Managing gene flow: A prerequisite for recombinant DNA biotechnology

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Submitted in fulfilment of requirements for the degree

Philosophiae Doctor

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Bloemfontein

South Africa

2008

A humble offering, placed at the Lotus Feet of

Bhagavan Sri Sathya Sai Baba

and so,

it is with love and servitude that I dedicate this research to the many

subsistence farmers of Africa. You are the ancient foundation of our

continent and this research was performed with a fervent hope of

enlightenment and as a modest attempt to lighten some of your plight.

ACKNOWLEDGEMENTS

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ABBREVIATIONS AND ACRONYMS

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
HT	Herbicide tolerance
i.e.	id est
IR	Insect resistance
k	Thousand
L	Litre
LOD	Limits of detection
M	Molar
m	Metre
m^2	Metre square
mg	Milligram
ml	Millilitre

min	Minute
mM	Millimolar
m/s	Metres per second
N	North
NaCl	Sodium chloride
NE	North-east
NNE	North-north-east
NNW	North-north-west
NW	North-west
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

ºC   Degree Celsius

%   Percent

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PREFACE

Genetically modified organisms (GMOs), refers to organisms that contains a transgene which was developed using recombinant DNA technology. This technology has mostly been applied to food crops such as maize and soybean, conferring transgenes with beneficial traits so as to increase crop yield, reduce input costs as well as reduce impact on the environment. In view of the substantial impacts agriculture has on biodiversity, GMO crop seemed a panacea. However, since its introduction, genetically modified (GM) crops have been surrounded by much controversy, as the unforeseen impacts in terms of environmental risks, human health, socio-economics and intellectual property rights to just name a few have plagued these crops. A great deal of research into the GM crop risk factors is required so that the safe use of this technology can be implemented.

Gene flow in GM crops, specifically pollen-mediated gene flow has been recognised as a potential area of risk in terms of the environment and human health. Adventitious commingling of GM maize or soybean with non-GM varieties, land races or wild relatives could result in compromised niche markets, carry health risks (pharmaceutical or industrial traits) or negatively impact the environment. Thus it is important to understand the factors affecting maize gene flow to be able to manage any potential negative impacts thereof.

Despite the commercial propagation of GM maize and soybean for 10 years in South Africa, which includes a high adoption rate, very little research and no published data is forthcoming on the potential impact of GM gene flow in maize and soybean in South Africa. In this thesis, I have endeavoured to provide basic data with regard to GM gene flow which in hindsight should have been used to inform regulatory decisions over the last 10 years, regarding the release and management of GMOs, in order to be able to manage the technology and minimise risks to the environment and human health.

The thesis contains a literature review, three research chapters and a concluding chapter in which I make specific recommendations on management practice to minimize gene flow where necessary. The Literature review contains four sub-sections that have been or are in the process of publication. In this chapter, all figures and tables are contained within the text to maintain an easy reading style. The research chapters are written in article format and the figures and tables have been placed after the reference list. When reading this thesis you will experience some repetition between the introductions in the different research chapters – the reason for this is to place each research question within the correct context. Furthermore, the soybean research on potential pollen-mediated gene flow and pollen-mediated gene flow has been combined into one chapter. However, I felt that the corresponding chapter for maize was too cumbersome, in terms of the volume of data, and have separated these aspects into distinct chapters.

Any research on the impact of genetic engineering is going to be controversial, depending on your point of view. In this thesis, I have attempted to traverse the path less known and provide some very basic yet essential answers to some of the most obvious, yet overlooked questions that should be asked including:

- **Does out-crossing occur in self-pollinating soybean?**
    - The basis of this question is that most if not all of the soybean varieties grown in South Africa are considered self-pollinating and gene flow is not considered significant. However, there is no evidence for this.

- **What are the factors affecting gene flow in maize and how much of an impact does it potentially have?**
    - There is very little consideration in South Africa on the need to minimize gene flow in maize – if only for niche non-GM markets. Most farmers do not apply any management strategies to minimize cross pollination and seed producers generally use regimes to ensure 96% to 99% seed purity.

- **What practical management practises could be applied to minimize GM commingling?**
    - The tolerance level of commingling often depends on the specific GM or its use. For example, for a field trial of a GM crop producing a pharmaceutical, commingling should not be allowed. However, depending on GM labelling requirements for approved GM crops, low levels of commingling might be acceptable.

When you read this thesis, please consider for a moment the importance of trying to answer the very basic yet most fundamental questions regarding the introduction of GMOs into our environment: What is the impact of this technology considering the simplest of biological process – gene flow?

CHAPTER 1: GENERAL INTRODUCTION

In the 2008/2009 planting season, South Africa entered the 11th year of growing GM (genetically modified) crops (James, 2007). The continued increase in the adoption of biotech crops is an indication that GM crops have been well received in South Africa compared to the rest of the continent that chooses a more conservative approach (James, 2007).

South Africa has commercialized GM crops since 1997 and insect resistant (IR) maize and cotton, herbicide tolerant (HT) soybean as well as stacked traits (IR and HT) for maize and cotton have been approved for general release (James, 2007). Despite this, there are a number of concerns surrounding the introduction of GM crops that need to be addressed. The intention with GM crops is to have a positive impact in terms of production, food security and the environment compared to conventional agricultural practice that is widely acknowledged as damaging to the environment (Carvalho, 2006; Castle *et al*., 2006). However, GM technology has also introduced additional complexities that cannot be ignored:

- Intellectual property rights and royalties
- The impact of GM on non-GM crop production in terms of niche markets
- Environmental impacts of GM compared to conventional agricultural practice
- Coexistence of GM and non-GM crops

Coexistence of GM crops with its conventional counterpart is generally overlooked. Non-GM products have become a niche market due to the introduction of GM. Furthermore, the commingling of undesired second or third generations GMOs in the food or feed market would be unacceptable as it could have dire consequences on human and animal health as well as the environment (Marvier and Acker, 2005; Moschini, 2006; Spok, 2007).

One foremost aspect of coexistence is gene flow, in particular pollen-mediated gene flow (Jank *et al*., 2006; Moschini, 2006; Lee, 2008). This has been largely neglected, probably due to the lack of understanding its importance. The aim of this study was:

1) To combine molecular techniques with field trials to study the self-pollinating nature of soybean and determine the extent of maize pollen movement and out-crossing under South African environmental conditions.

2) To make recommendations based on the data generated, on how pollen-mediated GM gene flow to non GM varieties or landraces can be minimized where necessary.

CHAPTER 2: LITERATURE REVIEW

2.1 The overall impact of recombinant DNA biotechnology in agriculture

Three decades have passed since the development of recombinant DNA technology and its impact on various areas of science and society is evident (Cohen *et al.*, 1973). Recombinant DNA refers to a DNA construct that contains a fragment of DNA from a foreign source, which once incorporated into the genome of an organism, is known as a genetically modified organism (GMO). This breakthrough, a mere two decades after the discovery of the structure of DNA (Watson and Crick, 1953), has made ground-breaking advances in the medical and agricultural sciences. In agriculture, recombinant DNA technology has added a new dimension to crop improvement, giving rise to biotech crops.

Due to an expanding global population, the agricultural industry is under constant pressure to increase food production (Endo and Boutrif, 2002). Currently, the world population is approximately 6.5 billion and is predicted to soar to an approximate 8.9 billion by 2050 (UN Report, 2004). Furthermore, it is predicted that global warming will also adversely affect agricultural production especially in developing countries (Houghton, 2005, Mendelsohn *et al.*, 2006; Schlenker *et al.*, 2006). Since the implementation of recombinant DNA in agriculture, it has been strongly suggested that biotech crops will aid in the alleviation of hunger and poverty (Endo and Boutrif, 2002), by developing crops with increased yield and low input costs

such as insect resistance and herbicide tolerance. Whether this highly publicised benefit of GM crops will hold true for the impoverished, has yet to be determined.

In 2007, GM crops accounted for 114.3 million hectares in 23 countries (12 developing and 11 industrial) compared to 221.8 million hectares conventional crops (Fig. 2.1.1), representing 34% of global agriculture. Since its introduction in 1996, the area planted of GM crops has increased nine-fold in the world (James, 1997). Currently, the major GM crops are canola, cotton, maize and soybean. In the 2007 production season in South Africa, GM crops made up 80% of soybean (herbicide tolerance), 90% of cotton (insect resistance and herbicide tolerance) and 57% of white and yellow maize (insect resistance and herbicide tolerance) (James, 2007). South Africa remains the only country in Africa to commercially produce GM crops and in 2007 contributed approximately 1.8% to the global production of biotech crops. South Africa has annually increased GM crop production since 1997 and the adoption of second and third generation GMOs is imminent.

First generation GM crops are those with agronomic traits, for example, insect resistance or herbicide tolerance. Second generation GM crop have value-added traits for consumers such as enhanced nutritional value and third generation GMOs are aimed at producing pharmaceuticals or compounds for industrial use (Smyth *et al.,* 2002). Second generation GMOs have the potential to provide consumers with vitamin enriched food (Falk *et al.*, 2002) while third generation GMOs provide the prospect of low-cost drugs (Twyman *et al.*, 2003; Elbheri, 2005). The envisioned benefits of pharmaceutical GMOs are appealing, considering the possibility that a

plant-made pharmaceutical for infectious diseases may soon be a reality that South Africa would be amiss to ignore (Elbheri, 2005).

Despite the proposed benefits of biotech crops including the alleviation of poverty and hunger, there are many considerations surrounding GMO adoption. These include the impact on society, the environment, the economy, and agriculture (Fig. 2.1.2). Furthermore, the increased adoption of GM as well as the development of second and third generation GMOs presents a number of concerns regarding safety and challenges for coexistence. These concerns include lack of consideration for regulatory frameworks, intellectual property, cost benefit, the requirement for identity preservation in the development of niche non-GM markets, societal issues including acceptance, ethics and socio-economics as well as protection of the environment.

- ***Regulatory frameworks***: The purpose of a regulatory framework is to manage the development and introduction of GMOs into the environment and to be able to capitalize on the potential benefit of this technology, while curtailing possible risks to human health and the environment. Regulatory frameworks tend to be specific to the needs of each individual country and often differ in terms of approach and stringency. The Cartagena Protocol on Biosafety is an instrument to assist developing countries when introducing GM crop. It imposes minimum regulatory requirements that must be incorporated into the framework but leaves the application to each adoptive country.

- **Intellectual property rights:** The patenting of novel gene sequences and the requirement to pay royalties raises a concern at the farm level, with regard to seed saving and sharing which is culturally significant, especially in developing countries.

- **Cost benefit:** One of the primary aims of developing GM crops was to reduce input costs by among others reducing pesticide usage. Current GMOs provide potential cost benefits to farmers, including the subsistence farmer, but not to the consumer. However, the impact of farming subsidies in developed countries compared to the lack thereof in developing countries is seldom taken into consideration during agricultural cost analysis. Furthermore, the reaction of the market to GM crops is also not considered.

- **Identity preservation:** The introduction of GM crops into existing agricultural practice has resulted in the need for a management system known as identity preservation (IP). The importance of IP is to maintain GM traits as well as ensure that conventional varieties remain non-GM in terms of market requirements. IP includes the farm level management of coexistence and segregation of which one of the most significant considerations is pollen-mediated gene flow.

- *Human health*: At a societal level, many concerns have been raised, regarding the safety of recombinant DNA technology and the long term effect of human health are still largely unknown. Health concerns include: the potential allergenicity of GM food, transgene transfer from GM food to intestinal micro-flora, the occurrence of unintended effects as well as altered nutrition value (Kuiper *et al.*, 2002). Although, Biotech companies perform risk assessments on the safety of GM food, the long term effects on human health are still unknown.

- *Consumer acceptance*: Current GM crops do not provide any benefit to consumers, except for the promise of cheaper food. Consumer rights are well established in most countries including South Africa and in many countries GM products are labelled in the same way as additives and colourants. In contrast to consumers in Europe, consumers in South Africa are largely unaware of the existence of GM, let alone the presence of GM products in the food chain (Rowland, 2002; Viljoen *et al.*, 2006). Consumers determine what drives the market and consumer attitudes to GM food will prove the final determinant in the GM debate.

- *Ethics*: GM crops are often marketed on their potential to alleviate starvation in the developing world with specific reference to Africa. The marketing promises food security and cheaper food (Cohen, 2005). However, these promises have not yet been realized. Furthermore, it is incorrect to make

comparisons between developing and developed countries in terms of food security and the impact of technology thereon since farmers in Africa do not receive subsidies like their counterparts in developed countries in order to make "cheaper food" a reality. In addition to this, the patenting of genes resulting in a technology fee makes the GM technology unaffordable for the "starving "masses.

- **Socio-economics:** Agriculture forms such an important aspect of South Africa's economy that it is important to consider the socio-economic impact of introducing GM crops on farmers and small scale farmers. GM crops have the potential to improve the economic status of farmers through increased production and lower input costs but it can also have negative impacts in terms of the requirement to acquire chemical inputs for traits such as herbicide tolerance (Cohen, 2005). In addition, the development of GM seed has created niche markets in commodity trading for non-GM and organic products. It is also envisaged that value added GM traits such as vitamin-enriched products may prove desirable to consumers. However, most farmers in South Africa are not aware of the impact that planting GM versus conventional crops may have on their ability to sell their produce and issues of market acceptance, safety and patents are not even considered.

- **Environment:** It is argued that GM crops can do no more harm in terms of the environment compared to conventional farming. However, as this

technology is relatively new, it is important to ensure that the environment is protected and biodiversity conserved.

- Non-target organisms: Very little is known on the impact of GM, especially those producing endotoxins, on non-target organisms including microbes, non-*Lepidoptera* species and small vertebrates.

- Target insects: The recent development of resistance in the target organism in South Africa may have important environmental implications (Van Rensburg, 2007).

- Weediness: The introduction of GM traits such as herbicide tolerance and the subsequent increased use of herbicides may contribute to the development of weediness in crops as well as other plants such as Johnson's grass (Clements *et al.*, 2004).

- Gene flow: Pollen-mediated gene flow impacts more than just the diversity of genes in landraces and/or wild relatives. GM gene flow to conventional non-GM or organic crops has important economic consequences due to the loss of market value for such products (Zepeda, 2006; Demont and Devos, 2008; Lee, 2008). A further important but little considered impact of gene flow, is its contribution to the development of resistance in the target insect through potential exposure to sub-lethal doses of toxin as a result of low levels of GM in saved seed or maize refugia where out crossing has occurred (Chilcutt and Tabashnik, 2004). Furthermore, there is the possibility of transgene escape via horizontal gene flow into soil bacteria which could alter the genetic capabilities of beneficial soil bacterium. Thus GM biotechnology

could have serious impacts on the environment and this should not be taken lightly.

