higher wind speeds were observed in a north, north-north east and easterly direction at a lesser frequency (Fig. 3.10). At Lichtenburg, the predominant frequency of wind was of a low wind speed in a north-north east to easterly direction while higher wind speeds were observed in various directions (Fig. 3.11).

DISCUSSION

The results of this study have demonstrated that the extent of out-crossing of white maize by pollen from adjacent yellow Bt maize varied in terms of wind direction, during flowering, and distance. The highest level of out-crossing over the two geographic locations was 15.2% and 39.1% between adjoining white and yellow

maize rows, with a sharp decline to 1.0% at approximately 25 m, similar to results by Ma *et al*. (2004) (Table 3.2 and 3.3). With the exception of the first row, there was no significant difference for data between the different geographic locations ($P = 1.38 \times 10^{-6}$). After the sharp decline up to 25 m, the average mean percentage out-crossing averaged 0.36% up to 81.6 m. However, out-crossing may have exceeded beyond the furthest row in terms of trial layout at both locations.

The out-crossing of the Bt yellow maize to the white maize was further confirmed and quantified by PCR and Real-time PCR detection. The yellow seeds from white cobs contained approximately 50% of the transgene found in the yellow Bt parent. White seeds from the white cobs, that contained yellow seeds, also tested positive for the presence of the transgene. Although quite low, less than 1% transgene content for the closest distance interval, the implication is that phenotype did not detect the full extent of out-crossing. This is a noteworthy discovery as previous studies determined the extent of potential recombination based solely on phenotypic data (with the exception of Henry *et al*. (2003) who utilised only genotype. The detection of the Bt transgene in white seeds from the white cobs, that contained yellow seeds, may possibly be attributed to meiotic recombination. The parameters of this study did not allow for further investigation. Notwithstanding, this finding adds a new dimension into the perception of the potential out-crossing abilities of maize pollen. Future studies should include a combination of phenotypic and genotypic markers to determine the effect of meiotic recombination.

The rate of out-crossing to distance was represented by an exponential function with R > 0.9 (Fig. 3.5 and 3.6) (Table 3.7). Based on the equation for the exponential function, the estimated zero over both locations would be between 110 and 128 m in Delmas and Lichtenburg, respectively.

For the regression analysis the R values were, 0.73 and 0.37, respectively, lower than for the exponential function suggesting that additional factors, other than just distance played an important role in the extent of out-crossing, similar to the findings of Ma *et al*. (2004). This was also confirmed by the lack of randomness of these data and that transformation of the distance data had no obvious effect on randomness (Fig. 3.5 and 3.6). The regression analysis for distance, square-root of distance and log of distance indicated that even though a definite relationship between distance and recombination exists, distance is not a sole causal factor for the percentage mean out-crossing observed. The pattern observed in the residual plots indicated a lack of randomness and that factors, other than distance still influenced recombination. Thus distance data on its own can not account for the level of out-crossing observed. These data suggest that combined environmental conditions including relative humidity, temperature, wind speed and direction contribute to the frequency of out-crossing observed.

Temperature is an important function for pollen viability, especially in conjunction with relative humidity (RH) (Luna *et al*., 2001; Aylor, 2003). Aylor *et al*. (2003) determined that at 23°C and 50% RH, the viability of maize pollen was 60 min. They determined that pollen viability increased with an increase in RH. During the

present study, the average daily temperature during pollination was below 20°C, especially morning temperature and the overall relative humidity was above 70%. Thus, conditions at both locations were well suited for pollen survival. However, the distance viable pollen can move is also determined by prevailing wind.

Compared to other grasses, maize pollen is amongst the largest and heaviest (Jones and Newell, 1948) and is thought to have a greater tendency to settle down than to move upward and downward (Raynor *et al*., 1972; Ma *et al*., 2004). There was a statistically significant difference in relevant wind direction over the two locations used in this study. There was also a statistically significant difference between low (<2 m/s) and high winds (>2 m/s), with low winds being predominant. These data suggest that high winds would not necessarily facilitate out-crossing over greater distance. However, the effects of the higher wind speeds may have been more noticeable if larger field trials were used.

The temporal isolation implemented in this study was effective as the data did not show any deviation that would indicate external pollen effects. While temporal isolation is effective for synchronised silking and anthesis but perhaps not viable due to the seasonal nature of maize as well as the dependence on sporadic rainfall patterns. Seed producers would be disadvantaged by temporal isolation due to time constraints of seed production especially if no irrigation facilities are available.

It is apparent that environmental conditions specific to maize breeding areas is an imperative consideration in IP of maize. Plot design is an equal variable, especially

for the analysis of pollen distribution from a specific pollen source and pollen load. In this study, the insignificant slope at Delmas and Lichtenburg, respectively, will have had a negligible impact on out-crossing.

Other methods, mainly molecular, to contain transgenes preventing gene flow have been suggested, these are maternal inheritance, male sterility, seed sterility and cleistogamy (Daniell, 2002). These techniques may find use in specific breeding application but not for normal agricultural practice due to the costs involved. Farmers will have to be made more aware of the importance of the proper implementation of IP, especially for GM crops.

Irrespective of the answers previous studies, this study or any future study can provide on pollen-mediated gene flow (PMGF), and the establishment of the necessary regulations thereafter, the challenge lies in educating and providing a support structure for farmers to implement IP. The general lack of awareness of the importance of IP in South Africa and Africa in general is alarming. The attitude from the first world smacks of a lack of insight into the needs of Africa's people, while the attitude of African's is that of apathy towards their situation. Serious deliberation in the form of African directed research is required to make the developments of modern biotechnology a reality in Africa.

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Table 3.1 A description of the trial layout for the different locations used in the study.

Field trial details	Delmas	Lichtenberg
Field dimensions	45 x 31.5 m	171 x 67 m
Field area	1417.5 m^2	11,457 m^2
Plant density (plants/Ha)		30,000
Soil type		clay, loam
Soil pH		5.82
Distance between rows	0.75 m	0.80 m
Planting dates	08 December 2003	28 January 2004
Days to flowering	61	71
Flowering period	16 - 24 February 2004	4 - 12 April 2004
Evaluation dates	June 2004	July 2004

Table 3.2 Transformed data for the out-crossing observed in Delmas.

Row number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mean percentage	15.208	12.216	11.376	9.368	7.839	7.159	6.150	4.690	4.332	3.595	3.777	2.412	2.548	2.424	2.582
Std Dev	3.0254	3.2917	3.6527	3.353	3.462	3.278	2.431	2.31	2.559	2.247	1.926	1.306	1.77689	2.2012	1.969
count	59	43	42	60	49	53	56	62	63	59	52	55	61	55	49
DOF	58	42	41	59	48	52	55	61	62	58	51	54	60	54	48
Null Hypoth Mean	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-test	5.0269	3.711	3.1144	2.794	2.265	2.184	2.53	2.031	1.693	1.6	1.961	1.847	1.43385	1.1014	1.312
T-critical	3E-06	0.0003	0.0017	0.004	0.014	0.017	0.007	0.023	0.048	0.058	0.028	0.035	0.0784	0.1378	0.098
Reject Null	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No	No	No
Distance (m)	0.75	1.50	2.25	3.00	3.75	4.50	5.25	6.00	6.75	7.50	8.25	9.00	9.75	10.50	11.25
Sqrt(dist)	0.86603	1.22474	1.5	1.7321	1.9365	2.1213	2.2913	2.4495	2.5981	2.7386	2.8723	3	3.122499	3.24037	3.3541
Ln(dist)	-0.28768	0.40547	0.81093	1.0986	1.3218	1.5041	1.6582	1.7918	1.9095	2.0149	2.1102	2.1972	2.277267	2.35138	2.4204

Row number	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Mean percentage	2.198	1.840	1.986	2.616	2.285	1.372	0.955	0.944	0.917	1.100	1.101	1.137	0.995	1.044	1.211
Std Dev	2.088	1.444	1.361	2.077	1.794	1.086	0.559	0.596	0.521	0.814	0.696	0.669	0.624	0.636	0.633
count	56	65	59	58	53	60	65	52	50	42	47	43	36	51	44
DOF	55	64	58	57	52	59	64	51	49	41	46	42	35	50	43
Null Hypoth Mean	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-test	1.053	1.274	1.459	1.259	1.274	1.263	1.709	1.585	1.761	1.352	1.582	1.699	1.595	1.64	1.913
T-critical	0.148	0.104	0.075	0.107	0.104	0.106	0.046	0.06	0.042	0.092	0.06	0.048	0.06	0.054	0.031
Reject Null	No	No	No	No	No	No	Yes	No	Yes	No	No	Yes	No	No	Yes
Distance (m)	12.00	12.75	13.50	14.25	15.00	15.75	16.50	17.25	18.00	18.75	19.50	20.25	21.00	21.75	22.50
Sqrt(dist)	3.4641	3.5707	3.6742	3.7749	3.873	3.9686	4.062	4.1533	4.2426	4.3301	4.4159	4.5	4.5826	4.6637	4.7434
Ln(dist)	2.4849	2.5455	2.6027	2.6568	2.7081	2.7568	2.8034	2.8478	2.8904	2.9312	2.9704	3.0082	3.0445	3.0796	3.1135

Table 3.3 Transformed data for the out-crossing observed in Lichtenburg.

Row number	1	2	3	4	5	6	8	9	10	12	14	16	18	20
Mean Percentage	39.14	27.77	16.37	9.24	9.58	9.40	6.56	5.42	3.87	3.43	3.61	2.62	3.14	1.85
Std Dev	6.70	5.61	4.82	1.98	3.14	2.27	1.57	2.15	1.39	1.24	1.61	1.01	1.43	1.11
count	12	12	12	12	12	12	12	12	12	12	12	12	12	12
DOF	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Null Hypoth Mean	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-test	5.8416	4.951	3.3934	4.657	3.054	4.14	4.176	2.522	2.788	2.758	2.239	2.589	2.191	1.674
T-critical	6E-05	0.0002	0.003	3E-04	0.005	8E-04	8E-04	0.014	0.009	0.009	0.023	0.013	0.025	0.061
Reject Null	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Distance (m)	0.85	1.7	2.55	3.4	4.25	5.1	6.8	7.65	8.5	10.2	11.9	13.6	15.3	17
Sqrt(dist)	0.922	1.3038	1.5969	1.844	2.062	2.258	2.608	2.766	2.915	3.194	3.45	3.688	3.912	4.123
Ln(dist)	-0.1625	0.5306	0.9361	1.224	1.447	1.629	1.917	2.035	2.14	2.322	2.477	2.61	2.728	2.833

Row number	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50
Mean Percentage	2.14	1.82	1.62	1.52	0.92	0.83	0.78	0.75	0.89	0.56	0.51	0.50	0.50	0.56	0.33
Std Dev	1.27	0.75	0.99	1.02	0.79	0.56	0.60	0.54	0.85	0.58	0.31	0.33	0.31	0.44	0.29
count	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
DOF	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Null Hypoth Mean	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-test	1.6832	2.4331	1.6482	1.49	1.16	1.47	1.3	1.38	1.04	0.96	1.67	1.51	1.61	1.27	1.16
T-critical	0.0602	0.0166	0.0638	0.08	0.14	0.08	0.11	0.1	0.16	0.18	0.06	0.08	0.07	0.12	0.14
Reject Null	No	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No
Distance (m)	18.7	20.4	22.1	23.8	25.5	27.2	28.9	30.6	32.3	34	35.7	37.4	39.1	40.8	42.5
Sqrt(dist)	4.3243	4.5166	4.7011	4.88	5.05	5.22	5.38	5.53	5.68	5.83	5.97	6.12	6.25	6.39	6.52
Ln(dist)	2.9285	3.0155	3.0956	3.17	3.24	3.3	3.36	3.42	3.48	3.53	3.58	3.62	3.67	3.71	3.75

Row number	52	54	56	58	60	62	64	66	68	70	72	74	76	78	80
Mean Percentage	0.511	0.391	0.481	0.316	0.301	0.361	0.15	0.15	0.301	0.165	0.15	0.181	0.105	0.241	0.211
Std Dev	0.492	0.391	0.464	0.257	0.281	0.308	0.186	0.151	0.311	0.18	0.186	0.255	0.093	0.208	0.169
count	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
DOF	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
Null Hypoth Mean	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-test	1.04	1	1.038	1.23	1.07	1.173	0.809	0.998	0.967	0.92	0.809	0.707	1.133	1.155	1.245
T-critical	0.16	0.169	0.161	0.122	0.154	0.133	0.218	0.17	0.177	0.189	0.218	0.247	0.141	0.136	0.12
Reject Null	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Distance (m)	44.2	45.9	47.6	49.3	51	52.7	54.4	56.1	57.8	59.5	61.2	62.9	64.6	66.3	68
Sqrt(dist)	6.648	6.775	6.899	7.021	7.141	7.259	7.376	7.49	7.603	7.714	7.823	7.931	8.037	8.142	8.246
Ln(dist)	3.789	3.826	3.863	3.898	3.932	3.965	3.996	4.027	4.057	4.086	4.114	4.142	4.168	4.194	4.22

Row number	82	84	86	88	90	92	94	96
Mean Percentage	0.1655	0.09	0.15	0.15	0.09	0.0752	0.105	0.135
Std Dev	0.1798	0.094	0.215	0.169	0.122	0.1207	0.121	0.174
count	12	12	12	12	12	12	12	12
DOF	11	11	11	11	11	11	11	11
Null Hypoth Mean	0	0	0	0	0	0	0	0
T-test	0.9202	0.957	0.698	0.889	0.742	0.6232	0.873	0.777
T-critical	0.1886	0.179	0.25	0.197	0.237	0.2729	0.201	0.227
Reject Null	No	No	No	No	No	No	No	No
Distance (m)	69.7	71.4	73.1	74.8	76.5	78.2	79.9	81.6
Sqrt(dist)	8.3487	8.45	8.55	8.649	8.746	8.8431	8.939	9.033
Ln(dist)	4.2442	4.268	4.292	4.315	4.337	4.3593	4.381	4.402

Table 3.4 Comparison between different geographical locations at different distance intervals.