When GM crops were developed and subsequently first commercialised, it was not envisioned that it would impact so many aspects of society. The primary aim of GM crops as put forward by companies and protagonists was to alleviate hunger and poverty (Chetty and Viljoen, 2007). When initially released, the social, environmental, economic and regulatory implications of GM crops were not considered. Nonetheless, the impact in these areas is undeniable, and has to be dealt with in a proactive manner. Although it is often argued that many of the potential impacts are similar or more severe for current conventional farming practice, it must be noted that the introduction of GM technology has added a complexity that from published literature does not appear to have been considered.
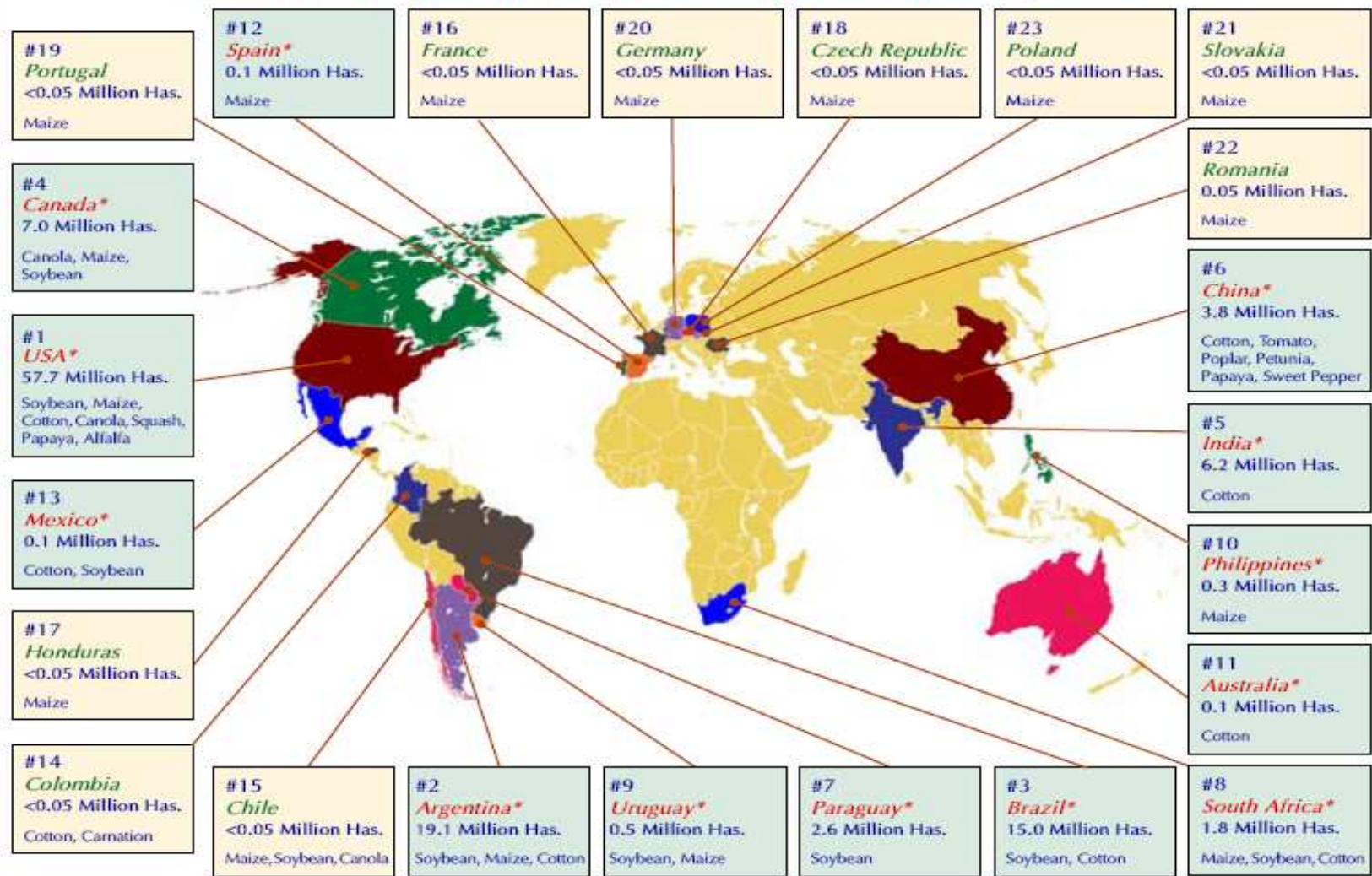
Figure 2.1.1 GM crop production (2007) in all 23 countries including the area and crop planted (reproduced from James, 2007).
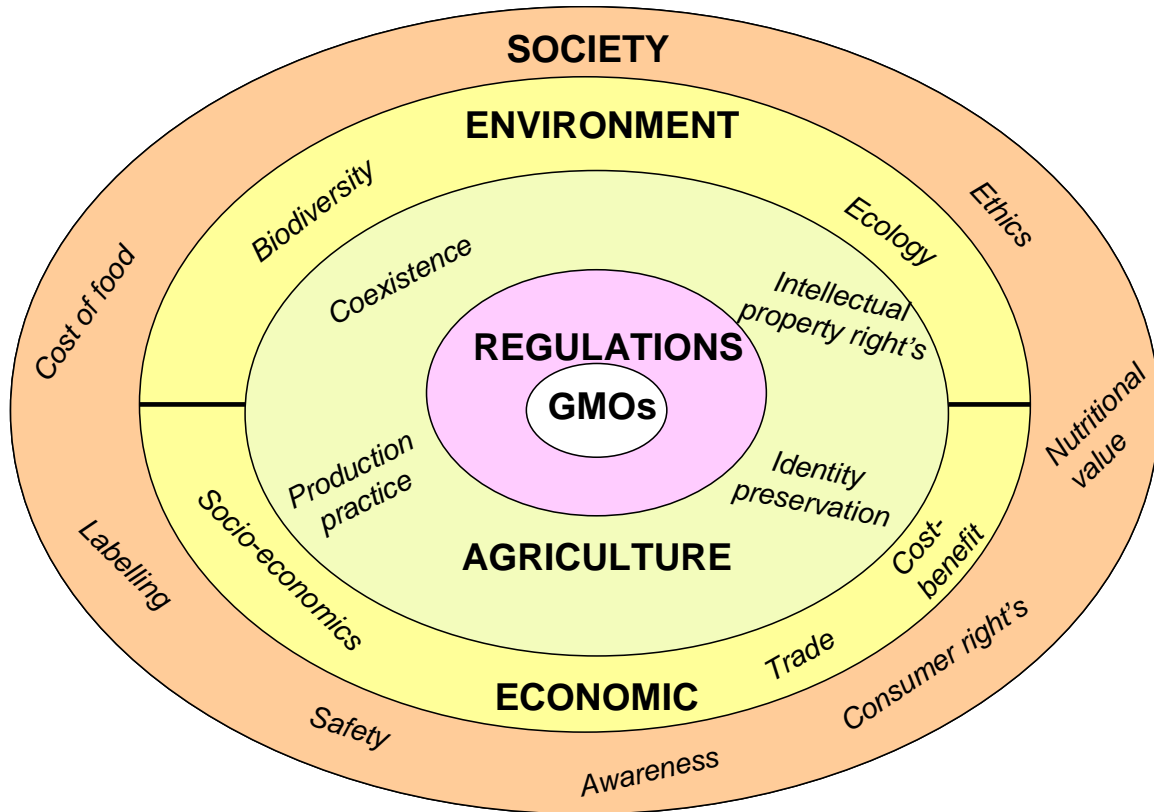
Figure 2.1.2 Diagrammatic representation of the impact of agricultural biotechnology in regulatory frameworks, agriculture, the economy, the environment and society.

2.2   GM biotechnology: friend and foe?

The opinion piece "Biotech's defining moments" indicates a frustration shared by many scientists (Miller, 2007).   This discontent stems from a perception that regulation of biotechnology in the name of biosafety is futile and biosafety research excessive (McHughen, 2006; Miller, 2007). At the same time advocates of biosafety, are too easily branded as anti-biotechnology, unscientific and unnecessarily short-sighted.   A number of important but contentious issues are currently being debated. These include:

1) A perception that Non-Government Organisations (NGOs) stigmatize genetic modification (GM).

2) Risk assessments do not make a positive contribution.

3) Distinguishing between GM and non-GM has no scientific basis.

4) Coexistence studies between GM and non-GM are unnecessary.

5) Some regulatory systems are scientific and others not.

6) The Convention on Biological Diversity (CBD) impedes genetic engineering research as well as its promotion in developing countries

7) Mandatory labelling is unscientific (Miller, 2007).

As a result, the GM biotech community appears to be at loggerheads with itself and sadly the potential benefactors of this technology in developing countries are the losers. It is therefore necessary to depolarize the debate so that the attempts to serve the interests of Africa are realised and make GM biotechnology a "friend".

Proponents of GM biotechnology are of the opinion that NGOs continually stigmatize and undermine public confidence in recombinant DNA technology (Miller, 2007). Ironically, there are as many NGOs that unscrupulously campaign that biotechnology is a "silver bullet" to alleviate hunger in developing nations without any scientific basis. Some of the unsubstantiated statements, referring to recombinant DNA technology, include: "The biggest threats that hungry populations currently face are restrictive policies stemming from unwarranted public fears." (Prakash and Conko, 2004), "a growing number of agricultural researchers, food experts and policymakers are pointing to plant biotechnology as a critical tool that can help increase food production and alleviate hunger without depleting natural resource."(Council for Biotechnology Information, 2007) and "As Kenya faces yet another famine, food experts say that irrigation and adoption of genetically modified (GM) crops could be the way out of the perennial hunger problem." (Opiyo, 2004).

Antagonists, equally, express negative sentiment towards GM biotechnology such as "Genetic engineering in its present form cannot form part of the solution; it is part of the problem." (South African Freeze Alliance on Genetic Engineering, 2007), "African countries are being targeted by the GM industry and its lobbyists with unprecedented backing from the US government. Even food aid has been used to push GM into Africa." (GM Watch, 2007) or "It is clear that GM crops offer no benefits and cannot feed the world." (Ho, 2007). Thus, propaganda on both sides of the argument contributes to a skewed public perception of GM

biotechnology, creates confusion, mistrust and cynicism amongst consumers and scientists alike.

Many scientists who develop GMOs (genetically modified organisms) believe that risk assessments are unnecessary and/or go beyond what is required to establish a lack of risk (Miller, 2007). Nonetheless, risk assessments remain vital to determining human safety. For example, a transgenic soybean engineered to contain a protein from Brazil nut would have been fatal for those with nut allergies, had the necessary allergy studies not been performed during the risk assessment (Nordlee *et al.*, 1996). However, there is a case where a risk assessment may have proved vital. In 1989, the Eosinophalia-Mayalgia Syndrome (EMS) epidemic in the US, caused by the GM dietary supplement L-tryptophan, resulted in 37 mortalities (FDA, 2001). It is not certain whether the risk assessment performed was insufficient or whether it was performed at all. Nevertheless, by suggesting that risk assessments are excessive, GMO advocates unwittingly impede biotechnology progress by implying that the technology is above risk or that they fear scrutiny. In addition to determining health safety, environmental risk assessment is just as important. The conservation of biodiversity, including the preservation of landraces is a global concern. A recent study in the US found an unreleased transgenic herbicide-resistant creeping bentgrass introgressed into wild populations (Reichman *et al.*, 2006). Clearly, risk assessments are imperative and not futile if performed with diligence.

There is a continued debate amongst scientists, as to whether a GMO is substantially equivalent to its non-GM counter-part. Substantial equivalence implies that a GMO, with the exception of the transgene, is not significantly different to its conventional counterpart. However, the application of Intellectual Property Rights (IPR) makes a clear distinction between GM and non-GM in terms of plant breeder's rights and patenting. In fact, GM and non-GM are biologically dissimilar (one has a transgene) and the GM variety is subject to patent rights and technology fees. Thus, GM and non-GM are seen as different on more than just a biological level. Whether the scientific community agrees or not, the legalities of transgene technology prohibit classification of GM and non-GM as substantially equivalent.

The numerous examples of "gene escape" over the last few years indicate that coexistence of GM and non-GM crop requires careful management. In Nebraska 2002, Prodigene's pharmaceutical maize commingled with soybean and in the same year in Iowa, cross-pollination with conventional maize occurred (Elbheri, 2005). Prodigene's financial losses were in excess of US$ 3 million which included fines and clean-up costs. Similar incidents of accidental transgenic entry into the food chain have occurred with Starlink maize (CRS Report for Congress, 2001) and Liberty Link rice 601 (FDA, 2006). Clearly, there is an urgent need for management to allow for coexistence and minimise commingling. The entry of a pharmaceutical crop into the human food chain would have devastating implications in Africa, where the resources to deal with such a situation do not

exist. Thus, the continued examples of gene escape suggest that more research is required to prevent transgene escape.

A sector of the biotechnology community believes that GMOs are unscientifically over-regulated while others feel that regulations are insufficient. The FDA procedure to regulate GMOs is not that of approval but rather a consultation process, which is voluntary. This involves an audit of a risk assessment based on information provided by the biotech company. "During the consultation process, the FDA does not conduct a comprehensive scientific review of data generated by the developer" (FDA, 1997). Whereas the European Commission requires verification of information provided and may additionally perform necessary food safety and environmental risk assessments before granting approval of a GMO (Official Journal of the European Union, 2003). In South Africa, the Department of Agriculture through the GMO Act 15 of 1997, also performs a risk assessment audit using independent scientific expertise (Department of Agriculture, 1997; Department of Agriculture, 2005). While some regulatory systems are more stringent than others, it is uncertain which of these is more scientific. In reality, bureaucratic requirements are no indication of scientific content.

The CBD and specifically the Biosafety Protocol are often seen as an attempt to hinder the spread and acceptability of biotechnology in developing nations (Miller, 2007). In reality, the Biosafety Protocol is a facilitation mechanism to help countries deal with the introduction of GM, through the implementation of GM regulatory frameworks (Convention on Biological Diversity, 2000). Thus, it would

seem short-sighted of biotech companies, NGOs and scientists to view the Biosafety Protocol in a jaded light when the CBD has proven to be an effective enabling mechanism in developing countries.

Mandatory labelling of GMO products is criticised as unscientific and an unnecessary expense (Miller, 2007). Food products are already being labelled with regard to potential allergens, ingredients and nutritional value. In addition, market directed labels including Kosher, Halaal, vegetarian, fat-free, low-fat, cholesterol-free and gluten-free are globally accepted. Thus labelling food products with regard to GM content is no less scientific than other current market directed labels. Additional information regarding the GM status of a product would allow for consumer choice and possibly contribute to an awareness of GM (Viljoen *et al.*, 2006). However, to deny consumers the right of choice, between GM and non-GM, in product selection is unreasonable and will taint biotechnology in the eyes of economically influential consumers.

In conclusion, biotechnology can potentially benefit developing countries, but within reason. To claim that starving millions will be saved and then charge a technology fee is paradoxical. In order for this technology to be beneficial, it is important that interested parties including NGOs, government organisations and scientists work proactively to resolve conflicts. In order to depolarise the current debate and fulfil the mandate of hunger alleviation in Africa a level of transparency and forthrightness from proponents as well as opponents of recombinant DNA

technology is required. This would inspire public confidence and perhaps make biotechnology more palatable to Africa.

2.3   Ten years of GM crops - can we coexist?

In 2007, South Africa was positioned eighth out of 23 countries producing genetically modified (GM) crops (James, 2007). GM crop was introduced in 1997 and a decade later South Africa now produces insect resistant and herbicide tolerant cotton and maize, as well as herbicide tolerant soybean contributing 1.8% to global GM crop production (James, 2007). Despite South Africa's positive adoption of GM and a decade of production, there is currently no emphasis on establishing management practices for effective segregation of GM and non-GM crop. Nonetheless, with the development of second and especially third generation GM crops, establishing systems for coexistence will become a necessity (Moschini, 2006).

Coexistence refers to the effective segregation of a specific GM trait from conventional and organic production. Furthermore, segregation from other GM traits in order to meet market requirements would allow farmers a production choice which in turn allows for consumer choice. Therefore, coexistence is about satisfying the rights of both producers and consumers in terms of niche markets (Brookes, 2004; Jank *et al.*, 2006).

Since before the introduction of GM, seed producers were, and still are, required to maintain seed purity levels. Seed purity levels typically range from 96 to 99% with an accepted varietal difference of 1 to 4% (Karrfalt, 2004; Zhou *et al.*, 2006). However, after the introduction of GM, the definition for "varietal difference" has now also been expanded to reflect the adventitious presence of GM. However, due to trade regulations, requirements for organic and non-GM production and GM labelling, the tolerance levels for GM in non-GM seed is usually set at a lower threshold and can even be zero depending on the nature of the genetic modification (Demont and Devos, 2008). Non-GM or GM purity levels have to be strictly adhered to as any infringement could result in serious economic loss to the seed producer and/or farmer. The development of pharmaceutical and industrial crop GMOs has added an additional complexity to seed production and coexistence that may require zero tolerance in terms of adventitious GM to ensure human and environmental safety.

GM crop segregation is required as a result of the different types of GM crop approval (including trial release), the requirements of consumers and the use of GM crop for food or feed, respectively. This is due to the development of niche markets to maintain trait segregation, especially in the case of GM pharmaceuticals, industrial compounds and biofuels (Figure 1.3.1). Thus, there are various levels of segregation for organic and conventional crops, as well as first, second and third generation crops.

Prior to the green revolution, "organic" production was applied but not characterized as such. While initially requiring the absence of typical inputs used in the green revolution, organic crop production now also includes a requirement for the absence of GM. Organic crop production in the European Union (EU) currently stipulates 0% GM (Demont and Devos, 2008). From 2009, regulations in the EU will allow the adventitious presence of up to 0.9% GM in line with the threshold level for GM labelling (Demont and Devos, 2008). In the United States, the accepted level of GM commingling for organic production is 5% according to USDA guidelines (United States Department of Agriculture, 2002). Currently in South Africa there is draft legislation for organic production that allows 0% of adventitious GM. Thus, due to these requirements, segregation systems have to be established and require some form of certification or verification to ensure compliance.

Ironically, GM crop production is having a similar impact on conventional production similar to what the green revolution did to establish organic. GM production has established non-GM conventional production as a niche market. The adventitious presence of GM in a conventional non-GM system could either occur due to contaminated seed, unintentional farm-level commingling or post-harvest mixing (Demont and Devos, 2006). In the EU, a crop may be considered non-GM if it contains less than 0.9% GM. Currently in South Africa there is no prescription regarding the adventitious commingling of GM crop. However, the Department of Agriculture applies a 1.0% threshold for the non-GM status certification of agricultural exports.

First generation GM crops with input traits (insect resistant and herbicide tolerant) are regulated in terms of their application for either for food or feed. In the case of first generation GM crops, identity preservation is at the level of the GM event. Thus GM crops regulated for human consumption may enter the feed market without contravening regulation. However, GM events regulated for feed may not enter the food market and require segregation (Fig. 1.3.1).

Similarly, second generation GM crops which have been developed with value-added traits (vitamin-enriched) in food and feed are also regulated per GM event. Second generation GM feed crops will probably not be permitted to enter the human food chain. However, a value-added trait specifically engineered for human consumption may not have the same benefit for animals and it is likely that this type of GM event may also require segregation from animal feed unless it is shown that they are safe for animal consumption. Although as yet no second generation GM crops have been approved for commercial release, these will require segregation to maintain the value-added trait as well as ensure that it does not commingle with other food or feed.

The use of third generation GMOs to produce pharmaceutical and industrial compounds as well as for biofuels, is the natural progression of GM technology, but adds significant complexity to GM segregation practice. The slightest possibility of this type of crop commingling with food destined for human or animal consumption would be considered unacceptable. The safety implications and economic

consequences could be disastrous. Therefore, strict segregation of third generation GMOs from all other crop production systems should be mandatory.

Compared to conventional agricultural systems, there is less tolerance for the environmental impacts of GM. The prospect of transgene transfer, to landraces and wild relatives is a great concern. The conservation of biodiversity is a global issue and GM crops can compromise the genetic integrity of wild relatives or landraces via gene flow. Although gene flow from GM crops to wild relatives or landraces is just as much a reality with conventional crops, the latter are not under the control of patents and the genes involved have originated from wild relatives. Unfortunately, gene flow has already been observed with maize landraces in Mexico and Bentgrass in the United States (Quist and Chapela, 2001; Reichman *et al.*, 2006). In Africa, indigenous crops such as sorghum and cassava are an important genetic resource and must be protected from transgene introgression. Although not indigenous to Africa, landraces of maize have acquired cultural importance and are an important aspect of agro-biodiversity – especially among rural farmers. Thus, just as maize germplasm must be preserved in Mexico, the centre of origin for maize, maize landraces require preservation in Africa and it is important to establish the necessary measures to achieve coexistence.