Delmas			Lichtenburg	F-test	T-test
Average percentage	Distance		Average percentage	P-value	P-value
22.29	0.75	0.85	39.14	5.12E-25*	1.373E-6*
6.35	6.75	6.8	6.56	0.003*	0.751
3.22	12.00	11.9	3.61	0.025*	0.536
2.91	13.50	13.6	2.62	0.018*	0.459
1.61	18.75	18.7	2.14	0.752	0.188

*Significant difference

Table 3.5 Random sampling of white maize cobs. Due to the difference in the size of trials, at Delmas sections A to E were sampled and at Lichtenburg sections A to K were sampled.

Area	Distance from yellow maize field	Number of cobs sampled
A	2	10
B	7	10
C	12	10
D	17	10
E	22	10
F	32	10
G	42	10
H	52	10
I	62	10
J	72	10
K	82	10

Table 3.6 The number of seeds in 10 randomly selected cobs and the average number of seeds per cob in Delmas and Lichtenburg, respectively.

	Lichtenburg	Delmas
1	480	286
2	546	377
3	560	336
4	588	518
5	574	385
6	680	276
7	532	462
8	540	384
9	480	396
10	560	360
Total	**5540**	**3780**
Average	554	378

Table 3.7 The R^2 and P-values for the regression analysis for distance, Square-root of distance and Ln of distance.

	Delmas		Lichtenburg	
	R^2	P-value	R^2	P-value
Distance	0.733420048	4.90995E-13	0.371377866	9.39245E-09
Square-root distance	0.851136072	1.82054E-15	0.518356793	2.51695E-11
Ln distance	0.944745402	1.97094E-21	0.718342313	5.77596E-17

Table 3.8 Temperature and relative humidity data for Delmas during flowering.

Temperature (℃)																								Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	15.5	15.1	15.1	14.7	14.3	14.2	14.4	16.3	18.4	18.5	19.8	22.1	23.3	24	24.1	23.5	20.2	20.8	19.2	18.4	17.3	16.9	16.6	16.3	18.28
Day 2	16.4	16.4	16.2	16.2	15.5	14.8	15.1	17.7	19.1	20.1	21	21.7	22.7	23.4	24	24.2	23.9	23.1	21.6	19.8	19.5	18.4	18	18	19.44
Day 3	16.9	16.5	15.9	15.2	14.9	14.3	14.7	17.1	19.6	20.4	21.8	22.6	24	24.8	22.6	17.9	16.5	16	16.2	16	15.8	15.5	15.3	14.9	17.71
Day 4	14.4	13.9	13.7	13.2	12.9	12.9	12.7	14.7	17.2	19.7	21.1	22	22.5	23.1	23.8	23.9	24	23.5	21.2	18.7	19	17.9	17.2	16.1	18.30
Day 5	15.9	15.4	14.5	14.4	13.8	12.8	13.4	17.5	19.3	20.4	22	22.5	23.7	24.2	24.6	25.6	25.3	24.2	21.9	19.7	18.1	17.7	17.2	17.3	19.22
Day 6	16.1	15.5	15.1	14.8	14.7	14.7	15.5	16.8	18.7	19.9	21.1	22.6	23.8	24.4	25.1	24.9	25.3	24.9	22.3	19.9	18.9	18.6	18.2	17.6	19.56
Day 7	16.7	16.3	15.6	15	14.9	14.1	14.7	17.4	20.2	22.1	23.5	24.2	24.7	25.3	24.9	23.2	23.1	22.9	20.6	19	18.5	17.1	16.3	15.9	19.43
Day 8	15.5	15.3	15.4	15.6	15.4	15.3	15.1	15.1	15.6	16.3	17.4	18.3	19.1	19.6	19.4	19.3	19	17.8	16.8	16.8	16.8	16.5	15.7	15.4	16.78
Day 9	15.2	15.1	14.9	14.8	15	15	15.2	15.4	15.9	16.1	16.3	16.7	16.9	17.6	17.9	18.4	17.6	16.8	15.6	15.6	15.8	15.6	15.7	16.4	16.06
Ave.	15.83	15.50	15.15	14.9	14.60	14.22	14.53	16.43	18.23	19.29	20.44	21.42	22.31	22.93	22.93	22.32	21.66	21.12	19.49	18.21	17.74	17.12	16.69	16.42	
Max.	16.9	16.5	16.2	16.2	15.5	15.3	15.5	17.7	20.2	22.1	23.5	24.2	24.7	25.3	25.1	25.6	25.3	24.9	22.3	19.9	19.5	18.6	18.2	18	
Min.	14.4	13.9	13.7	13.2	12.9	12.8	12.7	14.7	15.6	16.1	16.3	16.7	16.9	17.6	17.9	17.9	16.5	16	15.6	15.6	15.8	15.5	15.3	14.9	
Ave. (am)	16.7																								
Overall ave.	18.3																								
Relative Humidity (%)																									
Day 1	95.7	96.5	97.5	98.3	98.7	99.1	99.4	96.2	93.8	91.8	79.2	74.6	61.5	61.6	67	77.8	68.3	78.5	87.8	93	95.4	94.9	96.9	97.5	87.54
Day 2	97.8	98.7	99.1	98.9	98.1	97.9	90.4	86.7	80.4	78.6	74.7	66.2	61.6	62.5	57.9	58.2	59.5	68.5	81.1	83	80.5	92	90	92.3	81.44
Day 3	95.1	95.9	92.7	98.2	98.9	99.2	98.1	89.3	80.6	70.2	71.7	59.2	52.6	51.7	85.8	87.7	92.6	93.2	94.7	97.5	98.3	98.9	99.4	99.7	87.55
Day 4	99.6	100	100	99.7	99.9	99.7	100	97.8	84.5	65.8	61.7	61.7	56.6	52.9	52.8	50.5	51.6	56.9	70.1	74.3	71.4	79.2	79.5	87.6	77.27
Day 5	88.9	92.7	95.8	96	97.5	97.8	97.4	87.1	76.2	67.1	60.1	60.4	57.8	52.1	47.2	47.4	46	59.7	67.6	75.6	78.9	76.9	83.1	86.4	74.82
Day 6	88.3	93.2	94.4	96.6	98.1	98.7	97.4	91.9	83.6	75.8	66.1	63.4	59.6	57.1	51.8	54	47.4	51.6	73	67.8	70.1	72.2	76.6	82.3	75.45
Day 7	82.8	87.5	91.1	92.6	95	96.8	92.3	85.5	72.9	67.4	56	55.7	54.6	48.8	54.7	69.8	57	74.4	78.2	75.6	82.7	91.1	93	96.3	77.16
Day 8	97.6	98	98.5	98.8	98.9	99.5	99.8	99.8	99.8	99	92.6	86.2	84	84.4	87.2	83	90.8	96.1	97.7	98.9	99.4	99.5	99.8	100	95.39
Day 9	100	100	99.9	100	99.6	100	99.8	99.8	99.4	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	99.7	99.67
Ave.	94.00	95.88	96.57	97.69	98.31	98.75	97.19	92.68	85.69	79.46	73.59	69.73	65.36	63.39	67.09	69.35	68.07	75.39	83.37	85.05	86.30	89.38	90.93	93.5	
Max.	100	100	100	100	99.9	100	100	99.8	99.8	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	100	
Min.	82.8	87.5	91.1	92.6	95	96.8	90.4	85.5	72.9	65.8	56	55.7	52.6	48.8	47.2	47.4	46	51.6	67.6	67.8	70.1	72.2	76.6	82.3	

Table 3.9 Temperature and relative humidity data for Lichtenburg during flowering.

Temperature (°C)																									Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	15.5	15.1	15.1	14.7	14.3	14.2	14.4	16.3	18.4	18.5	19.8	22.1	23.3	24	24.1	23.5	20.2	20.8	19.2	18.4	17.3	16.9	16.6	16.3	18.28
Day 2	16.4	16.4	16.2	16.2	15.5	14.8	15.1	17.7	19.1	20.1	21	21.7	22.7	23.4	24	24.2	23.9	23.1	21.6	19.8	19.5	18.4	18	18	19.44
Day 3	16.9	16.5	15.9	15.2	14.9	14.3	14.7	17.1	19.6	20.4	21.8	22.6	24	24.8	22.6	17.9	16.5	16	16.2	16	15.8	15.5	15.3	14.9	17.71
Day 4	14.4	13.9	13.7	13.2	12.9	12.9	12.7	14.7	17.2	19.7	21.1	22	22.5	23.1	23.8	23.9	24	23.5	21.2	18.7	19	17.9	17.2	16.1	18.30
Day 5	15.9	15.4	14.5	14.4	13.8	12.8	13.4	17.5	19.3	20.4	22	22.5	23.7	24.2	24.6	25.6	25.3	24.2	21.9	19.7	18.1	17.7	17.2	17.3	19.22
Day 6	16.1	15.5	15.1	14.8	14.7	14.7	15.5	16.8	18.7	19.9	21.1	22.6	23.8	24.4	25.1	24.9	25.3	24.9	22.3	19.9	18.9	18.6	18.2	17.6	19.56
Day 7	16.7	16.3	15.6	15	14.9	14.1	14.7	17.4	20.2	22.1	23.5	24.2	24.7	25.3	24.9	23.2	23.1	22.9	20.6	19	18.5	17.1	16.3	15.9	19.43
Day 8	15.5	15.3	15.4	15.6	15.4	15.3	15.1	15.1	15.6	16.3	17.4	18.3	19.1	19.6	19.4	19.3	19	17.8	16.8	16.8	16.8	16.5	15.7	15.4	16.78
Day 9	15.2	15.1	14.9	14.8	15	15	15.2	15.4	15.9	16.1	16.3	16.7	16.9	17.6	17.9	18.4	17.6	16.8	15.6	15.6	15.8	15.6	15.7	16.4	16.06
Ave.	15.83	15.50	15.15	14.9	14.60	14.22	14.53	16.43	18.23	19.29	20.44	21.42	22.31	22.93	22.93	22.32	21.66	21.12	19.49	18.21	17.74	17.12	16.69	16.42	
Max.	16.9	16.5	16.2	16.2	15.5	15.3	15.5	17.7	20.2	22.1	23.5	24.2	24.7	25.3	25.1	25.6	25.3	24.9	22.3	19.9	19.5	18.6	18.2	18	
Min.	14.4	13.9	13.7	13.2	12.9	12.8	12.7	14.7	15.6	16.1	16.3	16.7	16.9	17.6	17.9	17.9	16.5	16	15.6	15.6	15.8	15.5	15.3	14.9	
Ave. (am)	16.7																								
Overall ave.	18.3																								
Relative Humidity (%)																									
Day 1	95.7	96.5	97.5	98.3	98.7	99.1	99.4	96.2	93.8	91.8	79.2	74.6	61.5	61.6	67	77.8	68.3	78.5	87.8	93	95.4	94.9	96.9	97.5	87.54
Day 2	97.8	98.7	99.1	98.9	98.1	97.9	90.4	86.7	80.4	78.6	74.7	66.2	61.6	62.5	57.9	58.2	59.5	68.5	81.1	83	80.5	92	90	92.3	81.44
Day 3	95.1	95.9	92.7	98.2	98.9	99.2	98.1	89.3	80.6	70.2	71.7	59.2	52.6	51.7	85.8	87.7	92.6	93.2	94.7	97.5	98.3	98.9	99.4	99.7	87.55
Day 4	99.6	100	100	99.7	99.9	99.7	100	97.8	84.5	65.8	61.7	61.7	56.6	52.9	52.8	50.5	51.6	56.9	70.1	74.3	71.4	79.2	79.5	87.6	77.27
Day 5	88.9	92.7	95.8	96	97.5	97.8	97.4	87.1	76.2	67.1	60.1	60.4	57.8	52.1	47.2	47.4	46	59.7	67.6	75.6	78.9	76.9	83.1	86.4	74.82
Day 6	88.3	93.2	94.4	96.6	98.1	98.7	97.4	91.9	83.6	75.8	66.1	63.4	59.6	57.1	51.8	54	47.4	51.6	73	67.8	70.1	72.2	76.6	82.3	75.45
Day 7	82.8	87.5	91.1	92.6	95	96.8	92.3	85.5	72.9	67.4	56	55.7	54.6	48.8	54.7	69.8	57	74.4	78.2	75.6	82.7	91.1	93	96.3	77.16
Day 8	97.6	98	98.5	98.8	98.9	99.5	99.8	99.8	99.8	99	92.6	86.2	84	84.4	87.2	83	90.8	96.1	97.7	98.9	99.4	99.5	99.8	100	95.39
Day 9	100	100	99.9	100	99.6	100	99.8	99.8	99.4	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	99.7	99.67
Ave.	94.00	95.88	96.57	97.69	98.31	98.75	97.19	92.68	85.69	79.46	73.59	69.73	65.36	63.39	67.09	69.35	68.07	75.39	83.37	85.05	86.30	89.38	90.93	93.5	
Max.	100	100	100	100	99.9	100	100	99.8	99.8	99.4	100	100	100	99.6	99.3	95.7	99.5	99.6	100	99.7	100	99.7	100	100	
Min.	82.8	87.5	91.1	92.6	95	96.8	90.4	85.5	72.9	65.8	56	55.7	52.6	48.8	47.2	47.4	46	51.6	67.6	67.8	70.1	72.2	76.6	82.3	

Table 3.10 Wind speeds and wind direction for nine days at Delmas during flowering.