Coexistence can best be achieved through segregation which can be implemented at various levels during crop production including cultivation, harvest and post-harvest (storage, transport and processing) (Jank *et al.*, 2006). For example, volunteer GM plants can result in commingling via gene flow through cross-

pollination or seed during harvest. Pollen-mediated gene flow is one of the major contributing factors that compromise coexistence. Unfortunately the effect of pollen-mediated gene flow is often underestimated due to a lack of understanding as a result of a lack of research. Therefore, in order to implement coexistence measures at the most basic level i.e. farm-level, a proper understanding of pollen-mediated gene flow is required to answer the question: is it possible for GM and non-GM or organic crops to coexist?
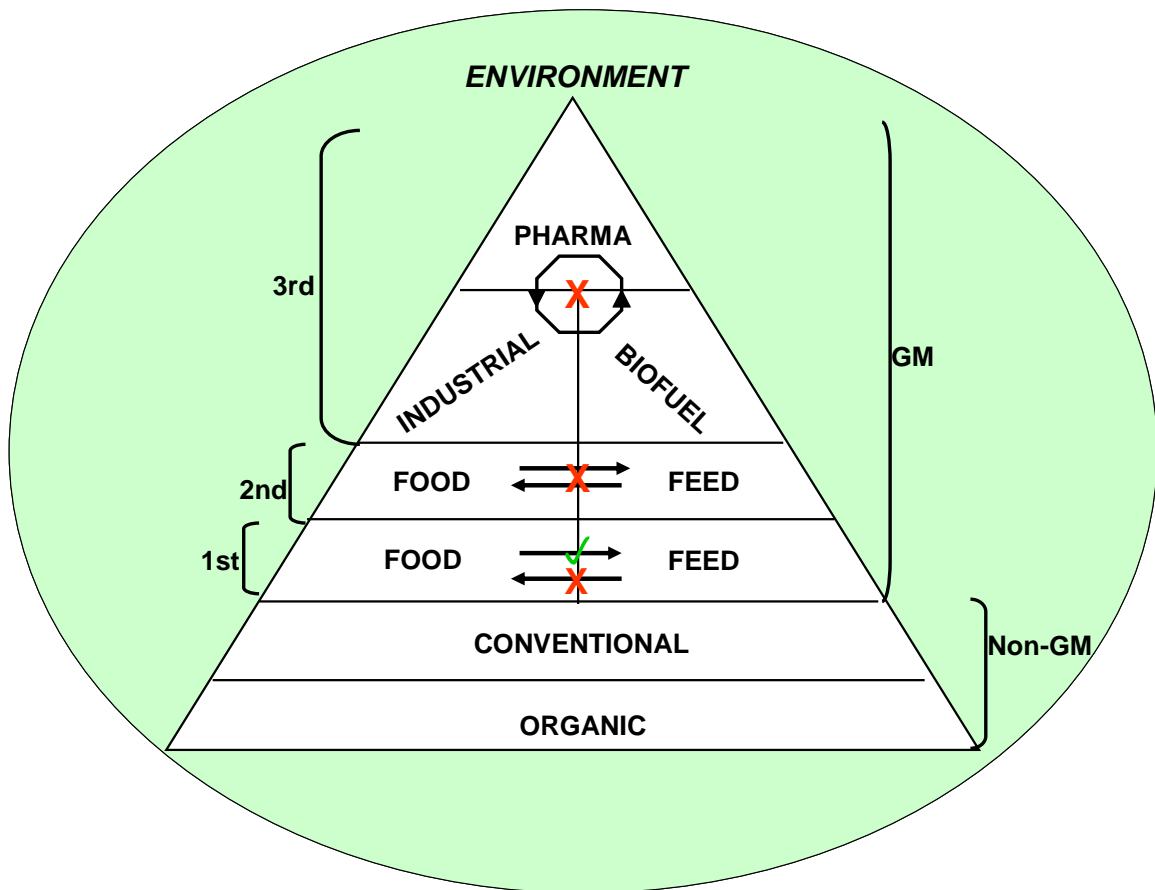
Figure 2.3.1 Diagram represents the various crop production systems and the levels of segregation.

2.4   GM gene flow: Much ado about nothing?

Genetically modified (GM) crops are currently produced in 23 countries and GM production contributed 34% of global agriculture in 2007. Currently, insect resistance and herbicide tolerance make up 72.2% and 20.3%, respectively of traits used (James, 2007). Although not yet produced at a commercial level, food crops have been genetically engineered for nutritional enhancement as well as for industrial and pharmaceutical traits (Moschini, 2006). This together with the rapid increase of GM crop production in many countries including South Africa and subsequent impact on trade with countries exhibiting a preference for non-GM has heightened the awareness of commingling between GM varieties and conventional varieties. The key contributor to commingling is gene flow which occurs at the farm level during crop production.

Gene flow is the movement of genes from one population to another. Vertical gene flow, with specific regard to GM crop is achieved via pollen. GM gene flow can occur through pollen from volunteer GM plants or from an adjacent GM variety with synchronous flowering (Huffman, 2004). Thus, pollen-mediated gene flow (PMGF) plays a key role in the management of coexistence between GM and non-GM crops.

In nature, PMGF is essential to maintain genetic variation and diversity. In crop improvement, plant breeders utilise PMGF to develop commercially viable varieties. After a variety has been established, PMGF has to be minimised to preserve the

genetic integrity of the new variety and maintain seed purity. Gene flow from GM crops can also result in an infringement of intellectual property rights for seed producers and compromise the integrity of non-GM or organic niche markets that would result in economic loss in terms of market rejection of the product (Demont and Devos, 2006; Lee, 2008). In addition to this, GM crops with pharmaceuticals and industrial compounds have to be managed and contained to ensure that the human and environmental safety is not compromised. Gene flow from a pharmaceutical GM crop, to a food crop could result in a major health risk as well as economic losses (Elbheri, 2005; Moschini, 2008).

There are various factors that influence pollen-mediated gene flow. The pollination mechanism relies on several vectors including wind, insects, birds and animals. Furthermore, the synchronous maturation of stigma and anther is required, as well as ample pollen production, that is viable and is dependent on environmental conditions (temperature and relative humidity) (Kerhoas *et al.*, 1987; Schoper *et al.*, 1987a; Schoper *et al.*, 1987b; Roy *et al.*, 1995; Aylor, 2004). In addition, for successful gene flow to occur, viable pollen must interact with a receptive stigma resulting in successful pollination and fertilization (Bhatia and Mitra, 2003). Thus, the diverse criteria required for out-crossing to occur makes studying PMGF extremely challenging.

The different criteria influencing PMGF has led to the utilisation of a diverse array of research methods. Potential pollen-mediated gene flow (PPMGF) is studied by performing pollen viability analysis, pollen dispersal and deposition, computer

modelling, mathematical simulation and pollen capture (Table 2.4.1) (Raynor *et al.*, 1972; Kerhoas *et al.*, 1987; Schoper *et al.*, 1987a; Schoper *et al.*, 1987b; Roy *et al.*, 1995; Fonesca *et al.*, 2002; Jarosz *et al.*, 2003; Aylor, 2004; Fricke *et al.*, 2004; Arrit *et al.*, 2007). Research into PMGF involves measuring the extent of out-crossing over distance (Paterniani and Stort, 1974; Garcia *et al.*, 1998; Burris, 2001; Jemison and Vayda, 2001; Luna *et al.*, 2001; Aylor *et al.*, 2003; Byrne and Fromhertz, 2003; Henry *et al.*, 2003; Ma *et al.*, 2004; Stevens *et al.*, 2004; Porta *et al.*, 2008; Bannert and Stamp, 2007). In addition, computer modelling has been used to predict theoretical distances at which PMGF can occur under different permutations of environmental conditions (Fricke et al., 2004). The purpose of these studies is to determine the factors affecting PMGF and establish isolation distances to minimise gene flow to within threshold levels (Lee, 2008; Demont and Devos, 2008).

Despite GM crops being produced in 23 countries, research into pollen-mediated gene-flow especially in maize and soybean (popular GM food crops according to production values) has been lacking (James, 2007). According to published data for maize, the furthest that out-crossing has been detected is 650 m (Henry *et al.*, 2003). However, a range of different distances has been recorded depending on the field trial design and the environmental conditions (Table 2.4.2). Similarly for soybean, generally considered to be a self-pollinating crop, very few published studies have determined the effect of the environment on PMGF (Table 2.4.3). Nonetheless, Ray *et al.*, (2003) found 0.3% out-crossing at 5.4 m and Abud *et al.*, (2007) found out-crossing of 0.52% at 1 m in soybean. One possible reason for the

lack of published data on out-crossing in genetically engineered crops, is that prior to the development of GM and hence a specific target sequence that could easily be identified, plant breeders relied mainly on morphological characteristics to determine seed purity. Thus, many of the recommendations to minimize gene flow such as isolation distances would have been based on less sensitive and robust non-molecular criteria.

After a decade of GM crops being commercialised in South Africa, there is still no published data (i.e. none which could be found after an extensive survey of the literature) regarding the extent of PMGF in either maize or soybean under South African conditions. Despite this *laissez-faire* (nonchalant) stance, the recent contamination of food crops with pharmaceutical GM maize in the US (Prodigene) (Elbheri, 2005) and the introgression of transgenes in Mexican landraces has most certainly created a sense of urgency for such research, especially in developing countries who have the most to loose in terms of niche markets (Quist and Chapela, 2001).

The introduction of biotech crops has most certainly added new complexities in a variety of areas that were not initially envisioned. The main areas of impact are in agriculture practice, regulatory frameworks, economic, environment and on society. Unfortunately, the polarized nature of the GM debate has distracted from scientific inquiry into these issues. With second and third generation GMOs on our doorstep, it is imperative to establish guidelines for coexistence and ensure GM and non-GM segregation where necessary.

Table 2.4.1   Potential pollen-mediated gene flow research in maize.

Description of methodology	Furtherest distance moved	Reference
Pollen dispersal and deposition	60 m	Raynor *et al.* (1972)
Effect of dehydration on pollen viability	None	Kerhoas *et al*. (1987)
Heat tolerance on pollen viability	None	Schoper *et al.* (1987a)
Water and heat stress on pollen viability	None	Schoper *et al*. (1987b)
Effect of temperature on pollen viability	None	Roy *et al*. (1995)
Pollen production and dispersal	None	Fonesca *et al*. (2002)
Airborne concentration and deposition	30 m	Jarosz *et al*. (2003)
Atmospheric exposure on pollen viability	None	Aylor (2004)
Computer simulation of pollen dispersal	880 m	Fricke *et al.* (2004)
Numerical simulation of pollen dispersal	None	Arritt *et al.* (2007)

Table 2.4.2   Pollen-mediated gene flow research in maize.

Description of methodology	Furtherest distance out-crossed	Reference
Out-crossing with phenotype detection	34 m	Paterniani and Stort (1974)
Out-crossing with detassling (phenotype)	184 m	Garcia *et al*. (1998)
Out-crossing with gentotypic detection	200 m	Burris (2001)
Out-crossing with phenotype detection	40 m	Jemison and Vayda (2001)
Out-crossing with phenotype detection	200 m	Luna *et al*. (2001)
Aerobiological framework to assess out-crossing	None	Aylor *et al*. (2003)
Out-crossing with phenotype detection	183 m	Byrne and Freomherz (2003)
Out-crossing with gentotypic detection	650 m	Henry *et al*. (2003)
Out-crossing with phenotype detection	48 m	Ma *et al*. (2004)
Out-crossing with detassling (phenotype)	300 m	Stevens *et al*. (2004)
Out-crossing with phenotype detection	56.7 m	Porta *et al*. (2008)
Out-crossing with phenotype detection	371 m	Bannert and Stamp (2007)

Table 2.4.3   Pollen-mediated gene flow research in soybean

Category	Description of methodology	Furtherest out-crossing distance	Percentage out-crossing	Reference
Insect-mediated	Out-crossing with phenotype detection	None	2.50%	Ahrent and Cainess (1994)
Insect-mediated	Out-crossing detected with enzymatic assay	None	9 -19%	Fujita *et al*. (1997)
Insect-mediated	Out-crossing detected with isozyme analysis	None	0.73%	Nakayama and Yamaguchi (2001)
PMGF	Out-crossing with phenotype detection	5.4 m	0.03%	Ray *et al*. (2003)
PMGF	Out-crossing with gentotypic detection	8 m	0.02%	Abud *et al*. (2007)

* This study was performed using wild soybean (*Glycine soja*)

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CHAPTER 3

POLLEN-MEDIATED GENE FLOW IN GM SOYBEAN IN SOUTH

AFRICA

3.1   INTRODUCTION

In 2007, genetically modified soybean (*Glycine max*) for herbicide tolerance (HT) overtook conventional global soybean production at 51% (58.6 million hectares), making it currently the premier biotech crop in the world (James, 2007). Soybean is an important food crop and a valuable source of vegetable oil and protein (Gardener and Payne, 2003; Lu, 2004). The high rate of GM soybean production is the result of high adoption rates in Argentina, Brazil and the United States. In 2007, South Africa planted approximately 144 000 hectares to GM soybean, comprising 80% of total production volume (James, 2007). Thus, it is not expected that GM soybean production will decrease especially since future developments include nutritional GM traits such as high oleic oil and input traits such as insect resistance (Cahoon, 2003; Kinney, 2003; Conner *et al*., 2004).

One of the major concerns surrounding the commercial production of HT soybean is the potential for it to cross-pollinate with wild relatives and landraces and consequently contribute to weediness by conferring herbicide resistance. However, this environmental concern pertains to areas where wild relatives (*Glycine soja*) of soybean are native such as China and Japan (Gepts and Papa, 2003; Kuroda *et al*,

2006). However, GM gene flow in soybean is also an important concern for commercial agriculture, especially since soybean farmers tend to save seed. The presence of GM in saved non-GM seed would contravene patent. Furthermore, GM gene flow in soybean is also an important concern for its market value if it is intended as non-GM, especially since soybean is an important source of protein for in the vegetarian food market which by default is mainly non-GM.

Ironically, pollen-mediated gene flow in soybean has been researched in countries where no wild relatives of soybean exist such as the United States and Brazil (Ray *et al.*, 2003, Abud *et al.*, 2007). The primary motivation for pollen-mediated gene flow research in these countries is to be able to determine the role that pollen-mediated gene flow plays in non-GM soybean production. South Africa, like the United States and Brazil is not native for wild relatives of soybean. However the coexistence of GM and non-GM soybean is an important component for GM management as well as maintaining seed purity levels for niche markets. Despite this, very few published data on soybean cross-pollination is available and specifically none for a South African environment. This is perhaps due to soybean being primarily considered a self-pollinating crop.

The few studies to investigate gene flow in soybean have none the less observed low levels of out-crossing of 0.03% and 0.52%, at 5.4 m and 1 m, respectively (Ray *et al.*, 2003; Abud *et al.*, 2007) (Table 1.4.2). The difference in percentage out-crossing is possibly the result of variance in field design or environmental

conditions. To date, studies on soybean out-crossing have only considered pollen movement in terms of insect pollinators.

The aim of this study was to determine the extent that soybean pollen movement and subsequent out-crossing that could occur in South Africa, given the varieties of soybean and environmental conditions including insect pollinators.

3.2. MATERIALS AND METHODS

3.2.1 Field trials

GM (PAN 737R) and non-GM (PAN 854) soybean seed were planted at two soybean breeding locations (Greytown and Delmas) over two seasons (2005/2006 and 2006/2007). The GM soybean contains the EPSPS gene (5-Enol-pyruvylshikimate-3-phosphate synthase) for tolerance to the gylphosate from the event GTS 40-3-2.

The trial design was a central GM plot with planted strips of non-GM soybean in four directions (Fig. 3.1). The fields were planted in duplicate at both locations in (2005/2006) but only one field in KZN for (2006/2007) due to a lack of available seed (Table 3.1, Figure 3.1 and 3.2). The non-GM and GM cultivars were selected for their similarity in flowering time. The Vantange Pro mobile weather station was positioned at each trial site for two days during the flowering period to capture wind speed (m/s), wind direction, temperature (°C) and relative humidity (%) data (Fig. 3.3).

3.2.2 Potential pollen-mediated gene flow

Pollen was trapped for two days during the flowering period (Table 3.1). The pollen trap was composed of a rod with a clamp attached. A glass slide coated in Tween 20 (pollen adherent) was placed in the clamp and the trap positioned in the centre of the GM field at a height of 0.5 m (Fig. 3.4). The Tween 20 coated slide was set at 7 am and removed at 4 pm daily. Upon retrieval, the slide was rinsed with 1 ml CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8) and the pollen suspension stored at 4℃. The samples were checked for the presence of soybean pollen (1:10 dilution) using a light microscope (10x magnification) and a haemocytometer.

3.2.3 Pollen-mediated gene flow

At maturity, soybean pods were sampled from the non-GM and GM plot of each field. Non-GM pods were sampled in 0.9 m intervals up to 5.4 m to the right and left of the GM plot and in 1 m intervals up to 3 m to the front and back of the GM plot. The seeds were separated into two batches for phenotypic and genotypic analysis.

3.2.3.1   Phenotypic analysis

Seeds (25 seed per petri-dish) corresponding to the different distance intervals, were germinated in four petri-dishes on filter paper moistened with ddH$_2$O at room temperature (25℃). Non-germinating seed (after a 5 day germinating period) were regarded as sterile and discarded whilst seed that developed a hypocotyl length of

approximately 2 cm was treated with 3% glyphosate solution for 1 min. The treated seed was placed in a separate Petri-dish with filter paper which was moistened daily. The seedlings that continued to developed secondary roots were rated as glyphosate-tolerant (GM) and seed that did not develop further were considered glyphosate-intolerant (non-GM).

3.2.3.2   Genotypic analysis

3.2.3.2.1   DNA extraction

A 2 g sub-sample of milled seed (36 samples) (granule size of less than 1.5 mm^2) was used in the DNA extraction by the addition of 10 ml of CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8) and 30 µl of Proteinase K [20 mg/ml]. The samples were agitated every 10 min. for 10 sec. during a 2 hour incubation period at 65℃. After the incubation, t he samples were centrifuged at 4k rpm for 5 min. at room temperature. The supernatant (1 ml) was incubated at 80℃ for 5 min. and 5 µl RNase A [100 mg/ml] added and incubated for a further 5 min. at 65℃. Chloroform:Isoamyl alcohol (24:1) (1 ml) was added to the sample, following centrifugation for 5 min. at 14k rpm and the aqueous layer retained. This step was repeated 3 times.   Thereafter, 1 ml of absolute ethanol was added and the precipitating sample kept on ice for 1 hour. The sample was then centrifuged at 14k rpm for 10 min., the supernatant discarded and the pellet retained. The pellet was washed twice by the addition of 500 µl 75% ethanol and centrifuged at 14k rpm for 5 min. The pellet was dissolved in 100 µl 0.2x T.E (1 M Tris, 0.5 M EDTA) and further purified using the GFX PCR DNA and gel Band Purification Kit according to the manufacturer's guidelines (Amersham Biosciences).