Wind speed (m/s)																								Daily average
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	0.39	0.3	0.2	0.2	0.2	0.22	0.2	0.28	2.63	2.3	1.92	2.04	1.48	1.55	1.78	2.74	3.53	1.78	0.82	0.24	0.36	0.25	0.39	0.29	1.09
Day 2	0.38	0.81	0.29	0.35	0.47	0.38	0.28	0.74	2.55	3.2	3.28	3.12	2.27	2.31	1.93	1.65	0.9	0.37	0.2	0.2	0.2	0.24	0.22	0.83	1.13
Day 3	0.5	0.33	0.31	0.72	0.3	0.23	0.32	0.91	1.42	1.01	1.18	1.86	2.08	1.28	2	2.2	3.96	2.93	2.42	1.29	0.74	0.2	0.2	0.2	1.19
Day 4	0.41	0.22	0.39	0.2	0.41	0.23	0.2	1.27	1.47	1.7	2.75	2.74	2.16	2.22	2.18	1.7	2.05	1.96	0.68	0.2	0.2	0.2	0.2	0.2	1.08
Day 5	0.21	0.2	0.2	0.29	0.2	0.2	0.2	0.4	2.03	2.53	2.17	1.1	1.84	2.27	1.54	1.96	2.01	1.45	0.31	0.2	0.2	0.21	0.26	1.04	0.96
Day 6	0.2	0.28	0.26	0.36	0.76	0.4	0.98	2.56	3	2.48	1.74	1.94	1.59	1.78	1.7	3.07	1.93	1.07	0.26	0.22	0.26	0.23	0.2	0.2	1.14
Day 7	0.2	0.27	0.21	0.2	0.2	0.2	0.2	0.69	2.21	1.94	2.15	1.78	1.65	1.36	1.21	0.92	1.11	0.5	2.16	1.55	1.6	1.44	1.61	0.32	1.07
Day 8	0.27	0.62	0.4	0.39	0.6	0.52	1.38	1.57	1.74	1.41	2.03	1.88	1.5	1.4	1.43	1.47	1.37	1.34	1.53	0.65	1.02	1.05	2.36	2.41	1.26
Day 9	2.3	1.86	1.73	1.46	1.48	1	1.74	1.96	2.21	2.06	1.53	0.91	0.95	1.03	0.76	0.54	0.2	0.33	0.2	0.2	0.2	0.22	0.22	0.62	1.07
Ave.	0.54	0.54	0.44	0.46	0.51	0.38	0.61	1.15	2.14	2.07	2.08	1.93	1.72	1.69	1.61	1.81	1.90	1.30	0.95	0.53	0.53	0.45	0.63	0.68	
Max.	2.3	1.86	1.73	1.46	1.48	1	1.74	2.56	3	3.2	3.28	3.12	2.27	2.31	2.18	3.07	3.96	2.93	2.42	1.55	1.6	1.44	2.36	2.41	
Min.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.28	1.42	1.01	1.18	0.91	0.95	1.03	0.76	0.54	0.2	0.33	0.2	0.2	0.2	0.2	0.2	0.2	
Wind Direction*																									
Day 1	ESE	SE	SE	SE	SE	SE	ESE	NNE	N	NE	NNE	NNE	NNE	NNE	NE	NNE	NE	NE	ENE	E	ESE	SE	SE	ESE	
Day 2	E	ESE	ESE	ESE	SE	ENE	E	NNE	NNE	NNE	NNE	NNE	NNE	NNE	N	N	NNW	WNW	SW	SSE	S	SE	SSE	SSE	
Day 3	NE	N	SW	SE	SSE	E	SE	E	ENE	N	NNE	NE	NNE	NE	SE	ESE	E	ENE	ENE	E	E	ESE	ESE	SE	
Day 4	SE	SE	ESE	SE	SE	ESE	SE	ESE	ENE	NE	NNE	NNE	NNE	NNE	N	NE	NE	NNE	NNE	E	NW	N	NW	N	
Day 5	ENE	E	N	N	SSE	WNW	W	NE	NE	N	NNE	NNE	ENE	NE	ENE	NNE	NE	NNE	ENE	NW	NW	NNE	N	N	
Day 6	N	NNW	NNW	NW	NNW	N	N	N	N	N	NNW	N	NNW	NNW	NW	N	NNW	NNW	NW	NW	W	W	WSW	WSW	
Day 7	WSW	WNW	N	NW	SSW	SE	ESE	N	NNW	NNW	NNW	NW	NW	NW	NW	N	NW	SSW	SSW	SSW	NE	ESE	SE	SSE	
Day 8	SSE	SSE	SSE	SSE	SE	SE	SE	ESE	ESE	E	ESE	E	NE	ENE	ENE	E	E	ENE	E	E	ESE	E	E	E	
Day 9	E	ESE	ESE	ESE	ESE	ESE	ESE	E	E	E	E	E	E	E	ESE	ESE	E	WSW	NE	ESE	ESE	ENE	ENE	ENE	

* Direction from which wind is blowing

Table 3.11 Wind speed and direction at Lichtenburg for nine days during flowering.

Wind speed (m/s)																								Average daily
Hour	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	
Day 1	1.24	1.73	1.44	1.48	1.46	1.72	1.4	2.56	4.11	4.46	4.16	3.84	3.71	3.32	2.79	2.78	2.99	2.46	1.66	1.6	2.22	2.92	1.07	1.42	2.44
Day 2	1.41	0.46	0.88	1.2	0.98	1.16	0.56	1.93	2.2	2.19	1.61	1.13	0.95	1.49	1.44	1.13	1.03	1.78	1.44	1.67	1.3	0.74	0.66	0.7	1.25
Day 3	0.31	0.36	0.62	0.53	0.38	0.94	0.52	0.7	1	0.8	0.92	0.6	0.86	1.66	1.68	2.93	3.03	2.06	0.69	1.11	2.61	1.12	0.77	0.58	1.12
Day 4	0.35	0.28	0.34	0.51	1.22	2.09	2.38	2.79	4.1	3.71	2.88	2.63	3	2.24	2.43	1.59	1.41	0.67	0.35	0.22	0.46	0.39	0.48	0.2	1.53
Day 5	0.2	0.31	0.2	0.24	0.33	0.31	0.71	1.14	1.99	2.33	1.86	1.56	1.04	1.52	1.11	0.89	0.97	0.34	0.26	0.2	0.2	0.34	0.2	0.2	0.77
Day 6	0.21	0.2	0.2	0.2	0.2	0.2	0.2	0.21	0.97	2.02	3.08	2.61	2.6	2.6	2.58	2.62	2.04	0.87	0.3	0.29	0.2	0.51	1.59	4.32	1.28
Day 7	4.85	4.7	4.61	4.5	2.89	2.25	2.29	3.27	3.94	3.4	2.98	3.18	3.55	3.82	3.74	3.63	3.42	2.88	1.68	1.7	1.57	1.18	1.37	2.11	3.06
Day 8	2.93	2.98	3.14	3.26	3.12	2.04	1.56	2.59	2.98	3.5	3.58	3.12	3.05	2.45	1.7	1.48	1.49	1.54	1.2	0.33	0.2	0.2	0.2	0.2	2.04
Day 9	0.2	0.2	0.2	0.84	1.86	1.55	2.12	2.72	3.02	3.53	2.95	2.25	2.6	1.92	2.51	2.7	2.2	1	0.28	0.22	0.21	0.54	0.26	0.2	1.50
Ave.	1.3	1.25	1.29	1.42	1.38	1.36	1.30	1.99	2.70	2.88	2.67	2.32	2.37	2.34	2.22	2.19	2.06	1.51	0.87	0.82	1.00	0.88	0.73	1.10	
Max.	4.85	4.7	4.61	4.5	3.12	2.25	2.38	3.27	4.11	4.46	4.16	3.84	3.71	3.82	3.74	3.63	3.42	2.88	1.68	1.7	2.61	2.92	1.59	4.32	
Min.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.21	0.97	0.8	0.92	0.6	0.86	1.49	1.11	0.89	0.97	0.34	0.26	0.2	0.2	0.2	0.2	0.2	
Wind direction*																									
Day 1	NE	ENE	NE	NE	NE	NE	NE	NNE	NNE	NNE	N	N	N	N	N	N	NNE	NNE	NNE	NNE	WNW	W	NW	N	
Day 2	NNE	NNE	NE	NNE	NNE	NNE	NE	NE	NE	NNE	NE	NNE	N	NNE	NNE	N	NNE	E	NE	NE	NE	NE	NE	NE	
Day 3	NE	NE	NNE	SSE	SSW	ENE	SSW	ESE	ESE	S	SE	SSW	NE	E	ENE	ENE	NNE	NNE	NNE	SE	SSE	SE	E	NE	
Day 4	E	ENE	ENE	E	ESE	E	E	ENE	ENE	NE	NE	NNE	NNE	N	N	NNW	SSE	SSE	ENE	ENE	NE	NE	NE	NE	
Day 5	ENE	E	E	ESE	E	E	ENE	E	ENE	E	E	E	ESE	SE	ESE	SE	SE	ESE	ESE	ESE	ESE	N	NNW	N	
Day 6	NNE	NNE	NNE	NNE	NNE	NE	NE	NNW	WNW	WNW	WNW	WNW	NW	WNW	WNW	WNW	WNW	WNW	WNW	NW	WNW	W	W	SSW	
Day 7	SSW	S	S	S	S	SSE	SSE	SE	SSE	SSE	SSE	SSW	SSW	SSW	SSW	SSW	SSW	SSW	SSW	S	S	S	S	SSE	
Day 8	S	S	SSE	SSE	SSE	SE	SE	SE	ESE	ESE	ESE	E	E	E	SE	ENE	ENE	ENE	NE	NE	SE	E	E	E	
Day 9	E	E	E	ESE	ENE	NE	NNE	NNE	NNE	NNE	NE	NE	ENE	ESE	ENE	NE	ENE	ENE	NNE	NNE	NE	NE	ENE	E	

* Direction from which wind is blowing

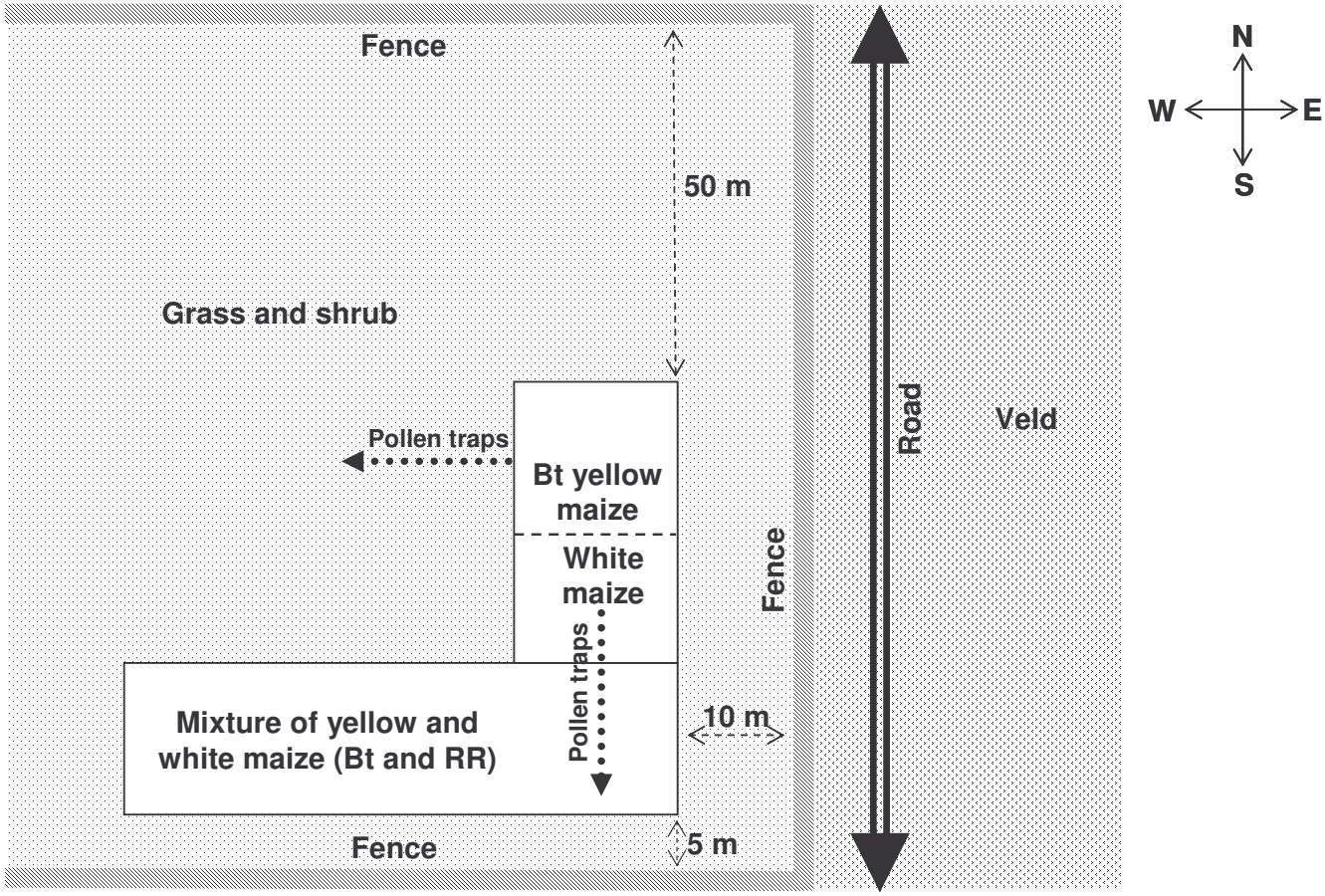


Figure 3.1 Schematic layout of field trial in Delmas; rows were planted parallel to the dotted line.