3.2.3.2.2   PCR detection for Roundup Ready

GMO screening was performed using the EPSPS gene sequence for soybean products according to the method of Lipp *et al.* (2001). A PCR master mix was prepared containing 19.9 µl Roundup Ready PCR buffer (GeneScan, GmbH) and 0.16 µl Ampli-Taq Gold for each reaction, including negative and positive controls. Each sample tube contained 20 µl of master mix and 5 µl of sample DNA while the negative control contained 5 µl 0.2x T.E buffer and the positive control contained 1.0% (in relation to non-GM) transgenic Roundup ready DNA (GeneScan, GmbH). The PCR was performed in an ABI 9700 and the cycling parameters were 95^oC for 10 min. (1 cycle), 95^oC for 25 sec, 62^oC for 30 sec., 72^oC for 45 sec. (50 cycles) followed by 72^oC for 7 min. and 25^oC (1 cycle). The limit of detection was 0.01%. The Roundup Ready amplicon (129 bp) was confirmed using a 2.0% Agarose gel run at 200 V for 40 to 50 min. and then visualised under UV light after staining in Ethidium Bromide [150 µl/1.5 L] for 30 mins.

3.4   RESULTS

3.4.1   Field trial

The soybean field trials in both seasons reached flowering and seed-set, despite poor rains especially in Delmas during the second season (2006/2007). Due to a seed shortage, only one field was planted in Greytown during (2006/2007).

In Delmas, the average temperature for the (2005/2006) season ranged between 20.4^{o}C and 22.4^{o}C and in (2006/2007) between 21.9^{o}C and 24.7^{o}C, for the 2 days during flowering, respectively. The average relative humidity for Delmas, ranged between 73.0% and 81.0% and 50.1% and 59.4% in the first and second season, respectively, for the two days during flowering. In Greytown, the average temperature ranged between 17.7 ^{o}C and 22.5 ^{o}C in the first season and 26.2 ^{o}C in the second season. The average relative humidity for Greytown in the first season was between 87.1% and 76.0% and between 65.6% and 52.8% in the second season. In Delmas during the (2005/2006) season, the predominant wind during the flowering period was north, east north-east, east and east south-east and in the second season for (2006/2007) it was north east, east north-east and south east (Fig. 3.5). In Greytown, in the (2005/2006) season, the predominant winds during the two days over the flowering period were north north-west, north, north north-east, north-east, east north-east, east and east south-east. In the second season, the prevailing winds were, north-west, west north-west, west south-west, south-west, south south-west, south, south south-east and south-east (Fig. 3.6).

3.4.2 Potential pollen-gene flow

No soybean pollen was visible from the pollen traps over the two seasons.

3.4.3 Pollen-mediated gene flow

3.4.3.1   Phenotypic analysis

Glyphosate resistance was not phenotypically detected.

3.4.3.2   Genotypic analysis

PCR detected the presence of the EPSPS gene for Roundup Ready in two seed samples. This was in Greytown, field B, during the (2005/2006) season in row 1 (0.9 m) to the right of the GM block and in Delmas during (2006/2007), in row 1 (0.9 m) to the left to the GM block.

3.5   DISCUSSION AND CONCLUSIONS

PPMGF was found not to play a role in terms of GM gene flow for the soybean varieties grown under South African environmental conditions as no pollen was detectable from pollen traps. From discussions with soybean breeders it appears that most if not all the soybean varieties planted in South Africa have closed flowers. However, this does not hold true for all soybean varieties, some of which are open-pollinating. Despite this, pollen-mediated gene flow was still observed up to 0.9 m. It was not possible to quantify the percentage out-crossing as we had pooled the seed. However, since no phenotypic out-crossing was detected we presume that the percentage out-crossing was low (less than 1 in 50 seed) (Table 3.3). These results are similar to studies by Ray *et al.* (2003) and Abud *et al.* (2007) who found out-crossing at 5.4 m and 8 m, respectively.

Since no pollen was observed in pollen traps, the gene flow observed in this study can most likely be attributed to insect-mediation. The generally accepted self-pollinating characteristic of soybean is offset by insects that may act as a pollinating vector (Chiari *et al.*, 2005). We therefore consider the environmental conditions

irrelevant for PPMGF. However, environmental conditions may play and important role in insect mediated pollination. Future studies regarding gene flow in soybean in SA should include a component of surveying pollination insects.

For this study we also refined a simple phenotypic method to screen for HT tolerance in soybeans. The method is a modification to that of Main *et al.* (2004) and Tillman and West (2004). In these studies the seed was treated with glyphosate prior to germination whereas in the current study, the seed was germinated and then exposed to glyphosate, so as to eliminate sterile seeds. The advantage of the latter approach is that it allows for a more accurate assessment of glyphosate tolerance.

Currently in South Africa, the recommended isolation distance for soybean is 5 m (South African National Standards, 2005). From the analysis of the data from this study, it appears that 5 m is sufficient. A possible exclusion to this would be the use of soybean varieties with open flowers. Thus, other management practices and not gene flow should be considered to be the main factor contributing to commingling of GM to non-GM soybean in South Africa. The practice of retaining seed is likely to be one of the greater contributors to GM soybean commingling in addition to seed storage, transport and processing.

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Table 3.1   Soybean field trial phenology for Delmas and Greytown in the (2005/2006) and (2006/2007) planting seasons.

2005/2006		
	Delmas	**Greytown**
Planting dates	02 December 2005	02 December 2005
No. of fields	2	2
Trapping dates	14-15 February 2006	Rained out
GM (PAN 737R)		
No. of rows	2	2
Length of row	6 m	6 m
Distance between rows	0.9 m	0.9 m
Non-GM (PAN 854)		
Right	6 rows	6 rows
Left	6 rows	6 rows
Front	16 m	16 m
Back	16 m	16 m
2006/2007		
	Delmas	**Greytown**
Planting dates	15 December 2006	14 December 2006
No. of fields	2	1
Trapping dates	23-24 February 2007	19-20 February 2007
GM (PAN 737R)		
No. of rows	2	2
Length of row	6 m	6 m
Distance between rows	0.9 m	0.9 m
Non-GM (PAN 854)		
Right	6 rows	6 rows
Left	6 rows	6 rows
Front	17 m	17 m
Back	17 m	17 m

Table 3.2   Pollen counts from traps for Delmas and Greytown in the 2005/2006 and 2006/2007 seasons.

Season	Area	Day	Amount of pollen
2005/2006	Delmas A	1	0
	Delmas B	2	0
	Delmas A	1	0
	Delmas B	2	0
	Greytown A and B	1 and 2	nd
2006/2007	Delmas A	1	0
	Delmas B	2	0
	Delmas A	1	0
	Delmas B	2	0
	Greytown	1	0
	Greytown	2	0

nd   not determined due to excessive rain in that location for that season

Table 3.3   Phenotypic and genotypic analysis for soybean seeds harvested from non-GM fields in Delmas and Greytown during 2005/2006 and 2006/2007 seasons.

				2006			
Sample #	Sample Name	Delmas A	row*/distance from GM field	Amount of seed for phenotype	Number of seed germinated	No of seed to survive 3% glyphosate	Genotype result (+/-)
1	DA-R1-2006	Right	row 1	70	57	0	negative
2	DA-L1-2006	Left	row 1	84	78	0	negative
3	DA-F1-2006	Front	1 m	65	59	0	negative
4	DA-B1-2006	Back	1 m	100	66	0	negative
		Delmas B					
5	DB-R1-2006	Right	row 1	100	84	0	negative
6	DB-L1-2006	Left	row 1	100	78	0	negative
7	DB-F1-2006	Front	1 m	57	49	0	negative
8	DB-B1-2006	Back	1 m	84	84	0	negative
		Greytown A					
9	GA-R1-2006	Right	row 1	100	93	0	negative
10	GA-L1-2006	Left	row 1	88	49	0	negative
11	GA-F1-2006	Front	1 m	51	51	0	negative
12	GA-B1-2006	Back	1 m	99	88	0	negative
		Greytown B					
13	GB-R1-2006	Right	row 1	100	86	0	**positive**
14	GB-L1-2006	Left	row 1	100	90	0	negative
15	GB-F1-2006	Front	1 m	66	60	0	negative
16	GB-B1-2006	Back	1 m	98	75	0	negative
				2007			
		Delmas A					
17	DA-R1-2007	Right	row 1	100	85	0	negative
18	DA-L1-2007	Left	row 1	100	69	0	negative
19	DA-F1-2007	Front	1 m	50	28	0	negative
20	DA-B1-2007	Back	1 m	50	39	0	negative
		Delmas B					
21	DB-R1-2007	Right	row 1	70	58	0	negative
22	DB-L1-2007	Left	row 1	100	76	0	**positive**
23	DB-F1-2007	Front	1 m	44	26	0	negative
24	DB-B1-2007	Back	1 m	65	49	0	negative
		Greytown A					
25	GA-R1-2007	Right	row 1	95	63	0	negative
26	GA-L1-2007	Left	row 1	89	75	0	negative
27	GA-F1-2007	Front	1 m	100	87	0	negative
28	GA-B1-2007	Back	1 m	90	60	0	negative
				Second Set of Phenotyping			
2-1	GA-F2-2006	Front	2 m	57	49	0	negative
2-2	GA-F3-2006	Front	3 m	56	52	0	negative
2-3	GB-F2-2006	Front	2 m	28	21	0	negative
2-4	GB-F3-2006	Front	3 m	58	41	0	negative
2-5	GB-B2-2006	Back	2 m	100	71	0	negative
2-6	GB-B3-2006	Back	3 m	62	53	0	negative
				Third Set of Phenotyping			
3-1	GB-R2-2006	Right	2 m	50	19	0	negative
3-2	DB-R2-2007	Right	2 m	40	18	0	negative

Table 3.4   Average temperature and relative humidity in Delmas and Greytown for two days in two seasons.

Delmas (2005/2006)						
Day	Temperature (°C)			Relative Humidity (%)		
	Min	Max	Ave	Min	Max	Ave
1	17	33	22	40	95	73
2	15	27	20	56	96	81
Greytown (2005/2006)						
1	17	19	18	73	95	87
2	16	33	22	48	96	76
Delmas (2006/2007)						
1	17	29	25	31	83	50
2	14	31	22	23	91	59
Greytown (2006/2007)						
1	19	32	26	41	93	66
2	16	37	26	14	95	53
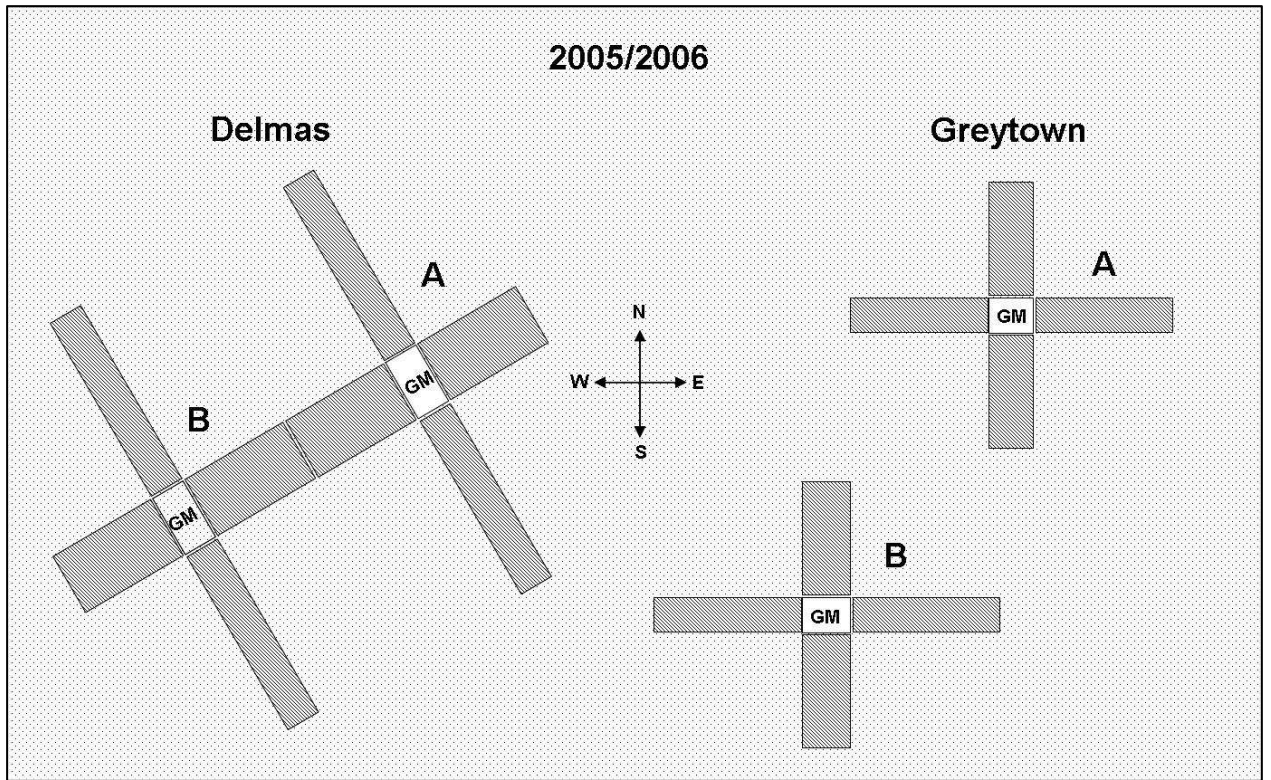
Figure 3.1   Schematic of the soybean field trials in Delmas and Greytown
               (2005/2006).   The cardinal directions are indicted for each
               location.
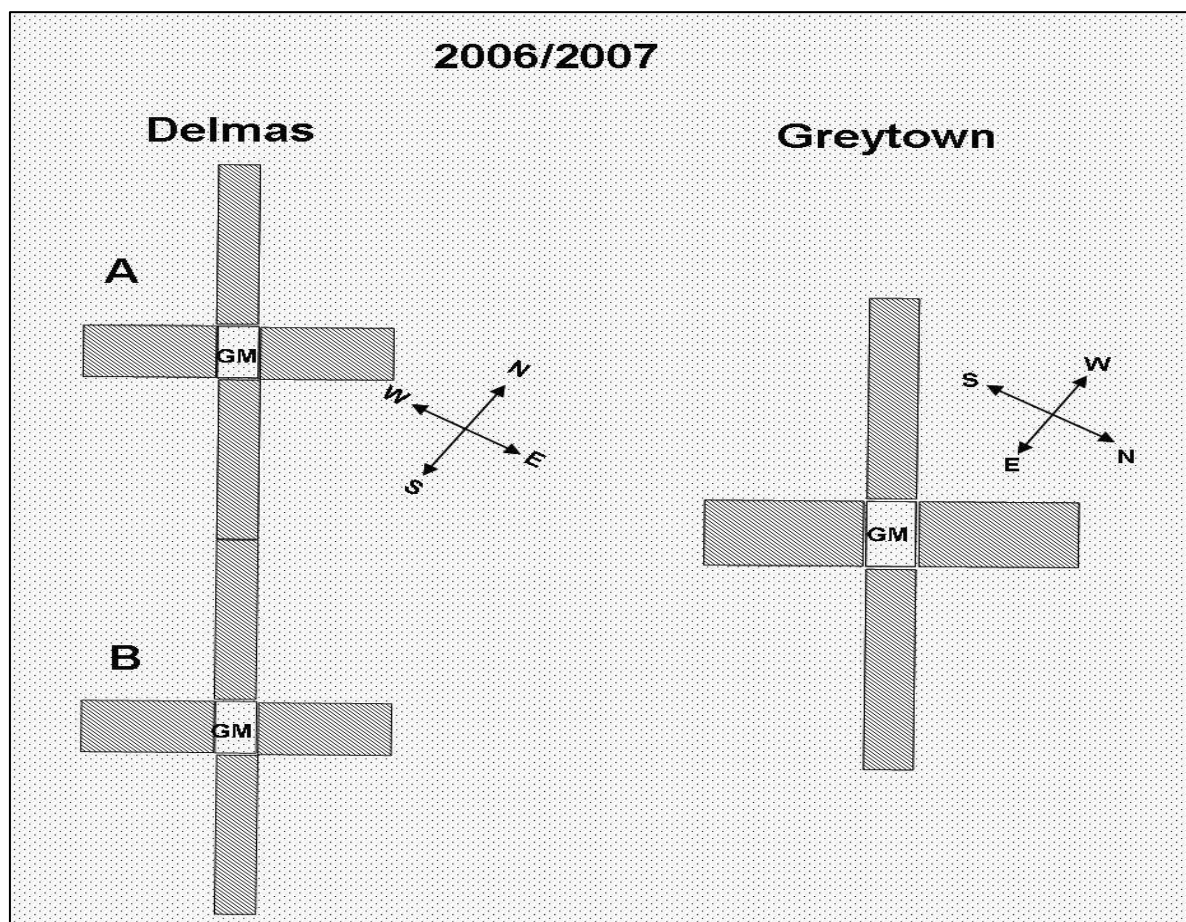
Figure 3.2   Schematic of the soybean field trials in Delmas and Greytown (2006/2007).   The cardinal directions are indicted for each location.

Figure 3.3   The Vantage Pro mobile weather station situated on the field during the flowering period.