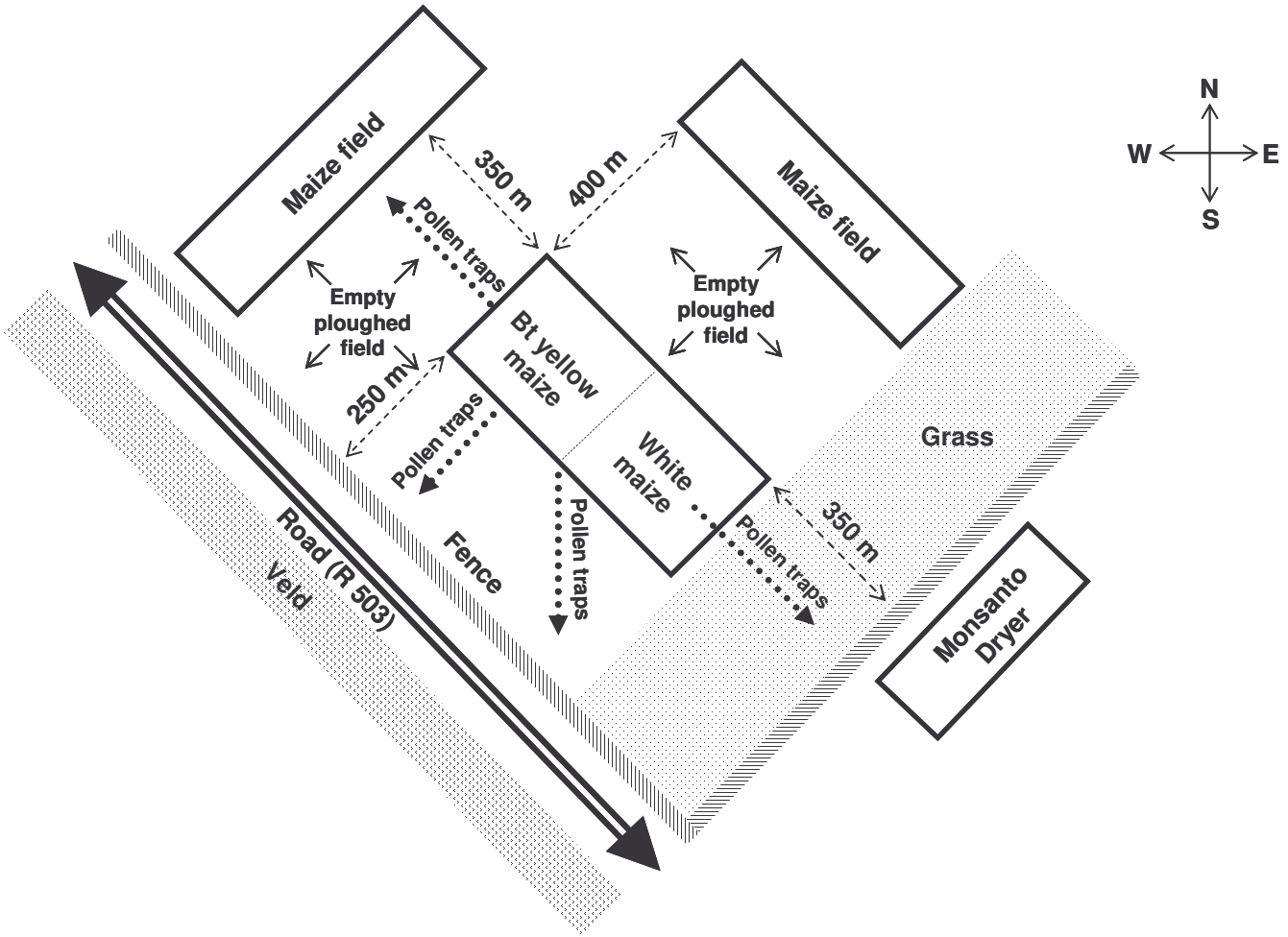


Figure 3.2 Schematic layout of field trial in Lichtenburg; rows were planted parallel to the dotted line.

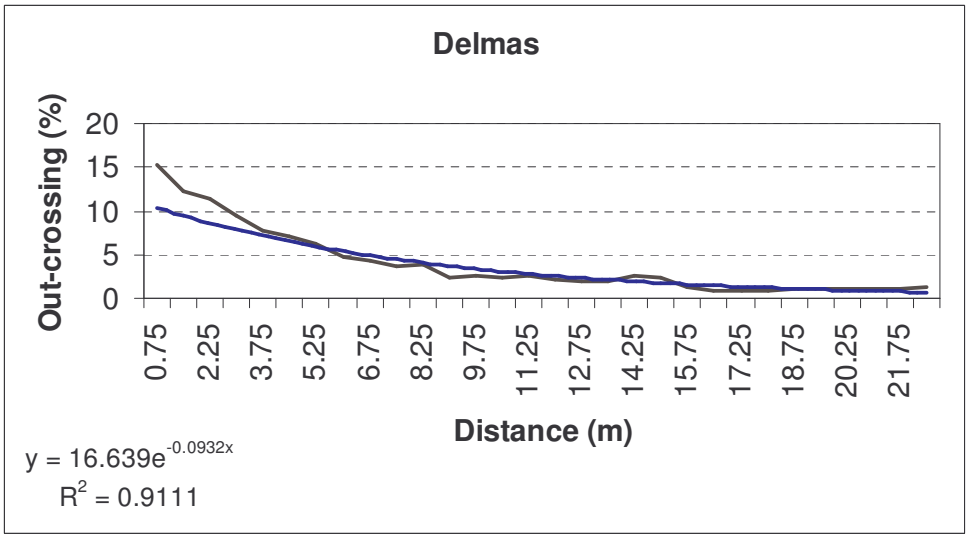


Figure 3.3 Percentage out-crossing over distance with an exponential fit for Delmas.

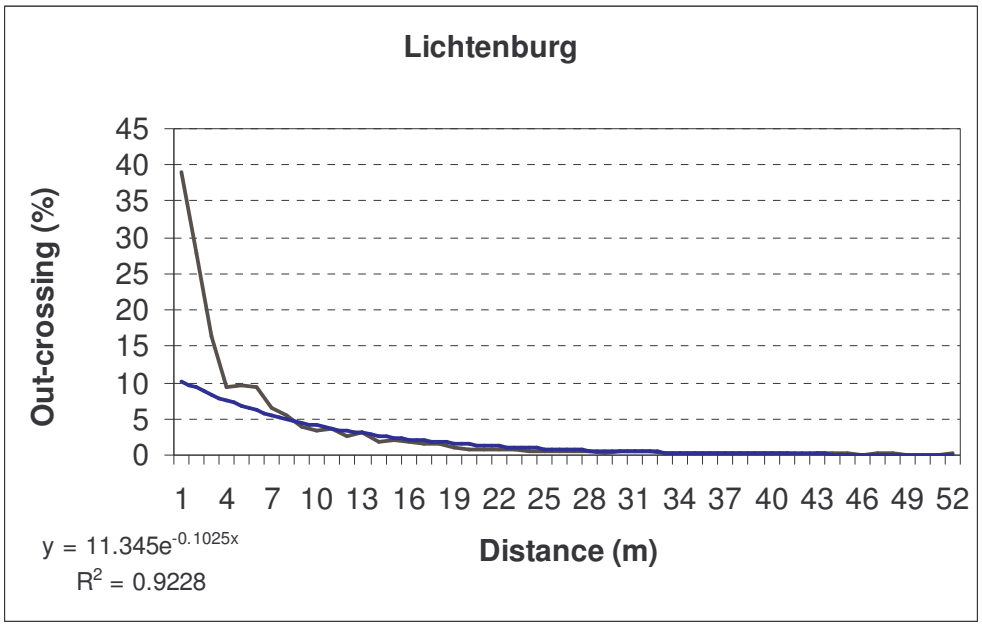


Figure 3.4 Percentage out-crossing over distance with an exponential fit for Lichtenburg.

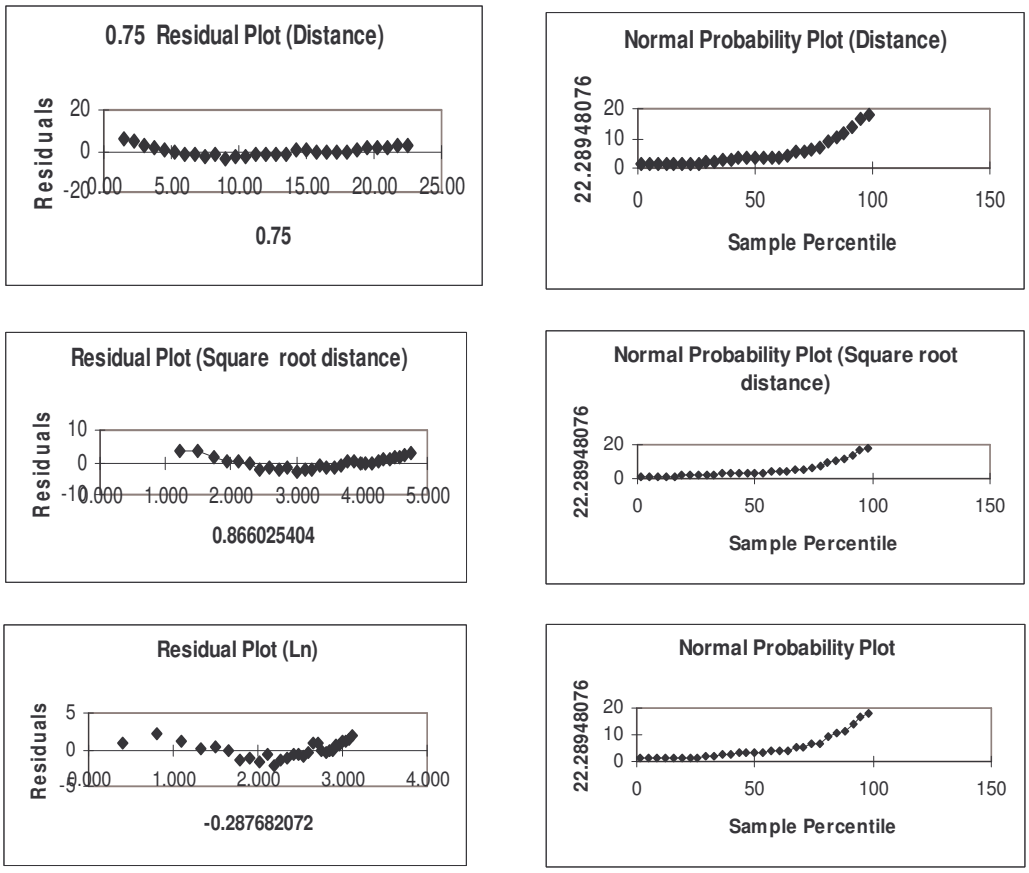


Figure 3.5 Residual and Normal probability plots for distance and transformed distance data for Delmas.

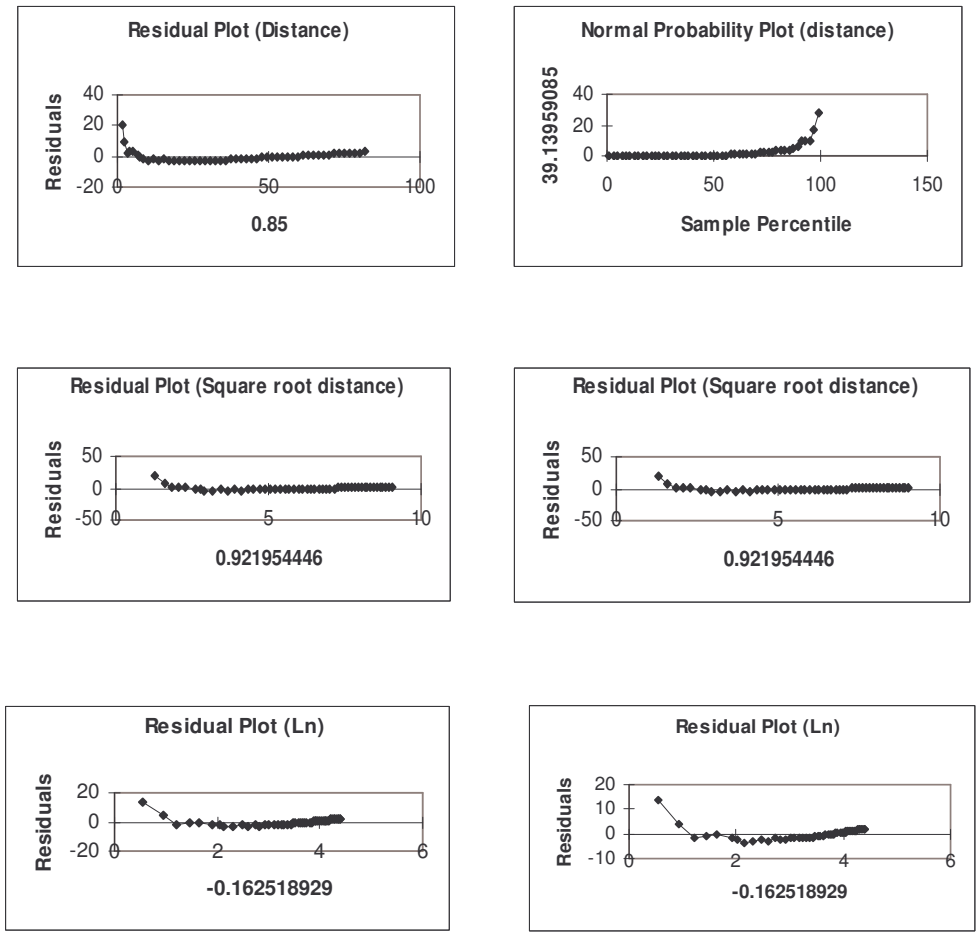
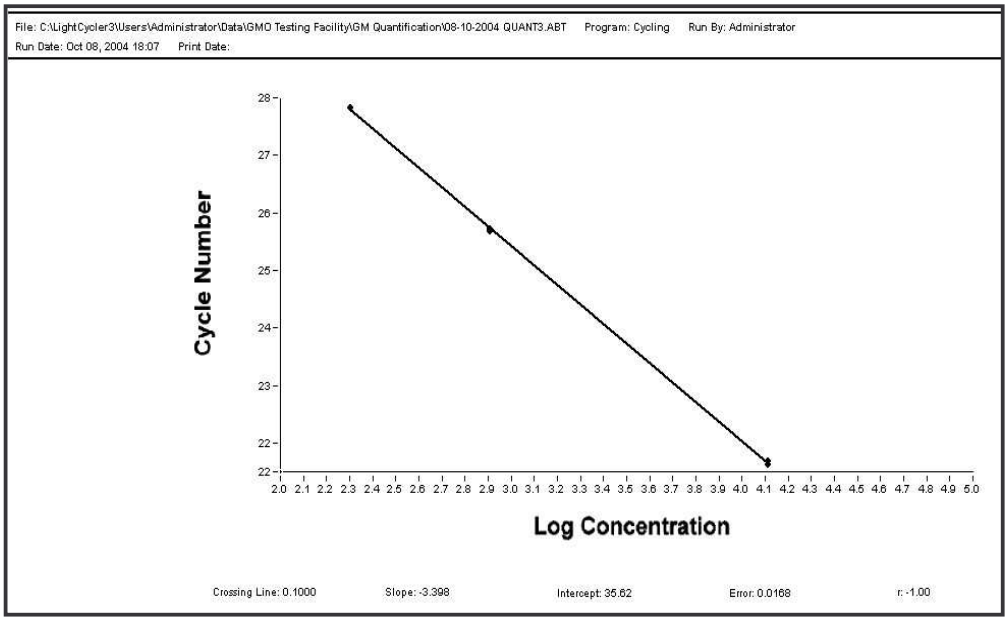