Figure 3.4   Soybean pollen trap with glass slide
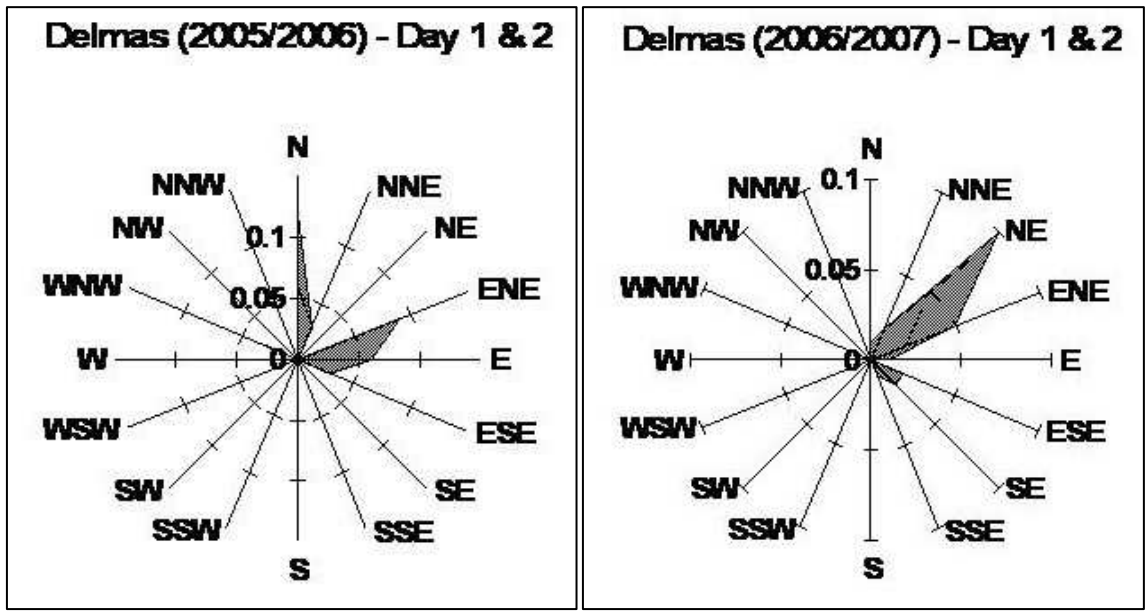
Figure 3.5   Wind rose indicating the wind frequency during the two flowering
               days in Delmas during the (2005/2006) and (2006/2007) seasons.
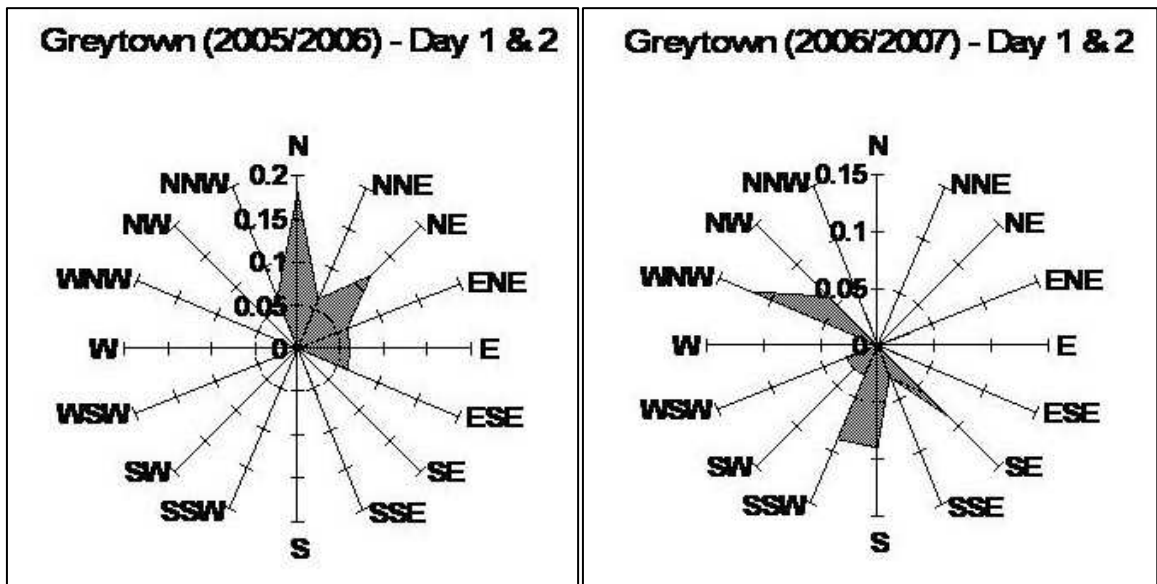
Figure 3.6   Wind rose indicating the wind frequency during the two flowering
               days in Greytown during the (2005/2006) and (2006/2007)
               seasons.

A

B

Figure 3.7   Control GM seed (A) and non-GM seed (B) after treatment with Glyphosate solution (3%).
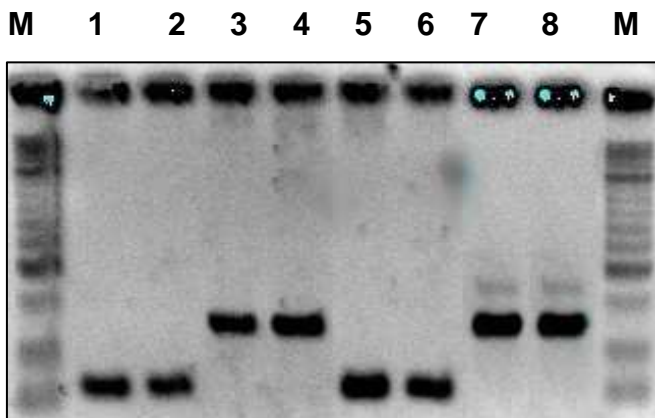
Figure 3.8   Genotype detection. Lane 1 and 2 (negative sample), Lane 3 and 4 (positive sample - 129 bp), Lane 5 and 6 (negative control) and Lane 7 and 8 (positive control).

CHAPTER 4

POTENTIAL POLLEN-MEDIATED GENE FLOW IN GM MAIZE IN A

SOUTH AFRICAN ENVIRONMENT

4.1   INTRODUCTION

South Africa first commercially released genetically modified (GM) crops in 1997. Since then, the adoption has steadily increased and currently 57% of maize produced in South Africa is GM (James, 2007). The GM traits grown in South Africa are insect resistance (IR) (MON 810) and herbicide tolerance (HT) (NK 603) as well as the stack for both IR and HT (MON810 x NK 603). Maize is a staple crop in South Africa and the rest of Africa and the continued adoption of GM crops including second and third generation GMOs is anticipated.

Maize is an open-pollinated species with the possibility of out-crossing via pollen-mediated gene flow (Miller, 1985). In South Africa, there is currently no formal requirement for farmers to segregate GM from non-GM production. Thus, unless farmers are specifically contracted, they do not apply either temporal or distance isolation when planting GM and conventional non-GM crops. However, the repercussions of pollen-mediated gene flow extend from compromising organic or non-GM niche markets, maize landraces that have cultural significance as well as unwanted second and third generation GM traits in the food chain (Moschini, 2006; Demont and Devos, 2008). Therefore, it is imperative to understand at a very basic

level, the factors affecting maize pollen movement or potential pollen-mediated gene flow (PPMGF).

There are very few published studies on maize pollen movement and none for South Africa (no literature was found after an extensive literature search) (Table 2.4.1). Some published studies have been theoretical, using computer simulation or mathematical modelling (Table 2.4.1) (Fricke *et al.*, 2004; Arrit *et al.*, 2007). Studying maize pollen is challenging due to its limited viability (one to two hours viability at optimum environmental conditions i.e. 28°C and 50% relative humidity) (Luna *et al.*, 2001). Most PPMGF studies have focussed on pollen load and viability (Table 2.4.1) (Raynor *et al.,* 1972; Kerhoas *et al.*, 1987; Schoper *et al.*, 1987a; Schoper *et al.*, 1987b; Roy *et al.*, 1995; Fonesca *et al.*, 2002; Aylor, 2004). Thus, despite a variety of approaches to analyzing the different aspects of PPMGF, there is still no conclusive evidence in support of minimum requirements to achieve specific levels of segregation. The furthest distance that maize pollen is hypothesized to able to effect out-crossing according to computer simulation, is 880 m (Fricke *et al.*, 2004).

In order to establish guidelines for isolation distances to manage PPMGF in South Africa, it is essential to determine the extent of maize pollen movement under South African environmental conditions. The aim of this study was to utilize molecular detection to determine the extent of GM maize pollen movement under South African conditions in maize planting regions.

4.2 MATERIALS AND METHODS

4.2.1 Field Trials

Yellow GM and white non-GM maize was planted at two typical commercial maize growing regions, Bainsvlei and Kroonstad, in the Free State during 2005/2006 and Bainsvlei and Waterbron during 2006/2007 growing seasons (Table 4.1). The cultivars were selected based on their similarity in flowering (74 to 76 days) (Table 4.1). The yellow GM maize contained the cry1Ab gene from event Mon810.

The trial design was a central GM maize plot surrounded by conventional maize (Fig. 4.1 and 4.2). The Waterbron and Kroonstad trials were planted with a four week temporal isolation from other maize plantings in the area. Weather data was captured (5 days during flowering) using a mobile weather station (Vantage Pro) positioned in the centre of the GM plot (Figure 3.3). In Bainsvlei, during the 2006/2007 season, the weather unit malfunctioned and weather data for the area was obtained from SA Weather. Weather data captured included wind speed (m/s), wind direction, temperature (°C) and relativ e humidity (%).

4.2.2 Pollen trapping

Pollen was trapped for 5 days during the flowering period (Table 4.1). Traps were set at 50 m distance intervals from the GM plot in four directions (Table 4.2). The pollen trap was composed of a rod with a clamp attached. The traps were adjusted to a height of 1.8 m, to match the height of flowering maize plants. A glass slide coated with Tween 20 (pollen adherent) was placed in the clamp at 6 am and

removed at 3:30 pm daily for five days. Collected slides were rinsed with 1 ml CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8) and stored at 4°C. Pollen was diluted (1:10) and c ounted using a haemocytometer under 10x magnification using a Light Microscope.

4.2.2.1   DNA extraction

Pollen suspensions were pooled for the 5 days per distance interval and direction and DNA extracted for genotypic GM detection. The pollen suspensions were centrifuged for 5 min. at 5k rpm and the excess CTAB buffer decanted. Fresh CTAB buffer (50 µl) was added to the pollen followed by homogenization using a plastic micro-pestle. Additional CTAB buffer (450 µl) and Proteinase K (30 µl) was added and the sample incubated for 2 hours at 65°C followed by 80°C for 5 min. Thereafter, 5 µl RNase was added and the sample incubated at 65 °C for 5 min. Chloroform: isoamyl alcohol (24:1) (500 µl) was added and the sample centrifuged for 5 min. at 14 k rpm. The aqueous layer was retained and the chloroform: isoamyl alcohol step repeated. Following this, absolute ethanol (1 ml) was added to the aqueous layer and the DNA precipitated overnight at 4°C. The supernatant was discarded and the pellet washed twice with 75% ethanol (500 µl) by centrifugation at 14k rpm for 5 min. The pellet was dissolved in 50 µl of sterile water and further purified using GFX PCR DNA and gel Band Purification Kit according to manufacturer's guidelines (Amersham Biosciences).

4.2.2.2 PCR analysis for 35S detection

GMO screening was performed using the 35S promoter sequence for CaMV according to the method of Lipp *et al*. (2001). A PCR master mix was prepared containing 19.9 µl 35S PCR buffer (GeneScan, GmbH) and 0.16 µl Ampli-Taq Gold for each reaction, including negative and positive controls. Each sample tube contained 20 µl of master mix and 5 µl of sample DNA while the negative control contained 5 µl 0.2x T.E buffer and the positive control contained transgenic 35S DNA (GeneScan, GmbH). The PCR was performed in an ABI 9700 and the cycling parameters were 95°C for 10 min. (1 cycle), 95°C for 25 sec., 62°C for 30 sec., 72°C for 45 sec. (50 cycles) followed by 72°C for 7 min. and 25°C (1 cycle). The limit of detection was 0.01%. The 35S (123 bp) was confirmed by resolving the amplicon on a 2.0% Agarose gel at 200 V for 40 to 50 min. followed by visualisation under UV light after staining in Ethidium Bromide [150 µl /1.5 l] for 30 mins.

4.3   RESULTS

4.3.1 Field trials

All maize trials reached flowering and seed set except the Kroonstad trial in the (2005/2006) season.

4.3.2 Pollen trapping

At Bainsvlei (2005/2006), the highest pollen count (155 820 pollen) was trapped 50 m north of the GM field (Fig. 4.3) with the highest amount of daily collected pollen (353 070 pollen) observed on day 4 (Fig. 4.4). The wind frequency on day four was

low compared to other days (Fig 4.9). The greatest incidence of wind was recorded in a southerly direction on day 5. During the second season (2006/2007) at Bainsvlei, the single highest pollen collected was the same as for 2005/2006 (50 m –north) (23 500), although not as high (Fig 4.5). The highest collective daily pollen count was on day 5 (176 750 pollen) with winds recorded from the West South East and East South East at high frequency compared to other days (Fig 4.10). In Waterbron (2006/2007), the highest pollen amount (178 500) was observed at 50 m south of the GM plot (Fig 4.7) and the highest daily pollen count (394 000) was observed on day 4. Northerly winds were observed on day 3 and day 5 (Fig. 4.11).

PCR analysis

PCR analysis detected GM pollen in four samples. GM pollen was detected at Bainsvlei (2006/2007) at 400 m west of the GM field (Table 4.3) and at Waterbron (2006/2007) at 50 m, 100 m and 200 m north of the GM field (Table 4.4).

4.4   DISCUSSION AND CONCLUSIONS

PPMGF is considered an important indication of the potential for out-crossing to occur (Jarosz *et al.*, 2003). Using a combination of pollen traps and PCR, it was determined that GM pollen could be detected up to 400 m from the source (Table 4.3). Thus, the current isolation distances (200 m or 250 m) recommended for seed production are not sufficient to prevent potential out-crossing (Devos *et al.*, 2008).

Environmental conditions also play a critical role in PPMGF, relative humidity and temperature for the production and viability of pollen and wind for its movement. What was interesting is that there did not appear to be a clear correlation between pollen movement and the frequency of wind when comparing total pollen counts per direction to the frequency (speed over time) of wind for the (2005/2006) and (2006/2007) seasons in both locations. One possible explanation for this is that both regions experience swirling winds. This is an additional consideration to wind speed and direction and is rarely taken into account in establishing isolation distances or in the design of gene flow experiments.

In this study it was found that genotypic detection is an effective way to determine the exact extent of GM pollen movement. However, this is a qualitative technique and it does not give any indication of the GM pollen load. Determining the relative load of GM pollen to non-GM pollen is possible using real-time quantification but determining actual pollen counts is more difficult. In a previous study (Chetty and Viljoen, 2004 unpublished), real-time PCR was effectively used to directly detect GM DNA in from 10 pollen grains under laboratory conditions (data not shown). However, it was also found that a loss of viability renders the DNA undetectable through PCR, presumably as a result of DNA degradation. Thus, the use of real-time PCR to determine GM gene copy number would result in an underestimation of GM pollen load.

Despite the high incidence of pollen counts on pollen traps, GM pollen was only detected at four traps. One possible reason is that the PCR assay limits of

detection (LOD) may not have been sufficient to determine the presence of low numbers of GM pollen. However, the laboratory LOD for the PCR assay used was 0.01%. Another possibility is competition between GM and non-GM DNA in terms of pollen load. However, this can be disregarded as GM pollen was detected in a pollen trap 400 m from the source. If pollen load was in itself a consideration one would expect the ability to detect GM pollen to decrease over distance which was not the case. Finally, an important consideration is the viability of the GM pollen. As previously mentioned, non-viable pollen does not result in PCR amplification. While the Tween 20 used to trap pollen does not affect the PCR assay, it does not necessarily preserve the pollen either. Thus it is possible that the PCR detection of GM pollen was underestimated as a result of pollen losing viability. Despite these considerations, we suggest that an increase in GM plot size would also increase the GM pollen load with a higher potential of PPMGF at further distances.

This is the first report of the use of a simple and inexpensive pollen trap combined with PCR detection to determine the movement of pollen of a specific genotype. The applications of this research include its use in crops indigenous to Africa, specifically sorghum and cassava, to study PPMGF. Furthermore, the genotypic detection of pollen would be most useful to monitor GM field trials, especially for pharmaceutical and industrial GMOs.

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Table 4.1   Maize field trial phenology for the 2005/2006 and 2006/2007 planting seasons in Bainsvlei,
              Kroonstand and Waterbron.

Details	2005/2006		2006/2007	
	Bainsvlei	**Kroonstad***	**Bainsvlei**	**Waterbron**
Planting dates	17 November 2005	17 February 2006	23/24 October 2006	05 December 2006
Trapping Dates	31 Jan to 06 Feb 2006	none	31 Jan to 4 Feb 2007	13 Feb to 17 Feb 2007
GM (PAN 6994B) - 74 days			**GM (PAN 6724B) - 75 days**	
Length	30 m	30 m	35 m	32 m
Breadth	20 m	20 m	17 m	18 m
No. of rows	11	11	10	9
Dist. between rows	2 m	2 m	2 m	2 m
No. of GM plants	1650	1650	1750	1440
Non-GM (PAN 6479) - 76 days			**Non-GM (PAN 6479) - 76 days**	
Length	230 m	230 m	N (320) S(294)	800 m
Breadth	180 m	180 m	W (186) E(177)	172 m
No. of rows	97	97	101	97
Dist. between rows	2 m	2 m	2	2 m
Dist. between plants	0.2	0.2	0.2	0.2
No. plants/m	5	5	5	5
No. plants/row	1150	1150	1470	4000

* Trial failed due to frost

Table 4.2   Distance intervals for the pollen traps at the two locations

Distance from GM field (m)

Bainsvlei						
North	50	100	200	300	n.d	n.d
South	50	100	200	300	n.d	n.d
East	50	100	200	300	400	500
West	50	100	200	300	400	n.d
Waterbron						
North	50	100	200	300	400	500
South	50	100	200	300	400	500
East	50	100	200	300	400	470
West	50	100	200	300	400	500

n.d.   Not Determined

Table 4.3   PCR results for 35S detection in trapped maize pollen for Bainsvlei (2005.2006) and (2006/2007).

Bainsvlei (2005/2006)		Bainsvlei (2006/2007)	
Pollen Sample	PCR result	Pollen Sample	PCR result
North - 50 m	negative	North - 50 m	negative
North - 100 m	negative	North - 100 m	negative
North - 200 m	negative	North - 200 m	negative
North - 300 m	negative	North - 300 m	negative
South - 50 m	negative	South - 50 m	negative
South - 100 m	negative	South - 100 m	negative
South - 200 m	negative	South - 200 m	negative
South - 300 m	negative	South - 300 m	negative
West - 50 m	negative	West - 50 m	negative
West - 100 m	negative	West - 100 m	negative
West - 200 m	negative	West - 200 m	negative
West - 300 m	negative	West - 300 m	negative
West - 400 m	negative	West - 400 m	positive
East - 50 m	negative	East - 50 m	negative
East - 100 m	negative	East - 100 m	negative
East - 200 m	negative	East - 200 m	negative
East - 300 m	negative	East - 300 m	negative
East - 400 m	negative	East - 400 m	negative
East - 500 m	negative	East - 500 m	negative

Table 4.4   PCR results for 35S detection in trapped maize pollen for Waterbron (2006/2007).