Figure 3.6 Residual and Normal probability plots for distance and transformed distance data for Lichtenburg.

A



B

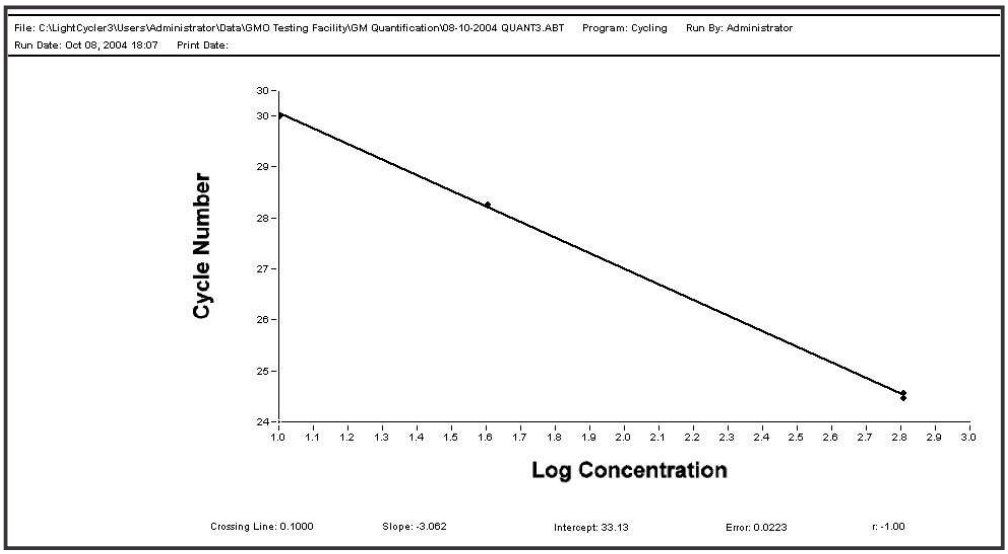
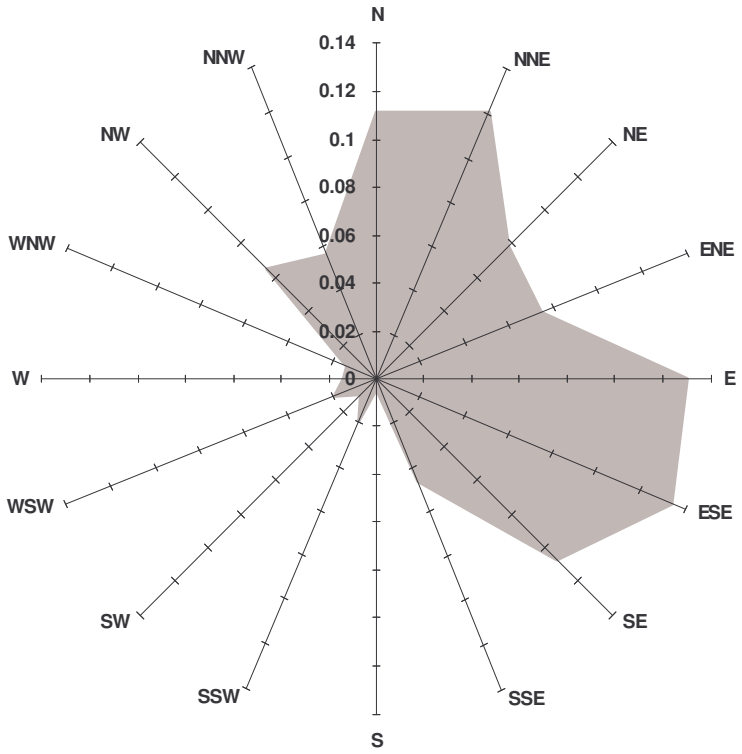


Figure 3.7 Standard curves for GMO quantification. (A) Reference standard curve and (B) GM standard curve.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S
SSW
SW
WSW
W
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0.08
0.06
0.04
0.02
0

Days 1-9

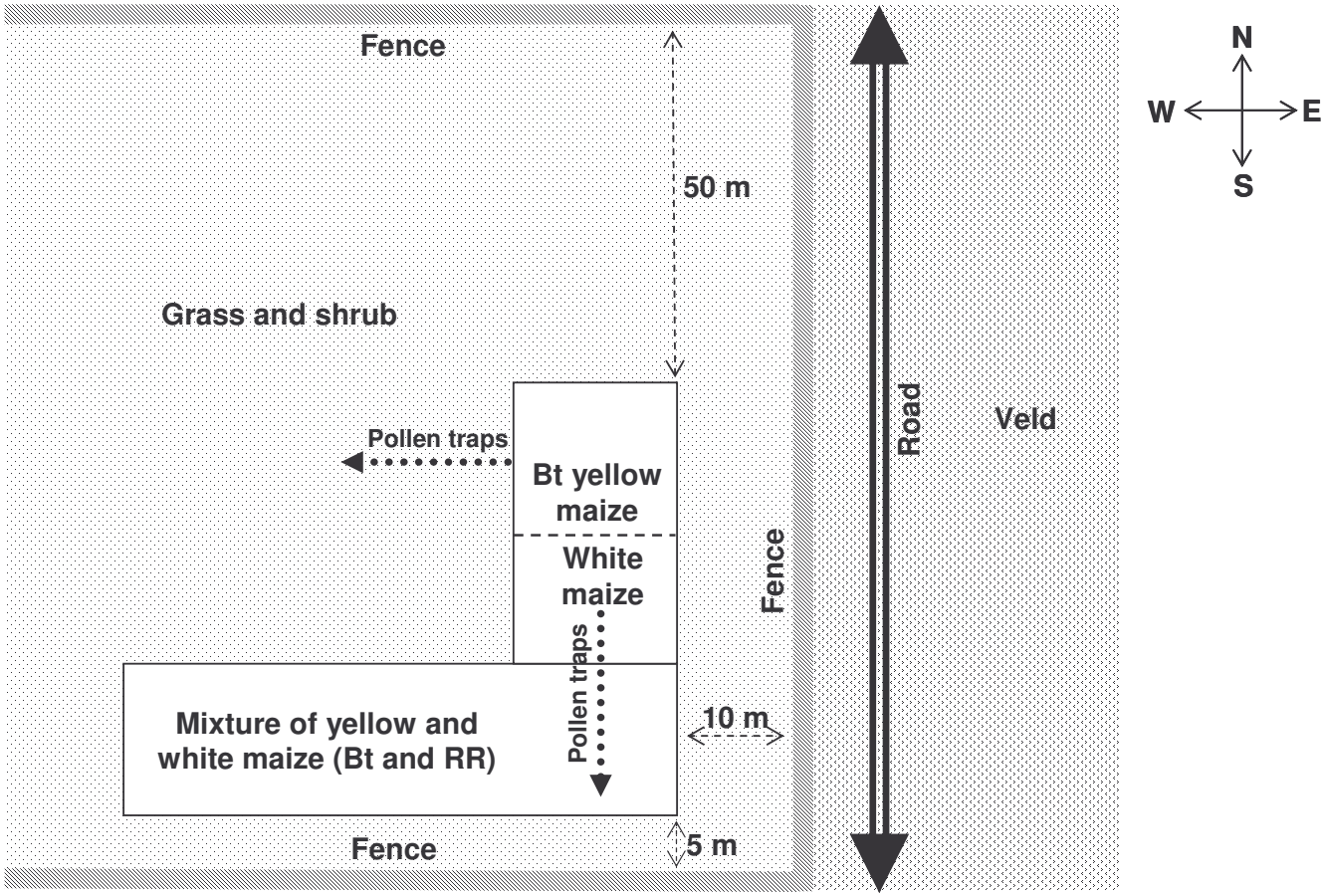
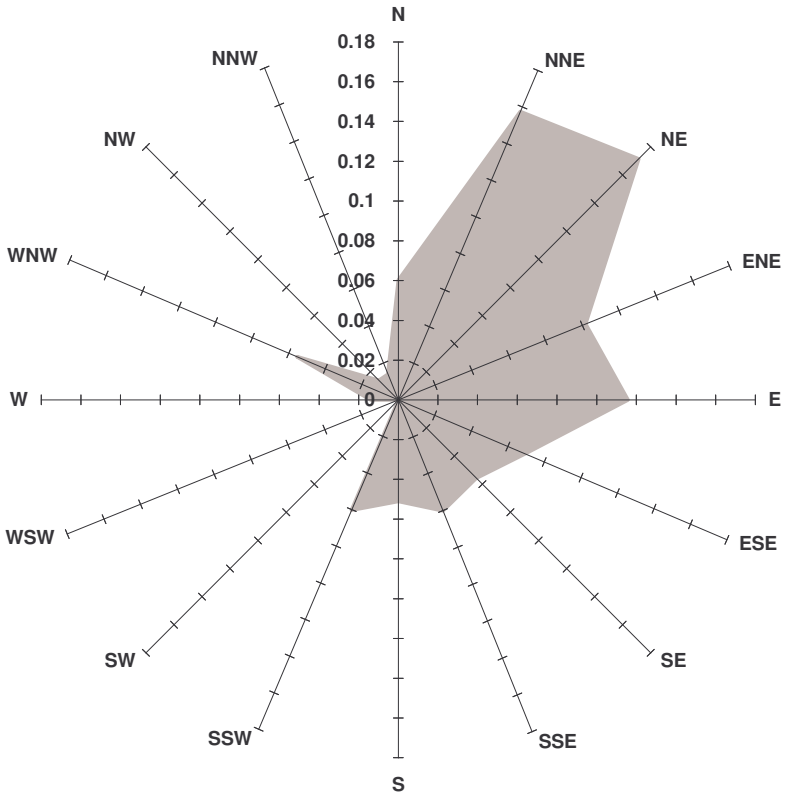


Figure 3.8 Wind rose for days 1 to 9 at Delmas.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0

Days 1-9

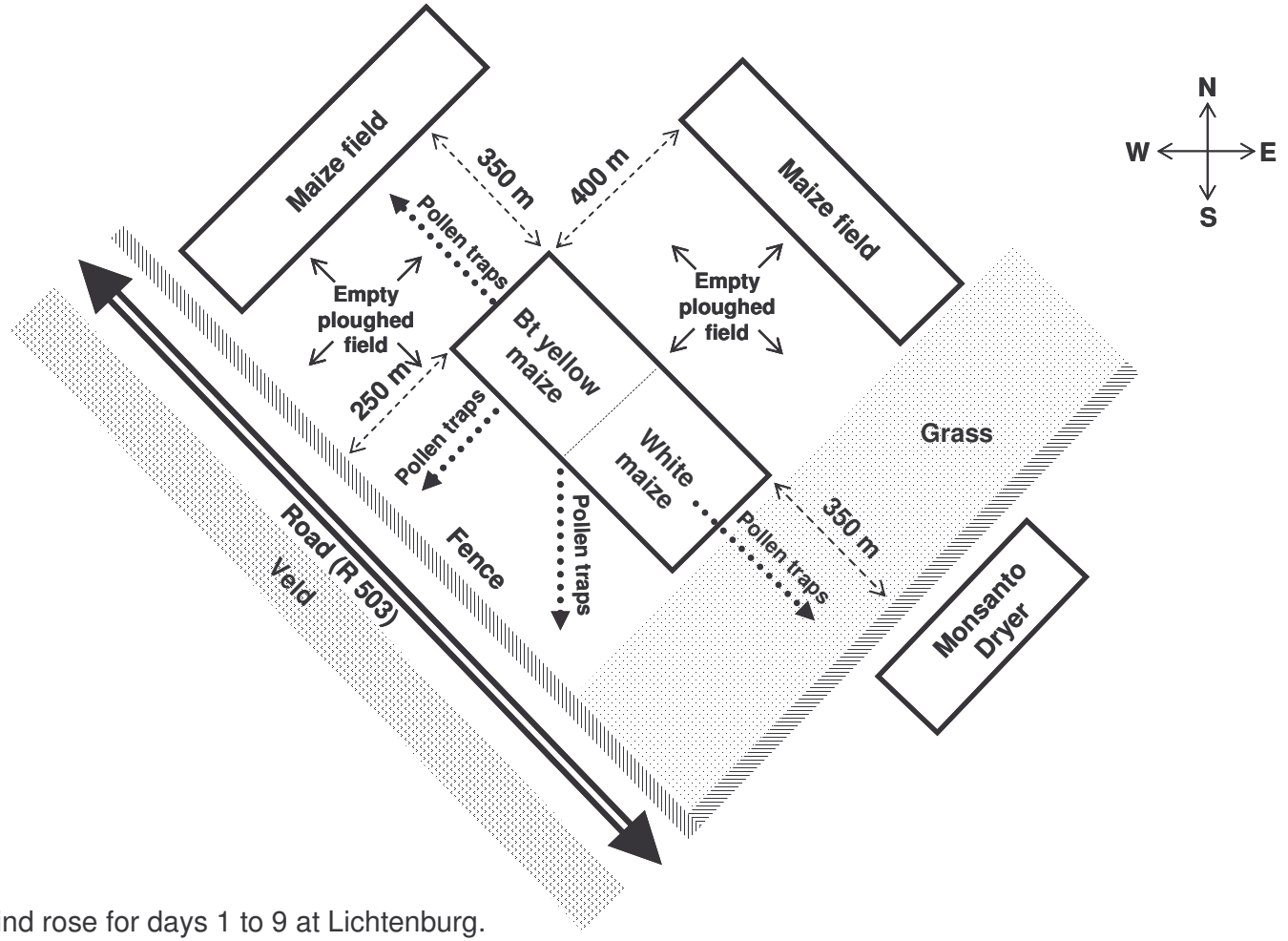

Figure 3.9 Wind rose for days 1 to 9 at Lichtenburg.

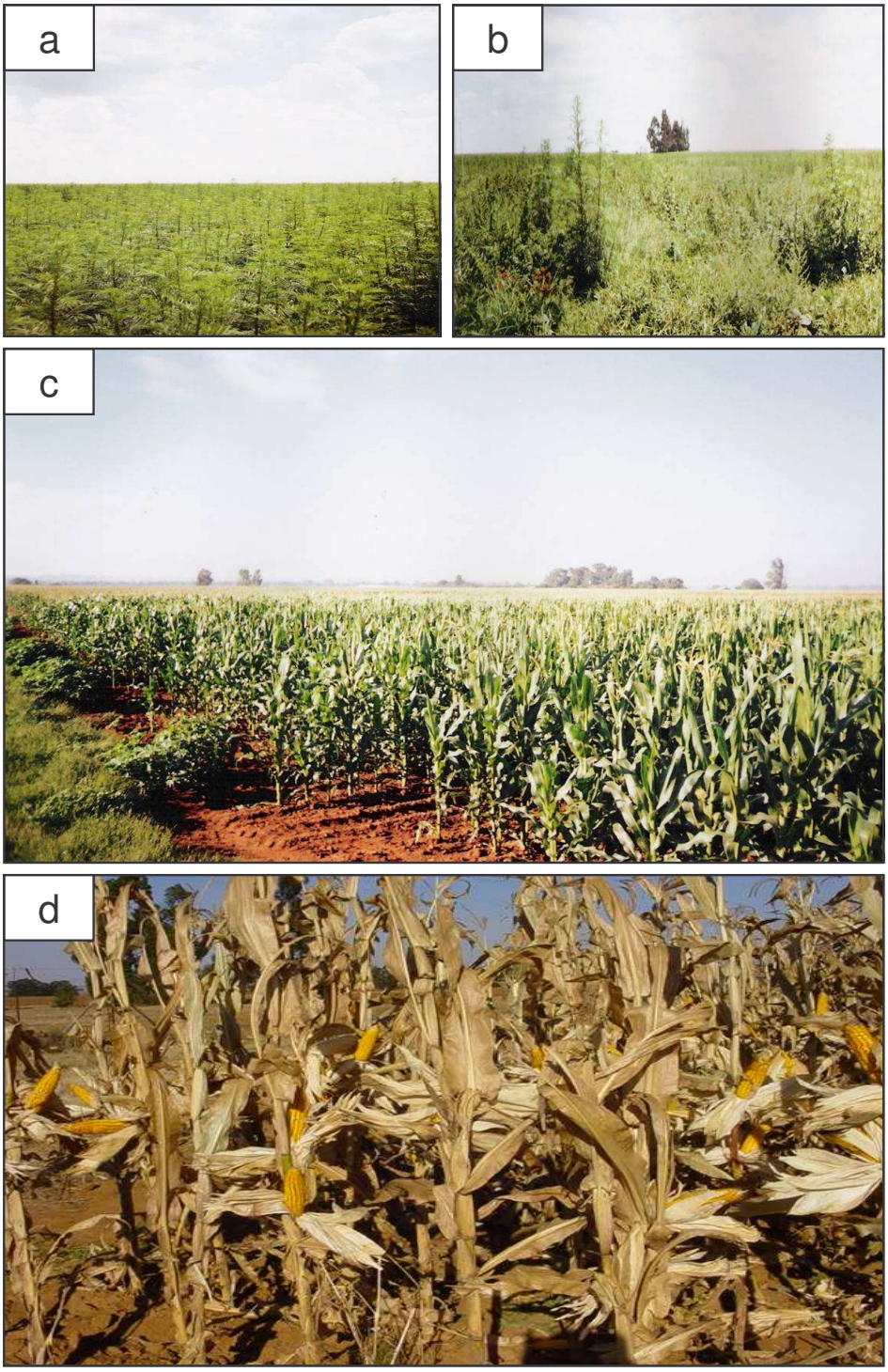

Figure 3.10 Photographs of Delmas field and surroundings; (a) and (b) surrounding grass areas, (c) and (d) maize during flowering and harvesting, respectively.

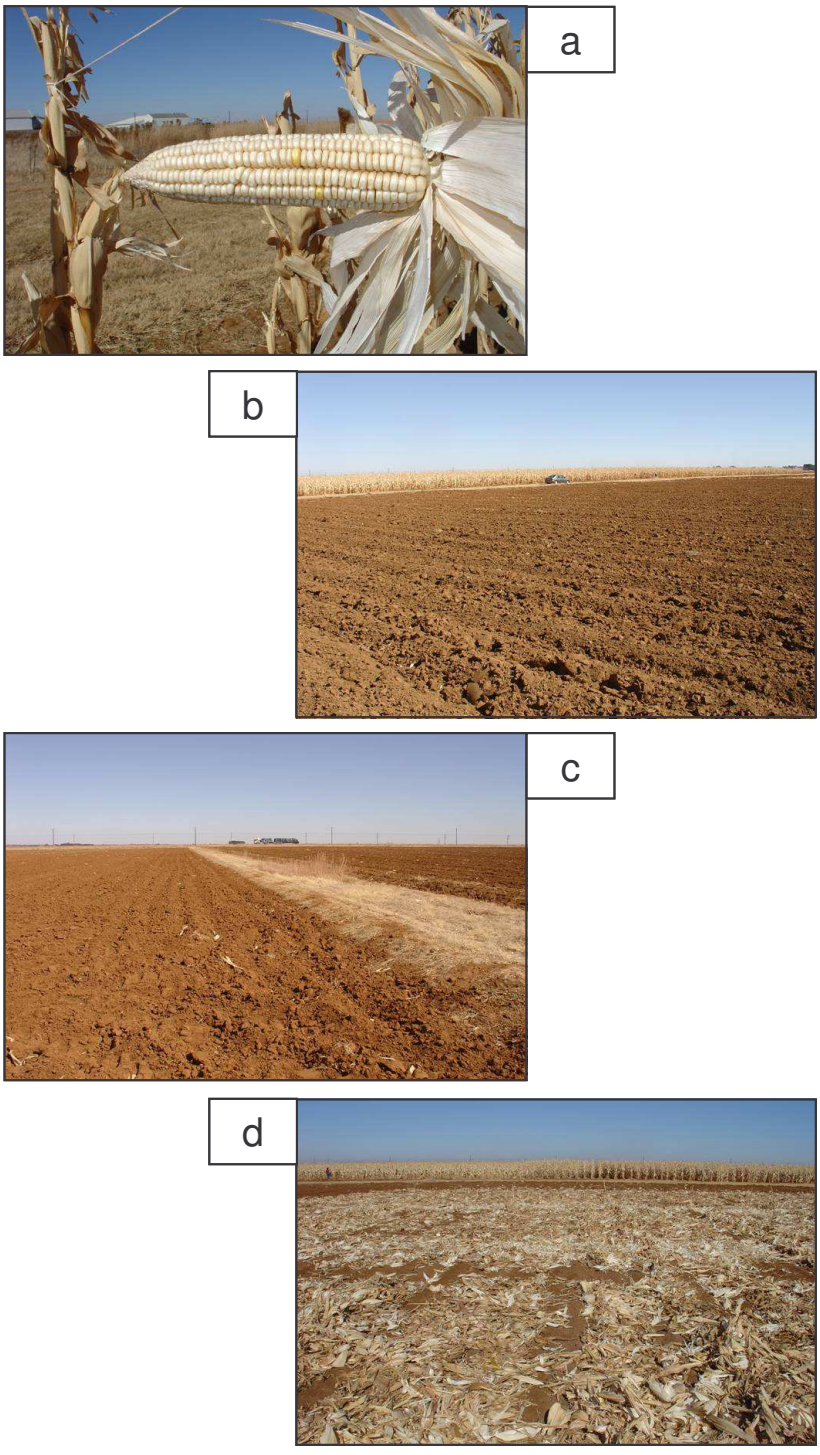

Figure 3.11 Photographs of Lichtenburg field and surroundings; (a) white cob in the furthest row of the white maize field, (c) view of the road from the field, (b) and (d) area around the fields.

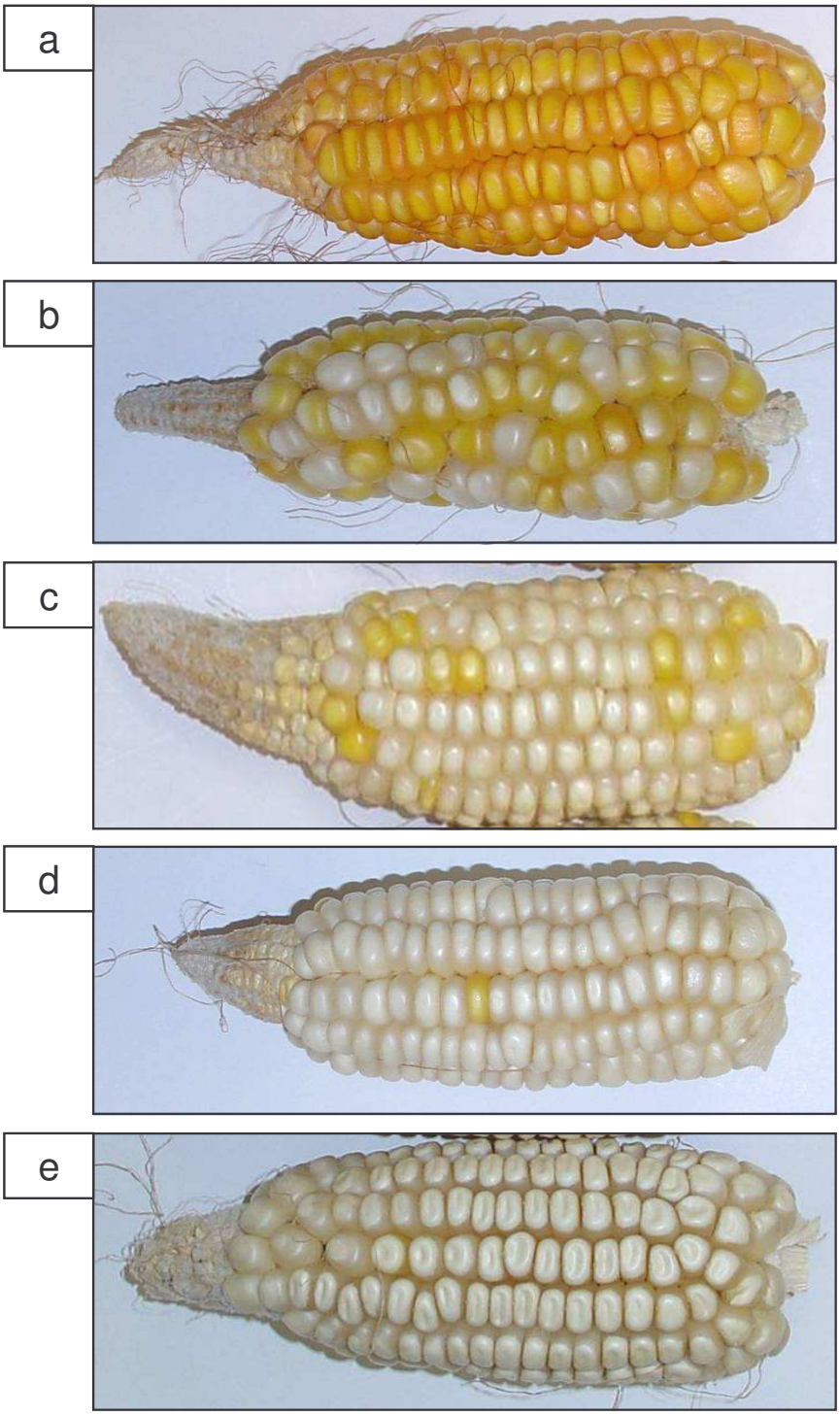

Figure 3.12 Example of out-crossing between yellow and white maize at Delmas. (a), (b), (c), (d) and (e), at increasing distances from the yellow maize field.