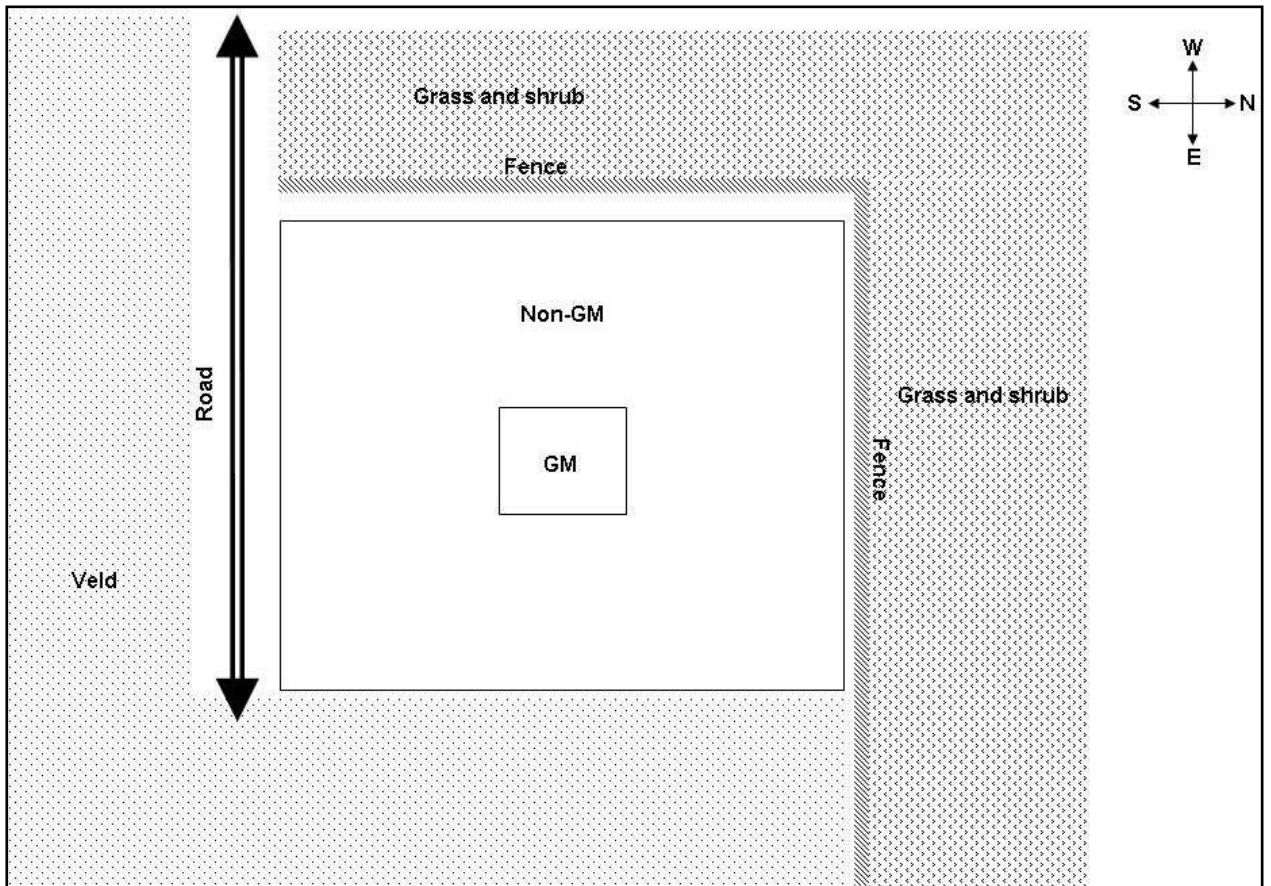| Waterbron (2006/2007)	
Pollen Sample	PCR result
North - 50 m	positive
North - 100 m	positive
North - 200 m	positive
North - 300 m	negative
North - 400 m	negative
North - 500 m	negative
South - 50 m	negative
South - 100 m	negative
South - 200 m	negative
South - 300 m	negative
South - 400 m	negative
South - 500 m	negative
West - 50 m	negative
West - 100 m	negative
West - 200 m	negative
West - 300 m	negative
West - 400 m	negative
West - 500 m	negative
East - 50 m	negative
East - 100 m	negative
East - 200 m	negative
East - 300 m	negative
East - 400 m	negative
East - 500 m	negative

Figure 4.1   Field trial schematic for Bainsvlei (2005/2006) and (2006/2007).
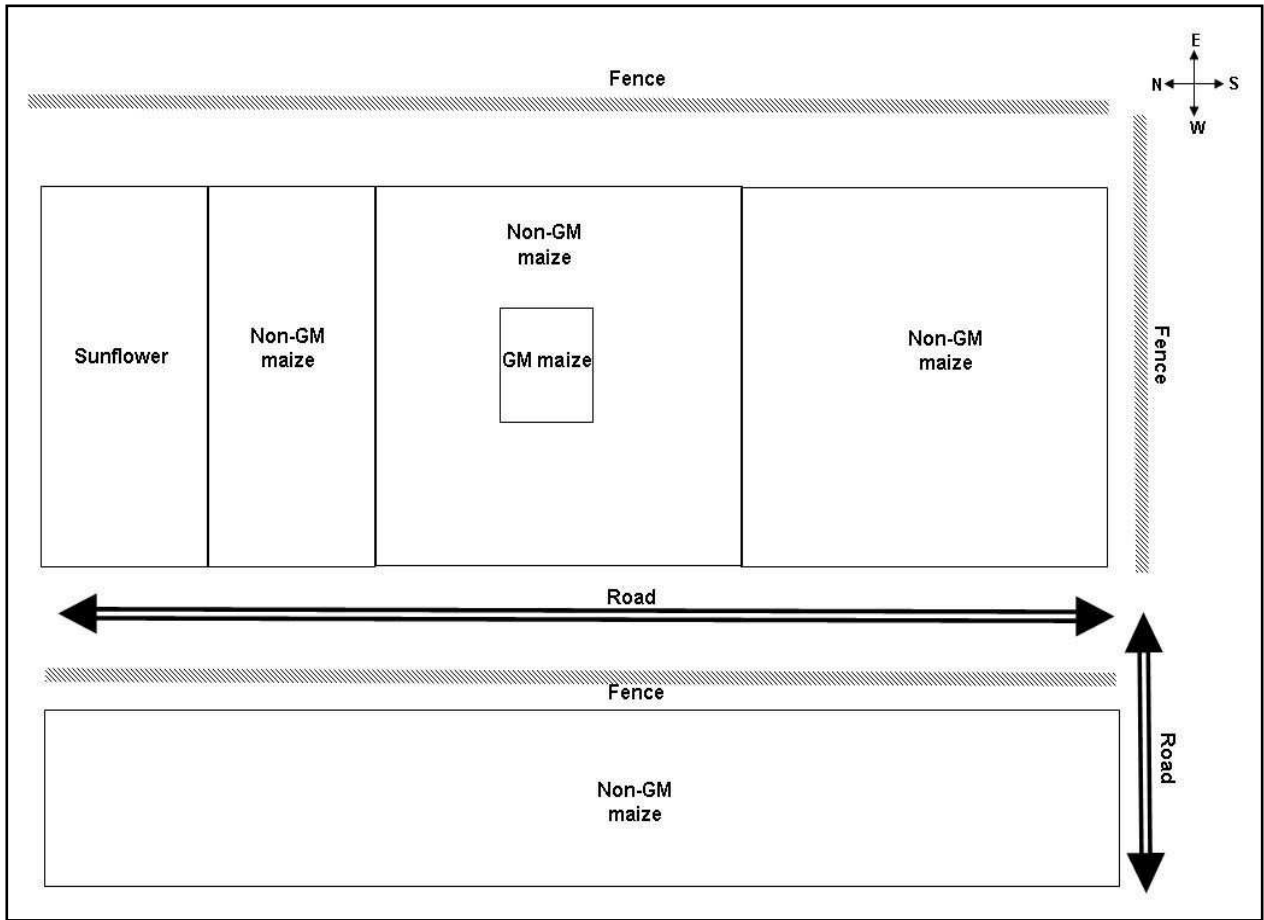
Figure 4.2   Field trial schematic for Waterbron (2006/2007). The surrounding

non-GM maize fields were planted, a minimum of 4 weeks prior to
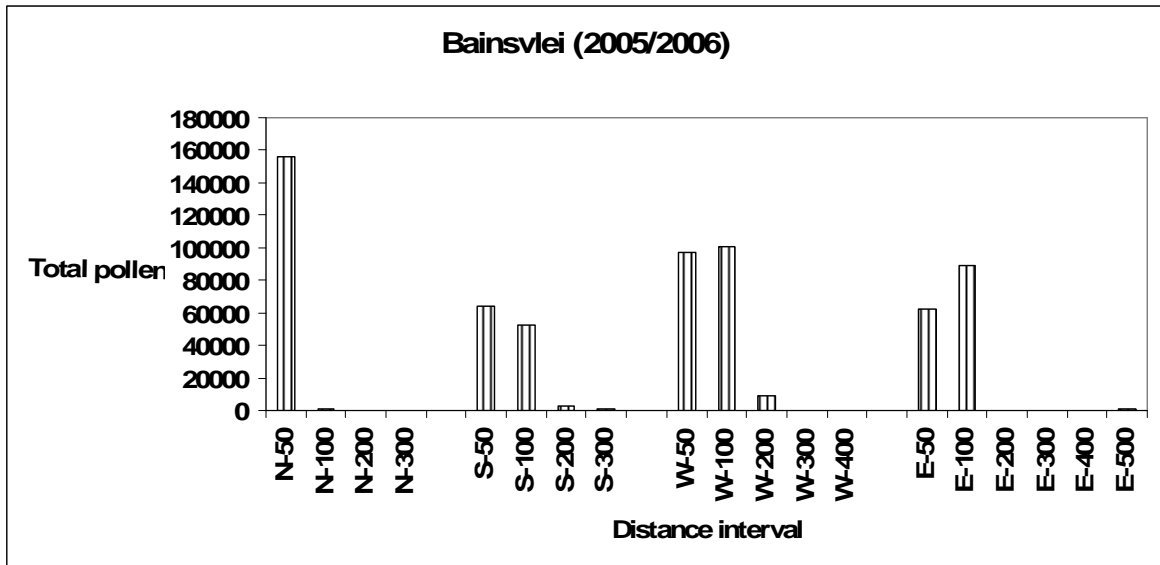
the study trial.

Figure 4.3   Total amount of pollen per distance interval over five days during

flowering for Bainsvlei (2005/2006).

Figure 4.4   Total amount of pollen per days for five days during flowering for

Bainsvlei (2005/2006).
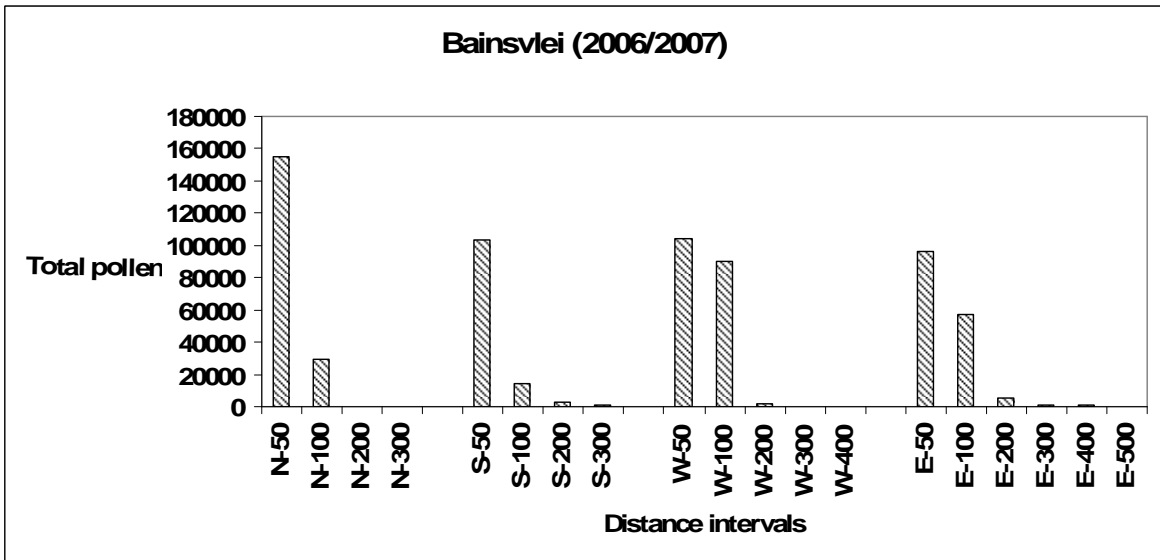
Figure 4.5   Total amount of pollen per distance interval over five days during

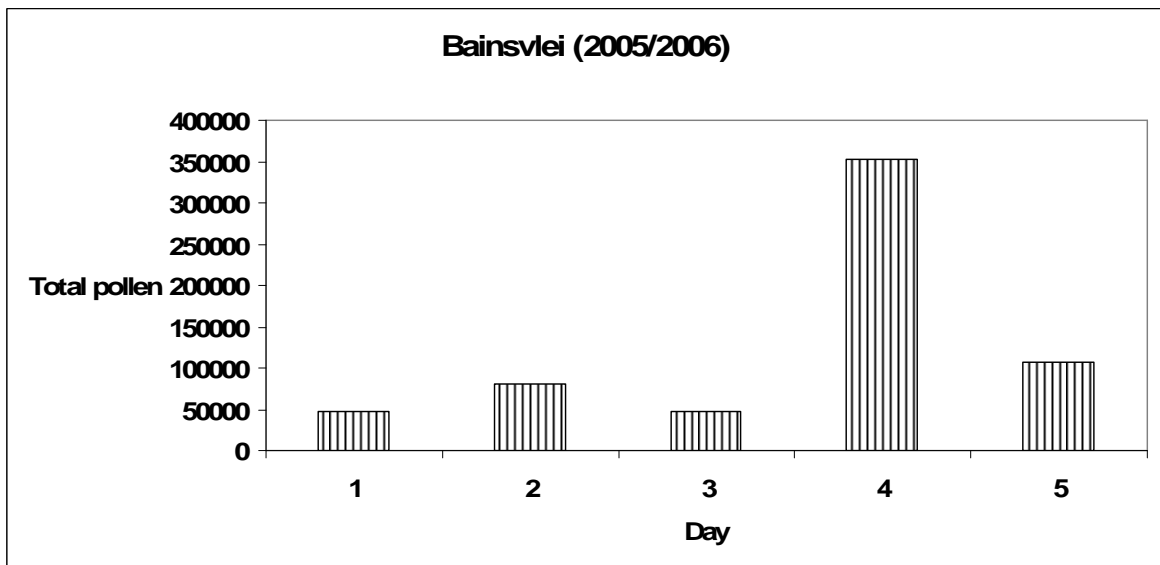flowering for Bainsvlei (2006/2007).
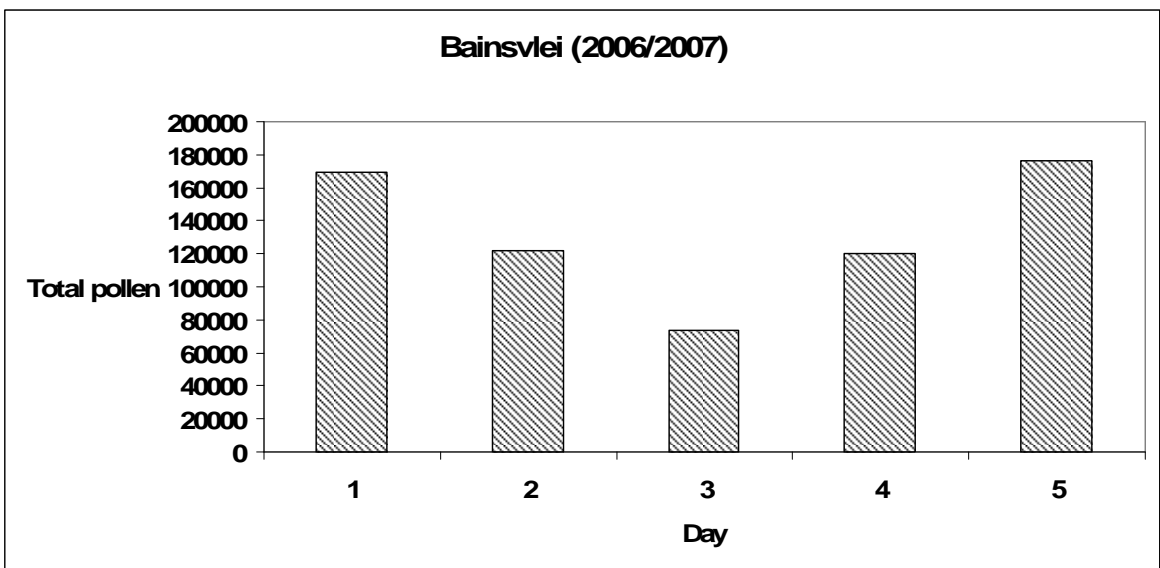
Figure 4.6   Total amount of pollen per days for five days during flowering for

Bainsvlei (2006/2007).

Figure 4.7   Total amount of pollen per distance interval over five days during

flowering for Waterbron (2006/2007).

Figure 4.8   Total amount of pollen per day for five days during flowering for

Waterbron (2006/2007).