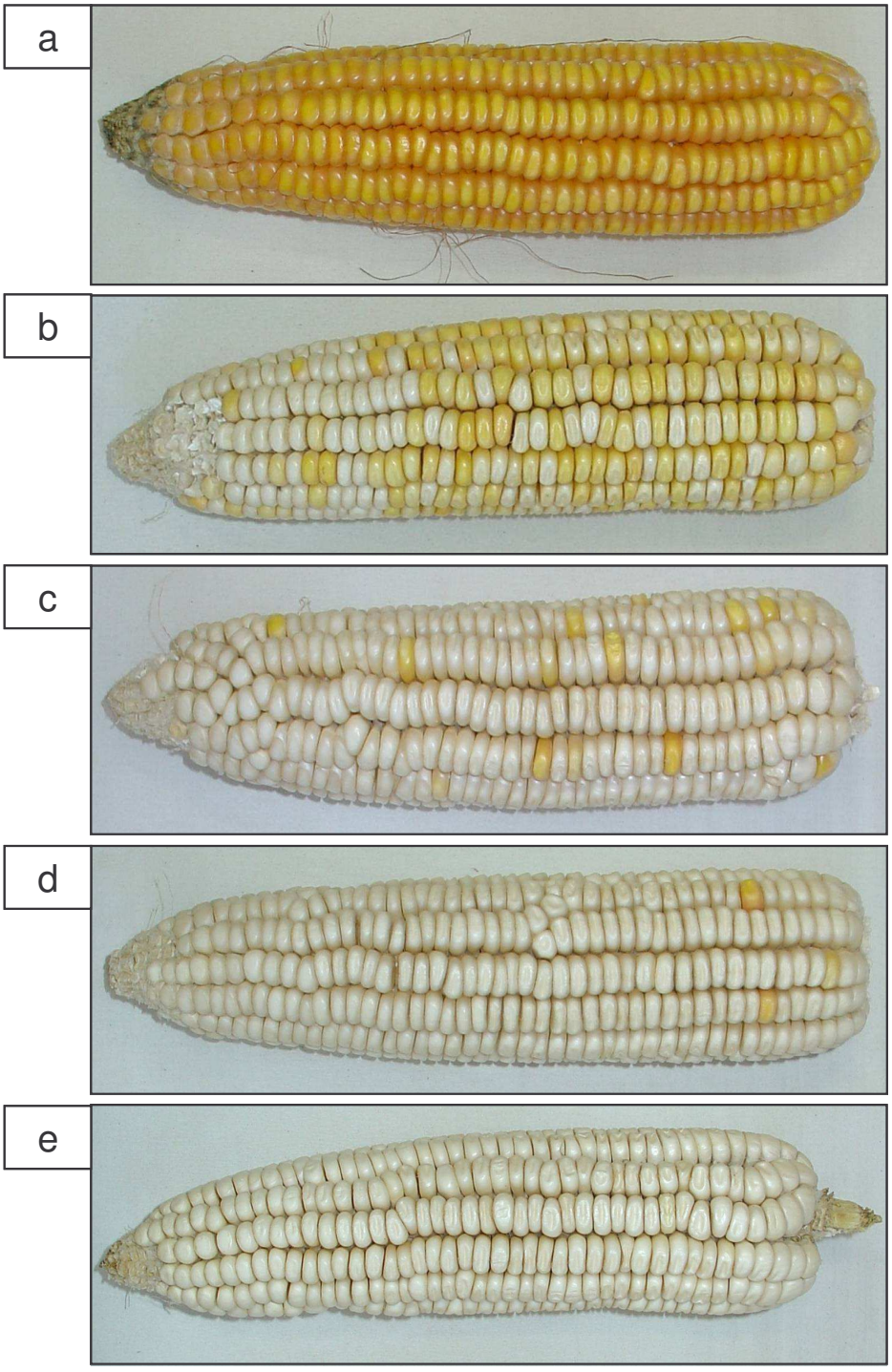

Figure 3.13 Example of out-crossing between yellow and white maize at Lichtenburg. (a), (b), (c), (d) and (e), at increasing distances from the yellow maize field.

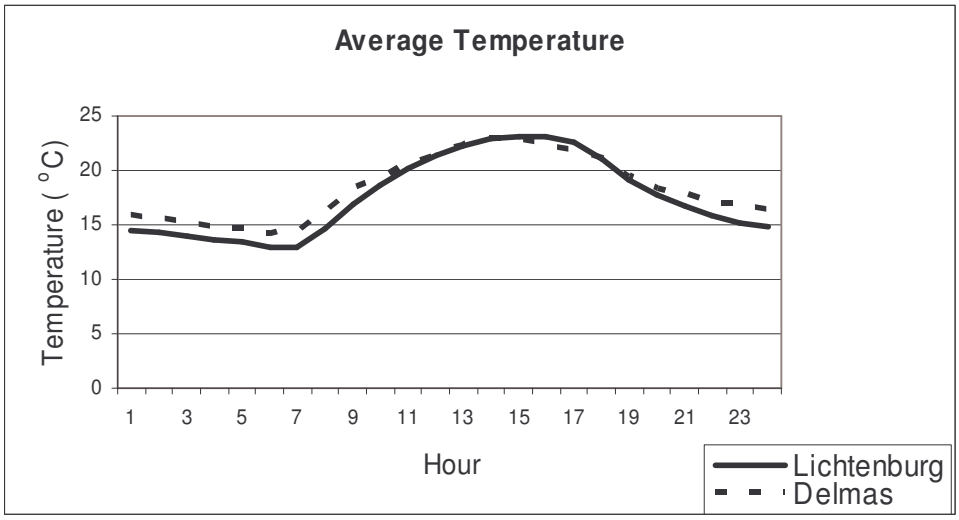

Figure 3.14 Average hourly temperatures in Delmas and Lichtenburg for 9 flowering days, respectively.

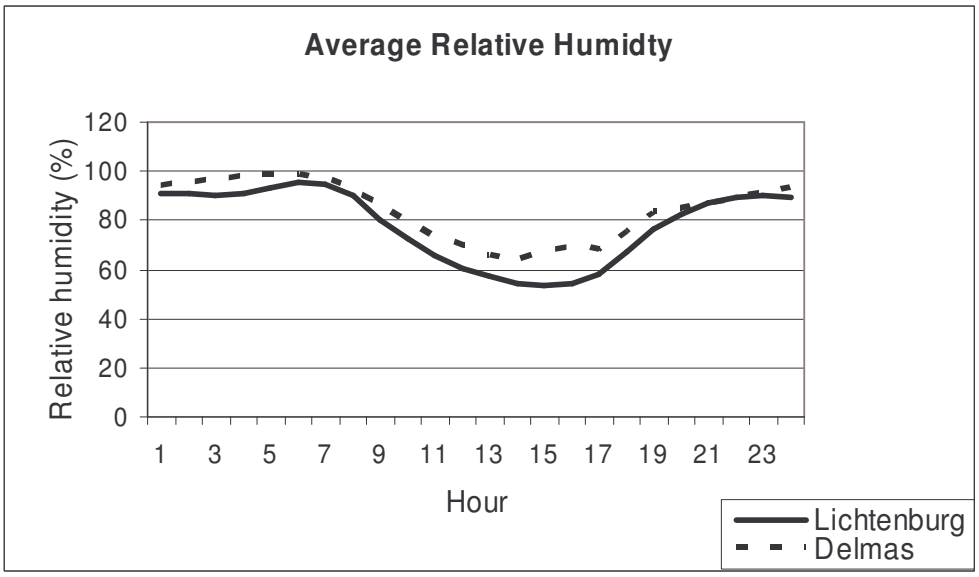

Figure 3.15 Average hourly relative humidity levels in Delmas and Lichtenburg for 9 flowering days, respectively.

Appendix A Yellow seed count in Delmas

	Row number																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	90			45	39	41	10	19	59		6	4		26	17	30	17	16	15	17	3	11	3	10	9	7	10	4		11
2	100		60	29	31	16	28	21	31	29	4		1	7	11	24	11	4	9	11	5	9	2	5	10		6	10	1	1
3	90	60	54	21	35	17	19	14	40		30				24	10	1	4	9	13	1	4		1	11	7	4	5	1	8
4	100		40	12	24	25	7	33	34	21	28	22		7		15	11			10	12	3		1	21	4	10	5	0	2
5	100			35	40	27		16	22	22	15		3	4	15	3	1		10	3	6	3	1		3	2	8		3	2
6	80	50	80	54			26	29	15	10	15		10	7	8	5	4	1	13	3	1	4	10	3	0	1		3	10	10
7	90	60	62			41	17	12	14	26	12	13	13	8	12	7	6	1		5		3	2	1	3	1	3	2	3	1
8	100	80	80	51	45	33	23	13	20	28		6	21	5	11	2	7	15		0	8	1	3		8		1		10	1
9	90	80		24	18	33	25	25	41	30		5	4	8	62	5	13	4	0			2	10	6	4		5	3	8	3
10	100	50		57	41	24	24	32	12	16	10	17	14	2		1	6		7		6	9				10		4	3	1
11	80	90	60	28	27	14	17	29	13	15	14	6	11	2	6	4	0		5	7	3	4	2			3		4	10	
12	80	100	50	18			43	14	12	5	23	5	7	4	10	6	1		13	0	9	3	6		3	0			6	5
13	50	50	50	38	48	16	15	13	9	1	14	35	8	4	3	5	1			19	1	3		8		7	6		3	6
14	80	60		35	64	35	34	7	51	0	21	13	2	24	8				4	3	4	5	2	10	1	6	10		2	10
15	50	80	55	26	29	75	41	17	13	16	15	5		6	3	8	5	12	14	4	9					5			8	10
16	80			58	27			23	21	10	10	5	13	7	4		2	6	7	11			13	1	1	4			6	7
17	80	60	50	66	18		31	55	25	12	17	16		7	4		0	20	5	35	4	4		6		3		1		10
18		50		55		39	28	17	17		11		4	9	5		17	2		43	10	4	2	6	2		4	2	3	10
19	90	80	50	24	34	48	58	26	9		5	8	60		9	24	7	9	3	4		4		3	2			3	1	10
20	90	50	31	57	53	46	45	19		16	12	13	5		9	50	5	8		9	4	6	12	6		16	4	9		
21	90		60	58	62	76	25	19	11	17		13	8		33	7	10	20	38			3	8	2		15	10	7		
22	90		60	59	33	40	38		24	14	33		6	16	40	38	5	7	17	5	4	5	4	5	5		0	10	8	
23		60		66	55	61	34	26	21	19	21	15	36	8	8	5	44	7	6	11	1	1	9	5		0		0	10	10
24		60	80	52			46	33	35	15	44	9	7	9	14	6	17	40	32	3	1	1	6	5			7	10	8	6
25	80		33	106			50	41	27	21	26	15	5	7	12	5	17	23	37	6			5	2	10		6	10	7	4
26	100	80	60				41	39	18	17	9	8		3	10	1	4	26	18	9			2		1	5	3	5	0	7
27	90	80	80	70		87	57		35	32	18	11	11	11	6	6		13	7	6		10	2	10		8		9	4	3
28	90		80	76	87	42	33	47	39	28	31		9	4		9	16	11	56	14	5	11	10	3		6	10	0	10	7
29	90	80	67	53	38	20		19	20	53	49	3	19	10	21	6	3	6	12	0	4	3	10	3		4		6	2	9
30	90		90	65	60	46	60	43	24	39	23	4		11	13	7	4	3	24	21	2		10		4	5		10		9
31	100	60		61	112		45	40	59	29	31	22	6	7		9		11	10	15	3	4	5	4			10	9	10	10
32	90	40	80	85	58	43	35	27	26	19	11		9		8	21	10	16	11		10	3	3	1		8	7	5	10	10
33	90	50		77	59	67	53	50	19	18	18	3	23	27		7	5	2	44			2	5	1		10	10	6	10	3
34	80		80	76	61	29	50	46	33	15	24	24	11	6	14	13	10	7	15		13		6		3	8	0	11		3
35	100	80	76	53	44	27	59		13	16	19	5	6	31	18	6	7		17		3		2	10	1	0	0	5	6	
36	100	80	80	92	72	73		46	24	16		25	19			23	7	9	24	10	6	3	12	4	9	6	5	2	3	6
37	90		90	60	72	66		25	17		13	20	6		30	57	11	13	9	8	6	6	4		7	8	10	10	10	10
38	100			56	68	48		45	28	26	14	13	19		13	15	17	14	3	11	5	5	4		3		10	0	5	3