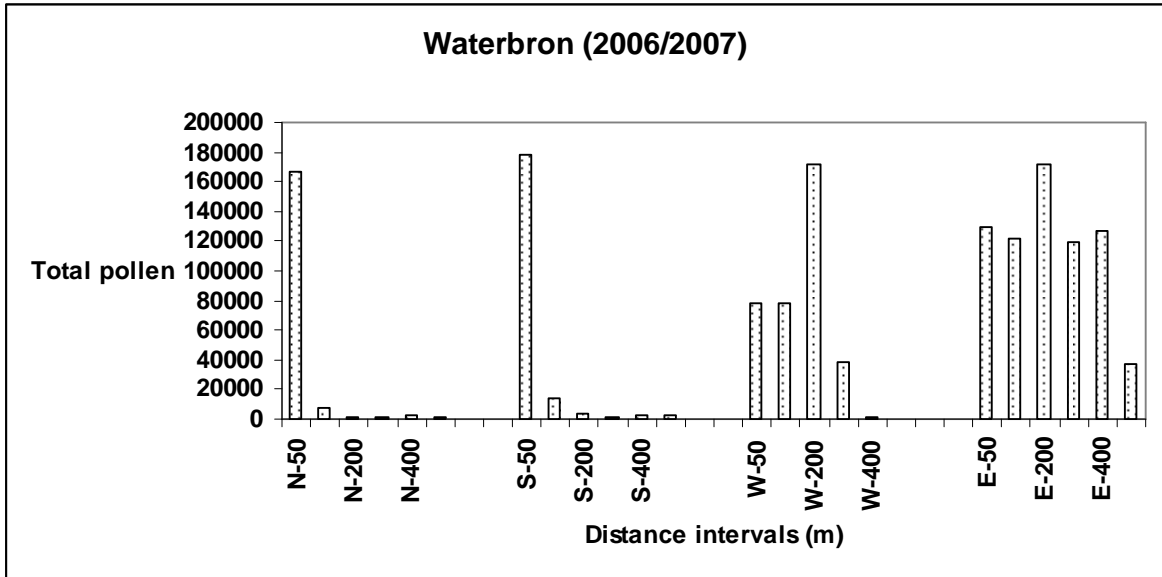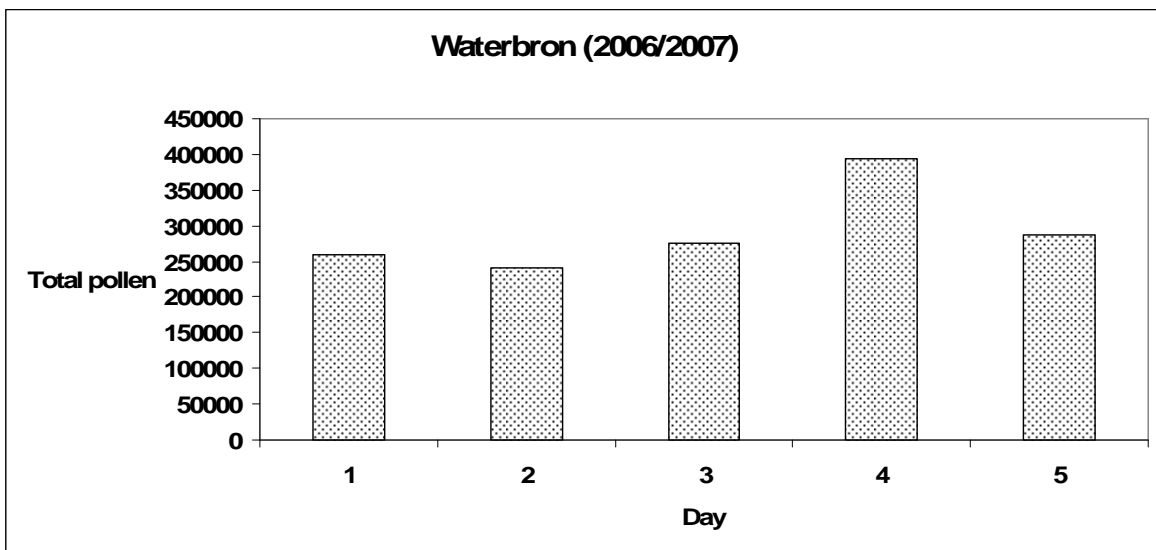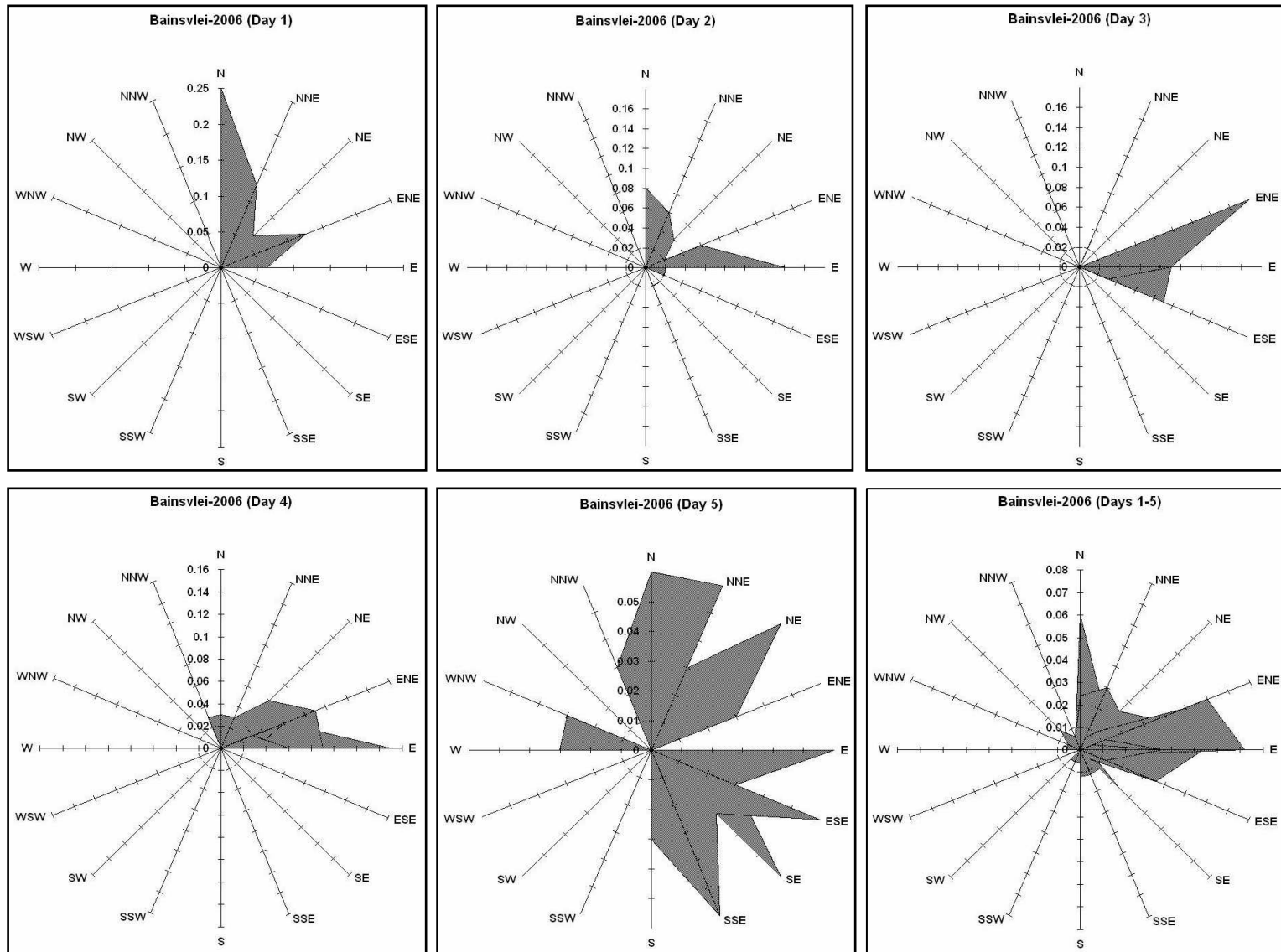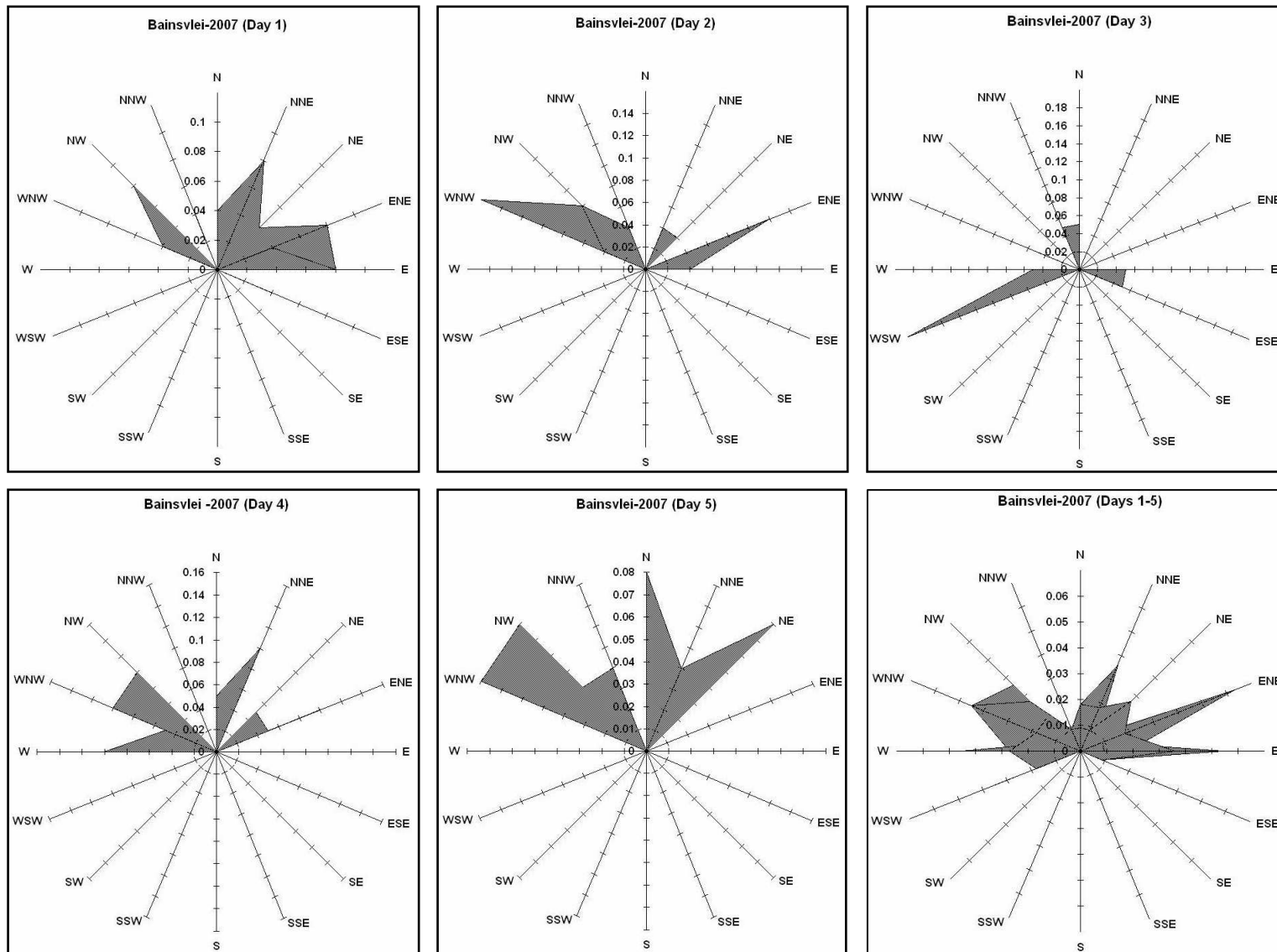Figure 4.9   Wind roses for five days during flowering in Bainsvlei (2005/2006).

Figure 4.10   Wind roses for five days during flowering in Bainsvlei (2006/2007).
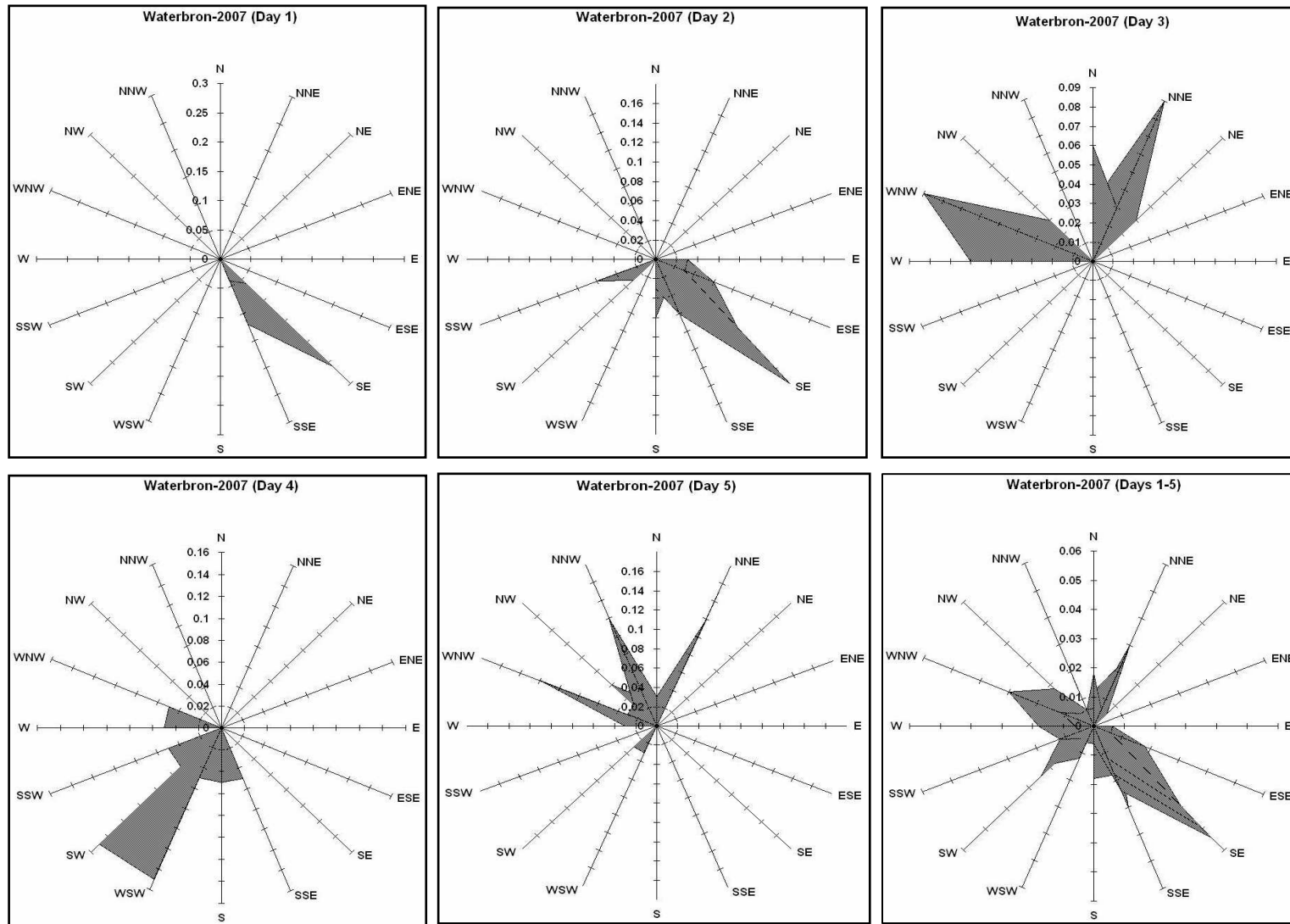
Figure 4.11   Wind roses for five days during flowering in Waterbron (2006/2007).

CHAPTER 5

AN INSIGHT INTO POLLEN-MEDIATED GENE FLOW OF GM MAIZE

IN SOUTH AFRICA

5.1   INTRODUCTION

A decade after the first commercialization of genetically modified (GM) crop (insect resistant cotton) (IR), more than half of the production of principle crops in South Africa is GM (James, 2007). The first generation traits are insect resistance in cotton and maize, herbicide tolerance in maize and soybean as well as stacks for maize (IR and HT) and cotton (IR and HT). Similar to trends in GM adoptive countries, the use of GM crops is expected to increase in South Africa making it a matter of time before second and third generation GM crops are also introduced.

Ironically, the increase in GM adoption, in South Africa and the rest of the world, has not made conventional farming redundant. To the contrary, non-GM niche markets have developed since the introduction of GM, especially in Europe and Asia (Demont and Devos, 2008, Lee, 2008). Although organic farming existed prior to the development of GM, it has gained popularity in recent years. Organic products are either GM free or non-GM with low levels of tolerance to GM (Lee, 2008). Thus as a result of non-GM niche markets, the coexistence of GM crops alongside non-GM crops has become a market imperative. In addition to meeting the requirements of niche markets, pharmaceutical and industrial GM crops require

strict segregation from other food or feed crops (GM and/or non-GM) (Moschini, 2006). Should coexistence fail and there be commingling between a pharmaceutical crop and food crop, as the Prodigene example, the ramifications would be dire (Chetty and Viljoen, 2007). Thus, it is important that the factors affecting coexistence are researched thoroughly and understood to ensure successful implementation.

One of the most significant factors that need to be minimized to achieve coexistence is pollen-mediated gene flow resulting in out-crossing from GM to other GM, non-GM or organic products as well as wild relative and landraces. In addition, although not considered important, one of the consequences of gene flow is the adventitious presence of the transgene, for example the Bt gene that could result in non-GM maize or landraces producing sub-lethal doses of endo-toxin. This would directly contribute to the development of resistance to the toxin in target insects as recently reported in South Africa (Van Rensburg, 2007). This may negatively impact the environment and agricultural sustainability since farmers would have to resort to costly additional insect control measures (Chilcutt and Tabashnik, 2004).

Despite its importance, there are very few studies that investigate PMGF in maize (Table 2.4.2). The studies performed thus far vary in trial design and out-crossing result. For example, out-crossing distances ranged from 34 m in one study to 650 m in another study, this discrepancy in the extent of PMGF in maize has added to the GMO debate and controversy surrounding segregation practices and

coexistence (Paterniani and Stort, 1974; Garcia *et al.*, 1998; Burris, 2001; Jemison and Vayda, 2001; Luna *et al.*, 2001; Aylor *et al.*, 2003; Byrne and Fromhertz, 2003; Henry *et al.*, 2003; Ma *et al.*, 2004; Stevens *et al.*, 2004; Porta *et al.*, 2008; Bannert and Stamp, 2007). Currently, there are no published studies to inform regulatory decisions in terms of science based isolation distances for environmental conditions in South African that can be applied to field trials of GM maize, segregation for non-GM maize or organic production systems. Thus, the aim of this study was determine the extent of PMGF from GM maize to non-GM maize under environmental conditions typical for commercial maize production areas in South African.