	Row number																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
39	90	90	90	59	53	50		27		19	17	9	22	21	25	10	18	6	16	9	9	15	7	5	5	5	4	3	1	4
40	80		100	80	52	60	57	33	35	5		13	5	28	10	42	8	14	9	14	10	3		3	10	5	10	4	10	4
41	90	80	100		29	36	42	46	48	22		25	1	70	34	9	4	15		16	4	6	5		10	10	20	1	10	10
42	90	90	80	45		51	17	63	23	19	12	11	13	0		16	8	6	15	16	5	1	6	3	4	4	5			10
43	90		60	50	46	82	33	6	13	7	17	12	17			11	17		37	37	10	4	3	9	3	7	8			10
44	100	80		50	18		47		14	22	35	5	29	30	19	13	3	4	1	27	35	3		9	20	7	6			10
45	90	90		43	33	43	42	20	21	32		12	8	26	12	7	15	6	12	27	9	7		1	8		6	7	3	6
46	90		40	60	45		36			24	36	13	29	0	20	11	42	9	14	14	15	4	2	5	7		6	9	6	10
47	90	80	71	50	40	24	34	27	26	25	28	14	20	35	13	5	2	3	15	12	13	7	3	6		6	6	2	0	10
48	90	80			13		33	19	16	21		16	10	36	14		6		20	6	7	10	10	4		9			5	0
49	90	90	80	50	39	19	43	34	17	11		9	12	12	7	7	8	17	12	11	13	4	5	4	8	0	7	10	7	10
50	11	80		56	46	36		25	32	19	43	14	26	11	12	9	8	25	13	20	12	4		9	7	7	10	9	9	10
51	50		50	52	37	40	34	31		13	13	8	28	0	14	13	10	7	8		7	5	3	4		5	9		0	
52	80	80	49	66		24		14	15			28	19	18	9	5	20	6	19	3	2	8		10	2	10	7		3	
53	90			30			45	14	17	21	16	15	18	17	5	11	11	17	13	10	14	10	4	9	10	5	1		10	
54		50	60	36	52		21	19	10		20	21	22	18	25	14	11	8	14	14	6	10	5	4	3	7	3		3	
55	90	50	50	42	34		33	26	3	8	14	18	11	9	7	11	11	14	11	12	5	1	1	3	8	5	7		4	
56			49	36		28	31	31	10	5		18	27	11	9	5	14	14	5	13	5	7	7	6	6	6	5		11	
57	80	50	0	53		30	19	38	6	10	48	31	15	9	5	5	10	4	7	13	7	4	1		4		5		10	
58	50	50	50	55	30	58		11	10	4	28	18	19	23		9	4	7	23	7	5	3	3	9	3	6	6		2	
59	50	50	43	39	21	29	9	12	16	8	16	15	23			1		11	1	9	12	10	7	5			6		5	
60	50	80		60		39	16	10	17	74	28	13	14	6		14	15	6	1	18	2	7	4	7		6	0		8	
61		10		50	33	29	27	24	12			10	9	11		14	13	17	43			7		9	7	3			9	
62	90	60		34	17	34	32	9	14	11	25	13	13	4			11	10	15	9	6	4		3	10	20			9	
63	90			40		16	39	13	32	29	26	11	13	8			7	4	8	10	7	6	8	5	6	8			4	
64				80	36	43	49	15	78	38	20		10	6			8	7	8	19	7	8	9			7			8	
65						25	24	20	21	32			19	9			16	11	11	47	5		2			4				
66	80					25	28	18	33	16			10	19			12	29	10		13	0	2							
67	80					26		26	42	10			12	33							10	5								
68										39			11	22			14	16			9	5								
69																	15	8			13	4								
70																	8	18			30	8								
71																	25					6								
72																	7					9								
73																						10								
74																						10								

Appendix B Yellow seed count for Lichtenburg

Transect	1	2	3	4	5	6	8	9	10	12	14	16	18	20
1	198	164	59	35	18	24	19	20	10	13	14	11	10	5
2	201	102	51	31	40	36	30	19	13	9	7	7	5	3
3	320	132	59	38	53	65	28	16	23	7	10	4	12	2
4	181	166	104	62	70	56	31	16	12	16	14	20	10	7
5	220	222	117	53	45	47	44	25	20	17	23	14	11	9
6	234	141	110	62	53	68	41	19	18	25	30	22	17	6
7	218	151	94	58	74	58	35	40	22	21	15	18	28	13
8	234	137	76	53	52	52	43	38	30	23	32	16	24	16
9	198	125	122	50	55	54	44	44	37	25	33	13	25	11
10	210	164	88	62	53	53	46	37	25	18	13	22	17	21
11	177	154	77	50	40	65	45	38	22	29	25	15	21	19
12	211	188	131	60	84	47	30	48	25	25	24	12	29	11

Transect	22	24	26	28	30	32	34	36	38	40	42	44	46	48
1	6	12	7	1	7	3	2	2	1	3	1	1	2	4
2	2	7	5	1	1	1	2	0	0	0	1	3	2	0
3	2	7	3	7	1	1	2	2	0	1	1	0	1	3
4	7	6	7	8	1	2	1	2	1	0	2	2	1	1
5	14	9	7	4	3	4	2	1	5	1	1	2	4	1
6	11	10	10	8	5	4	1	3	3	0	3	1	2	1
7	15	13	5	7	3	5	6	5	6	3	3	3	1	5
8	8	4	7	5	3	2	5	8	2	6	4	5	3	7
9	20	8	9	12	2	7	5	6	11	5	6	2	7	1
10	20	12	12	16	13	11	8	9	5	4	3	3	3	2
11	23	19	12	19	10	8	12	8	13	11	4	6	3	6
12	14	14	24	13	12	7	6	4	12	3	5	5	4	6

Transect	50	52	54	56	58	60	62	64	66	68	70	72	74	76
1	0	1	1	1	2	0	0	0	0	2	0	2	1	0
2	1	1	0	0	0	0	1	0	0	0	0	2	0	1
3	1	0	0	0	0	1	0	2	1	1	0	0	0	0
4	1	2	0	0	1	1	2	0	0	0	0	0	1	0
5	2	1	2	1	0	0	2	1	1	1	1	0	0	1
6	1	1	0	1	1	2	1	1	1	1	0	0	0	0
7	1	4	4	7	2	1	2	0	0	4	2	1	1	1
8	1	4	1	4	2	2	4	0	2	0	0	0	1	1
9	4	5	6	3	3	5	2	3	2	3	2	1	0	1
10	5	3	3	6	4	2	3	0	0	0	2	1	5	1
11	4	10	5	6	4	4	1	2	2	3	2	0	1	0
12	1	2	4	3	2	2	6	1	1	5	2	3	2	1

Transect	78	80	82	84	86	88	90	92	94	96				
1	1	0	0	1	1	1	1	0	0	1				
2	1	2	1	0	0	0	1	1	1	0				
3	0	1	1	0	2	0	0	0	1	1				
4	1	1	0	1	0	0	1	1	0	0				
5	0	1	0	0	0	0	0	1	0	0				
6	3	0	0	0	1	1	0	0	1	0				
7	0	1	1	1	0	1	0	0	1	1				
8	1	2	0	0	0	1	0	0	0	1				
9	1	3	2	1	0	2	0	0	1	0				
10	3	1	2	1	1	1	0	0	0	0				
11	2	0	1	0	1	0	1	2	0	2				
12	3	2	3	1	4	3	2	0	2	3				

Summary

Maize is an economically important crop in Africa including South Africa. To maintain and produce maize varieties with specific agronomic traits and qualities, management is required in the form of identity preservation (IP). Identity preservation is becoming increasingly important with the advent of modern biotechnology. The purpose of IP is to minimize gene flow of which the principal factor is pollen movement.

Maize pollen movement has been previously studied measuring out-crossing, measuring pollen concentrations and computer modelling. In this study, genotypic detection of trapped pollen as well as the phenotypic observation of out-crossing was used. Field trials were performed at two geographic locations in South Africa and spatial as well as temporal isolation was used to ensure that surrounding maize production did not influence the result of this study.

It was determined that although various methods of pollen preservation were not effective to maintain pollen DNA integrity, storage in CTAB buffer proved successful for up to nine months for PCR analysis. An inexpensive pollen trapping system was devised using Tween 20 coated on glass slides. From pollen trapping experiments it was determined that maize pollen, with a specific genotype, could be detected at up to 400 m from the source even though the pollen load on the traps was low.

Phenotypic evaluation of out-crossing revealed a very high incidence (between 22.3 and 39.1%) of out-crossing between yellow and white adjoining maize rows, and decreased to 1% at a distance of 25 m, and thereafter was an average of 0.36% up to 81.6 m. The two geographic locations were not significantly different in terms of out-crossing data. The analysis of out-crossing data over distance, determined that distance is not solely responsible for the pattern of out-crossing. The analysis of weather data taken during the flowering period indicated that temperature and relative humidity were not significantly different across the different locations. However, wind differed significantly between the different locations with more relevant wind, regarding the orientation to maize plots, in Delmas than in Lichtenburg.

Low levels of out-crossing were detected using PCR that would otherwise have remained undetected. The 35S promoter from the Bt gene was detected at 0.12 and 0.9%, respectively, across the different locations, in sampled cobs of white maize up to a distance of 2 m. The quantification of the Bt gene in yellow hybrid seed was consistent with expected values, taking the development of the yellow maize parent into consideration.

During the course of this study different areas of research were identified that have not been addressed adequately in this or other studies, this includes, in hind sight, several ways in which the experimental design of this study could have been improved. The assessment of the impact of individual environmental variables on pollen longevity warrants a more detailed study. The correlation between pollen

DNA viability and fertilisation potential should be further investigated. It would also be useful to have included data over multiple years but was not possible due to the time constraints of an M.Sc.

In conclusion, this study has determined that maize pollen of a specific genotype can be detected at 400 m from its source and that even though out-crossing declines rapidly up to 25 m, out-crossing events average 0.36% up to 81.6 m. Out-crossing is determined by distance in conjunction with environmental factors, making geographic specific data important for region specific identity preservation requirements. This study makes an important contribution to available data on pollen movement, as no other published data is available for South Africa.

Opsomming

Mielies is ekonomiese belangrik in Afrika insluitend Suid-Afrika. Identiteitspreservering (IP) is nodig om gewasproduksie te handhaaf met spesifieke agronomiese en kwaliteit eienskappe. IP het al hoe meer belangriker geword na die ontwikkeling van moderne biotegnologie. Die doel van IP is om die vloei van gene te beperk waarvan die grootste faktor stuifmeelbeweging is.

Mielie stuifmeelbeweging was al voorheen bestudeer d.m.v. oopbestuiwing, stuifmeelkonsentrasie bepalings en rekenaarmodellering. In hierdie studie is genotipering van gevange stuifmeel asook fenotipiese oopbestuiwing gebruik. Veldproewe is by twee geografiese gebiede in Suid-Afrika gedoen, en tyd en spasie-isolasie is gebruik om te verseker dat mielieproduksie in die omgewing nie die resultate van die studie beïnvloed nie.

Dit is vasgestel dat alhoewel verskillende metodes nie effektief is om stuifmeel DNA te bewaar nie, CTAB buffer die stuifmeel DNA vir tot nege maande vir PCR analise kan stoor. ’n Lagie Tween 20 op ’n glasplaatjie is gebruik as ‘n goedkoop stuifmeellokstelsel. D.m.v. stuifmeellokeksperimente kon dit vasgestel word dat stuifmeel van ‘n spesifieke genotipe tot op 400m van die bron gevind kon word al was die stuifmeellading laag.

Fenotipies evaluering het vasgestel dat daar hoë vlakke (tussen 22.3 en 39.1%) van oopbestuiwing tussen aangrensende geel- en witmielies is en dat dit afneem

tot 1% op 25m, met die gemiddeld daarna van 0.36% tot en met 81.6m. Daar is gevind dat die twee geografiese gebiede nie betekenisvol verskillend in terme van oopbestuiwingsdata is nie. Die analise van oopbestuiwingsdata het getoon dat afstand nie alleenlik verantwoordelik is vir die patroon van oopbestuiwing nie. Die analise van die weer tydens die blomtydperk oor die gebiede wat bestudeer is het getoon dat temperatuur en humiditeit nie noemenswaardige verskille het nie. Die wind was wel verskillend veral ten opsigte van die relevante winde, ten opsigte van die ligging van die proewe.

Lae vlakke van oopbestuiwing is bespeur deur die gebruik van PCR, wat nie andersins waargeneem sou word nie. Die 35S promotor van die Bt geen is aangedui teen 0.12 en 0.9%, onderskeidelik, oor die verskillende gebiede, in steekproewe van witstronke tot en met 2 m. Die kwantifisering van die Bt geen in geel baster saad was in ooreenstemming met die ontwikkeling van die geel ouer.

Gedurende hierdie studie is verskillende aspekte raakgesien wat nie hier of in ander studies voldoende aangespreek is nie. Dit sluit in, die gedagte aan verskillende maniere waarop die eksperimentele ontwerp van hierdie studie verbeter kan word. Die assessering van die impak van omgewingsveranderlikes op stuifmeel oorlewing verg verdere studie. Die korrelasie tussen stuifmeel DNA lewensvatbaarheid en bevrugtings potensiaal moet ook ondersoek word. Dit sou ook nuttig gewees het om oor meerjarige data te beskik, wat weens die tydsbeperking van die M.Sc. studie, nie moontlik was nie.

Gevolglik het hierdie studie getoon dat stuifmeel van ‘spesifieke genotipe tot en met 400m vanaf die bron bespeur kan word en dat alhoewel oopbestuiwing vinnig afneem tot 25m, dit teen 0.36% voorkom tot en met 81.6m. Dit is ook gevind dat oopbestuiwing deur die kombinasie van afstand sowel as omgewingsfaktore bepaal word. Dit beteken dat geografiese data die belangrike oorweging is met IP. Hierdie studie maak ’n belangrike bydrae ten opsigte van die beskikbare data oor stuifmeelbeweging, veral omdat daar nie ander gepubliseerde data vir Suid-Afrika beskikbaar is nie.