5.2   MATERIALS AND METHODS

5.2.1 Field Trial

The same trial referred to in Chapter 4 (Section 4.2.1) (Table 4.1, Figure 4.1 and Figure 4.2) was used in this study.

5.2.2 Phenotypic analysis

The non-GM field was divided into 16 radial transects from the GM field outwards (Figure 5.1). At Bainsvlei, white cobs were sampled at 2 m intervals up to 100 m. At Waterbron, white cobs were sampled at 2 m intervals up to 100 m and thereafter at 10 m intervals up to 400 m.

The number of yellow seeds per cob was counted and expressed as a percentage to total seed per cob (yellow seeds indicate out-crossing). The average percentage out-crossing over distance was represented graphically and subjected to a power trendline. The trendline equation, was used to calculate theoretical distances for 1.0%, 0.01%, 0.001% and 0.0001% out-crossing, for each wind direction observed.

The out-crossing data was also represented in a radial graph for correlation to wind data in a wind rose.

5.2.3 Genotypic analysis

Cobs with phenotypically visible out-crossing were selected from the furthest three distances that out-crossing was observed. The white seed was collected, DNA extracted and screened for GM content using PCR to determine whether partial introgression of the transgene occurred, where the yellow phenotype may not be expressed.

5.2.3.1 DNA Extraction

Sampled seed was milled using a Waring Blender to a granule size of less than 1.5 mm^2. A sub-sample of 2 g was taken and DNA extracted by the addition of 10 ml CTAB buffer (20 g/l CTAB, 1.4 M NaCl, 0.1 M Tris/HCl and 20 mM EDTA, pH 8) and 30 µl Proteinase K [20 mg/ml]. The sample was incubated for 2 hours at 65℃, with vortexing every 10 min. for 10 sec. The sample was centrifuged at 4 k rpm for 5 min. at room temperature and 1 ml of the supernatant retained and incubated for 5 min. at 80℃, followed by the addition of 5 µl RN ase A [100 mg/ml] and a further

incubation at 65℃. Thereafter, chloroform:isoamyl alcohol (24:1) (1 ml) was added to the sample followed by centrifugation for 5 min. at 14 k rpm. The aqueous layer was retained and the procedure repeated 3 times. The DNA was precipitated by the addition of absolute ethanol (1 ml) on ice for 1 hour. The precipitate was obtained by centrigugation for 20 min. at 14k rpm, the supernatant discarded and the pellet washed twice with 75% ethanol (500 µl) for 5 min. at 14k rpm. The pellet was dissolved in 100 µl 0.2x T.E. buffer (1 M Tris and 0.5 M EDTA). The DNA was further purified using the GFX PCR DNA and gel Band Purification Kit according to the manufacturer's guidelines (Amersham Biosciences).

5.2.3.2　PCR analysis

GMO screening was performed using the 35S promoter sequence for CaMV according to the method of Lipp *et al*. (2001). A PCR master mix was prepared containing 19.9 µl 35S PCR buffer (GeneScan, GmbH) and 0.16 µl Ampli-Taq Gold for each reaction, including negative and positive controls. Each sample tube contained 20 µl of master mix and 5 µl of sample DNA while the negative control contained 5 µl 0.2x T.E buffer and the positive control contained transgenic 35S DNA (GeneScan, GmbH). The PCR was performed in an ABI 9700 and the cycling 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) followed by $72^{o}C$ for 7 min. and $25^{o}C$ (1 cycle). The limit of detection was 0.01%. The 35S amplicon (123 bp) was resolved in a 2.0% Agarose gel at 200 V for 40 to 50 min. and then visualised under UV light after staining with Ethidium Bromide [150 µl /1.5 l] for 30 mins.

5.3 RESULTS

5.3.1 Field trial

All maize trials reached flowering and seed set with widespread out-crossing of yellow GM maize with white conventional maize except the Kroonstad trial in the (2005/2006) season. The Kroonstad trial failed due to winter frost as a result of late planting and late first rains.

5.3.2 Phenotypic analysis

Out-crossing was recorded between GM yellow maize and white conventional maize for all field trials (Fig 5.15). At Bainsvlei (2005/2006) the highest out-crossing (13.81%) was observed at 2 m and the lowest percentage out-crossing observed was 0.01% at 94 m (Fig 5.2). In the second season at Bainsvlei (2006/2007), the highest out-crossing observed was 18.76% at 2 m and the lowest percentage out-crossing (0.01%) was observed at 96 m (Fig 5.3). At Waterbron (2006/2007), the out-crossing at 2 m was 18.48% and the lowest percentage out-crossing (0.01%) was observed at 300 m (Fig. 5.4). At all three field trials the percentage out-crossing declined sharply up to 25 m with intermittent levels of out-crossing thereafter up to the end of the non-GM white maize field (Fig 5.2, 5.3 and 5.4).

At Bainsvlei (2005/2006), a theoretical level of 1.0% out-crossing was calculated at a distance of 9 m, 0.1% at 33 m, 0.01% at 113 m, 0.001% at 396 m and a theoretical zero (0.0001%) at 1382 m (r^2 = 0.9) (Table 5.1) (Fig. 5.5). During the

second season (2006/2007) at Bainsvlei, the theoretical 1.0% out-crossing was at 14 m, 0.1% at 44 m, 0.01% at 135 m, 0.001% at 418 m and the theoretical zero (0.0001%) was at 1295 m (r^2 = 0.92) (Table 5.2) (Fig. 5.6). At Waterbron (2006/2007), the theoretical 1.0% out-crossing was calculated at 16 m, 0.1% at 53 m, 0.01% at 177 m, 0.001% at 596 m and the theoretical zero (0.0001%) was calculated to be 2009 m (r^2 = 0.9) (Table 5.3) (Fig. 5.7) (Hurst *et al.*, 1999).

5.3.3 Genotypic analysis

The transgene was not detected in any of the white seed samples tested.

5.3.4 Weather data

In Bainsvlei (2005/2006), the wind was predominantly northerly and the predominant out-crossing was observed in a southerly direction. The average temperature ranged between 20℃ and 25℃ (Fig 5.9) and the average range in relative humidity between 56% and 72% (Fig. 5.10). In the second season (2006.2007) in Bainsvlei the prevailing winds were northerly, and out-crossing observed in the southerly region of the trial (Fig. 5.8). The average temperature ranged between 25℃ and 27℃ (Fig. 5.11) and the av erage relative humidity between 30% and 37% (Fig. 5.12).

In the Waterbron trial (2006/2007), the wind blew in all directions with the predominate wind in the northerly and southerly directions. The majority of out-crossing was in the northern and southern areas of the field (Fig. 5.8). The

average temperature ranged between 18°C and 23°C (F ig. 5.13) and the average relative humidity was between 29% and 40% (Fig. 5.14).

5.4   DISCUSSION AND CONCLUSIONS

Three maize trials used for this study flowered synchronously and achieved seed-set despite unfavourable climate conditions for maize planting. Nonetheless, out-crossing was detected at the furthest distance of 300 m at 0.01% in Waterbron (2006/2007). This result is similar to the PMGF study by Stevens *et al.* (2004), where PMGF was detected at 300 m at 0.02%. The pattern of out-crossing observed in all field trials showed, high levels of out-crossing at the distance intervals closest to the GM source plot with a sharp decline to 25 m. This was also observed by Ma *et al.* (2004). After 25 m, intermittent out-crossing was observed to the end of the white non-GM field and was probably due to non-horizontal wind types such as gust or swirling winds. Similar findings of intermittent out-crossing at long distances were reported by Bannert and Stamp (2007).

The determination of wind type was not within the scope of this study only the dimensions of horizontal wind flow (wind speed and direction) were captured. Thus, it is essential to fully understand the various parameters within the environment and its subsequent influence on out-crossing in a PMGF study.

When considering the effect of out-crossing based on the statistical analysis indicating that the average expected distance for a theoretical zero will be 1382 m,

1295 m and 2009 m for Bainsvlei (2005/2006), Bainsvlei (2006/2007) and Waterbron (2006/2007), respectively. The average expected distance ranged from 33 m to 53 m for 0.1% out-crossing, from 113 to 177 m for 0.01% out-crossing, 396 m to 596 m for 0.001% out-crossing and for the theoretical zero (0.0001%), the expected distance was 1295 m to 2009 m. However these values are based on the average out-crossing percentages for all wind directions. The data for actual out-crossing per wind direction indicates an entirely different scenario, for example in the ENE direction in Bainsvlei (2005/2006), the expected distance to achieve 0.01% admixture will be approximately 79 km. In the second season the distance is 956 m and in Waterbron (2006/2007), the distance is approximately 3.5 km. Therefore, it is important to note that out-crossing is favoured in a particular direction depending on the location.

These data have implications for farmers who want to achieve crop production below various threshold levels of percentage commingling. In a trial of this magnitude, 1% was below 25 m however at a commercial scale it would be much higher at greater distance. Therefore for GM to effectively coexist with organic and conventional crop the expected distance have to placed in context of the threshold levels required (Fig 2.4.3).

Besides the level of admixture that can occur as a result of out-crossing, another concern has arisen as a result of PMGF and that is the development of resistance in the target insect as a result of sub-lethal dosages of toxin produced in out-crossed seed (Chilcutt and Tabashnik, 2004). South African subsistence and

small-scale farmers share and save seed and grow traditional varieties alongside hybrid maize. This behaviour, although not allowed by the patent laws of GM seed, continue throughout the developed world, greatly contributing to gene flow. Target insect resistance has already been reported in South Africa and it is yet to be established whether this was as a result of lack of refugia or due to gene flow (Van Rensburg, 2007).

In terms of regulatory decisions especially with regards to second and third generation GMOs, this data can be considered when determining the limits of a field. The factors that have to be considered are the type of GM, crop type, threshold requirement, the size of field and typical environmental conditions for that area. And thus a decision can be made on whether actual or average expected distance should be utilised in establishing isolation distances. In addition, the methodology used in this study can be used as a blueprint for to monitor field trials of new GM in crops other than maize.

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Table 5.1   Calculated theoretical distances for 1%, 0.1%, 0.001% and 0.0001% out-crossing for Bainsvlei

(2005/2006).

	1	0.1	0.01	0.001	0.0001	Equation	r^2
N	2	291	35055	4225890	509429012	$y = 1.5267x^{-0.4805}$	0.80
NNE	8	59	445	3371	25569	$y = 10.218x^{-1.1365}$	0.79
NE	1	16	308	5824	110083	$y = 0.8906x^{-0.7834}$	0.57
ENE	1	209	79677	30430621	11622207095	$y = 0.7912x^{-0.3873}$	0.63
E	8	68	584	5032	43381	$y = 9.0534x^{-1.0689}$	0.77
ESE	8	64	505	3994	31578	$y = 10.245x^{-1.1136}$	0.65
SE	6	61	590	5686	54763	$y = 6.5637x^{-1.0166}$	0.55
SSE	10	37	134	492	1799	$y = 59.961x^{-1.7751}$	0.89
S	16	73	333	1527	7008	$y = 64.84x^{-1.5113}$	0.73
SSW	13	222	3877	67601	1178740	$y = 7.7719x^{-0.8055}$	0.27
SW	14	105	773	5705	42089	$y = 21.28x{-}^{1.1522}$	0.68
WSW	14	104	796	6093	46622	$y = 19.165x^{-1.1315}$	0.54
W	16	63	248	969	3788	$y = 110.73x^{-1.6891}$	0.87
WNW	4	92	2243	54906	1343732	$y = 2.5882x^{-0.7201}$	0.49
NW	1	21	53	85	117	$y = 0.442e^{-0.0717x}$	0.83
NNW	10	55	311	1743	9777	$y = 21.306x^{-1.3354}$	0.65
Average	9	33	113	396	1382	$y = 61.043x^{-1.8422}$	0.90

Table 5.2   Calculated theoretical distances for 1%, 0.1%, 0.001% and 0.0001% out-crossing for Bainsvlei (2006/2007).

	1	0.1	0.01	0.001	0.0001	Equation	r^2
N	3	196	11892	720727	43681942	$y = 1.9329x^{-0.561}$	0.36
NNE	12	64	357	1981	11001	$y = 26.818x^{-1.3432}$	0.74
NE	7	91	1191	15607	204479	$y = 5.6628x^{-0.895}$	0.62
ENE	11	103	956	8907	83007	$y = 11.873x^{-1.0316}$	0.65
E	7	82	962	11237	131250	$y = 6.2321x^{-0.9368}$	0.47
ESE	12	80	556	3867	26888	$y = 18.183x^{-1.1874}$	0.85
SE	14	119	1009	8527	72080	$y = 17.383x^{-1.0787}$	0.85
SSE	18	101	550	3005	16415	$y = 51.985x^{-1.356}$	0.73
S	28	107	412	1583	6080	$y = 298.83x^{-1.7113}$	0.86
SSW	23	108	499	2298	10592	$y = 116.31x^{-1.507}$	0.72
SW	17	76	344	1567	7139	$y = 71.207x^{-1.5187}$	0.71
WSW	20	96	447	2093	9798	$y = 90.032x^{-1.4919}$	0.85
W	14	129	1222	11578	109676	$y = 14.507x^{-1.0241}$	0.54
WNW	7	104	1514	21943	318030	$y = 5.4793x^{-0.8612}$	0.57
NW	15	100	674	4542	30603	$y = 25.961x^{-1.207}$	0.91
NNW	13	127	1205	11416	108140	$y = 14.299x^{-1.0241}$	0.54
Average	14	44	135	418	1295	$y = 216.91x^{-2.0359}$	0.92

Table 5.3   Calculated theoretical distances for 1%, 0.1%, 0.001% and 0.0001% out-crossing for Waterbron

(2006/2007).

|	1	0.1	0.01	0.001	0.0001	Equation	r^2
N	34	41	41	41	41	$y = -4.9683Ln(x) + 18.5$	0.37
NNE	13	150	1767	20803	244853	$y = 10.782x^{-0.9339}$	0.59
NE	11	121	1312	14173	153125	$y = 10.388x^{-0.9675}$	0.67
ENE	6	141	3479	85933	2122835	$y = 3.4892x^{-0.718}$	0.57
E	10	133	1731	22586	294636	$y = 8.0023x^{-0.8965}$	0.62
ESE	11	95	805	6844	58206	$y = 13.356x^{-1.0757}$	0.53
SE	7	48	328	2261	15589	$y = 9.9985x^{-1.1925}$	0.76
SSE	16	50	154	471	1442	$y = 317.14x^{-2.0581}$	0.83
S	23	107	503	2369	11163	$y = 102.75x^{-1.4852}$	0.81
SSW	33	159	780	3821	18714	$y = 155.62x^{-1.4494}$	0.82
SW	22	130	769	4537	26751	$y = 55.637x^{-1.2977}$	0.75
WSW	22	136	854	5358	33622	$y = 47.319x^{-1.2537}$	0.76
W	14	111	853	6578	50715	$y = 20.142x^{-1.1273}$	0.75
WNW	14	77	433	2443	13773	$y = 32.412x^{-1.3314}$	0.76
NW	6	66	708	7566	80864	$y = 5.8864x^{-0.9719}$	0.90
NNW	11	59	310	1625	8512	$y = 29.183x^{-1.3906}$	0.82
Average	16	53	177	596	2009	$y = 183.12x^{-1.8961}$	0.89
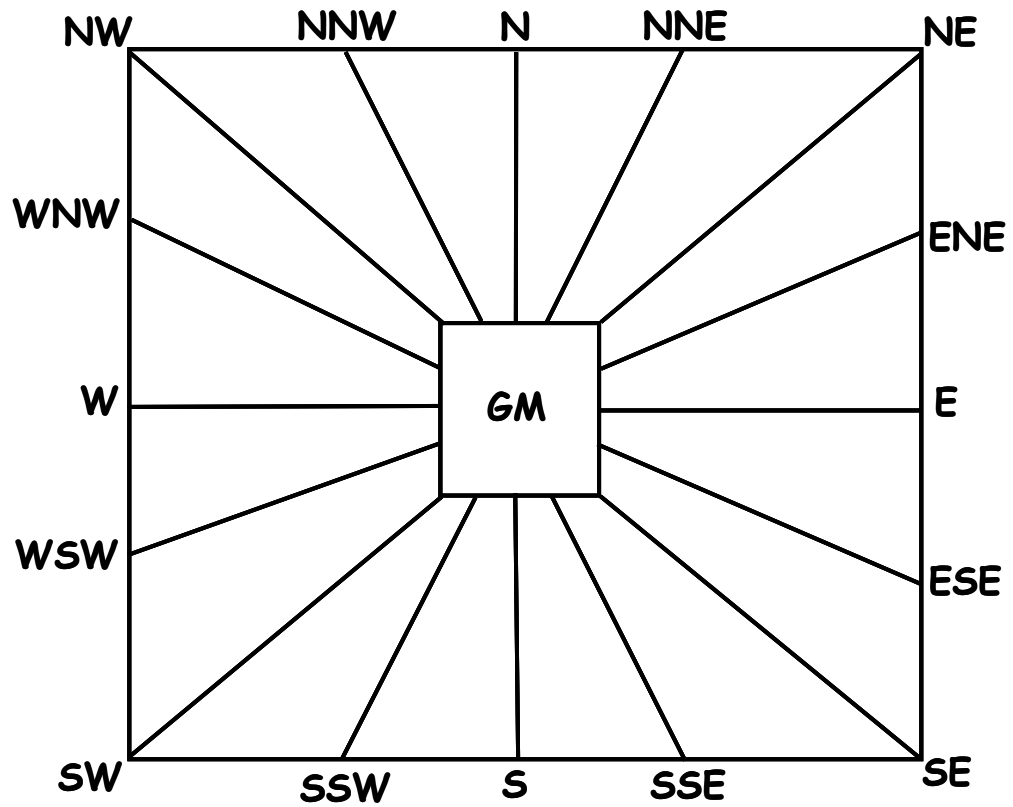
Figure 5.1   Diagram represents the cardinal directions that sampling was performed in all the field trials
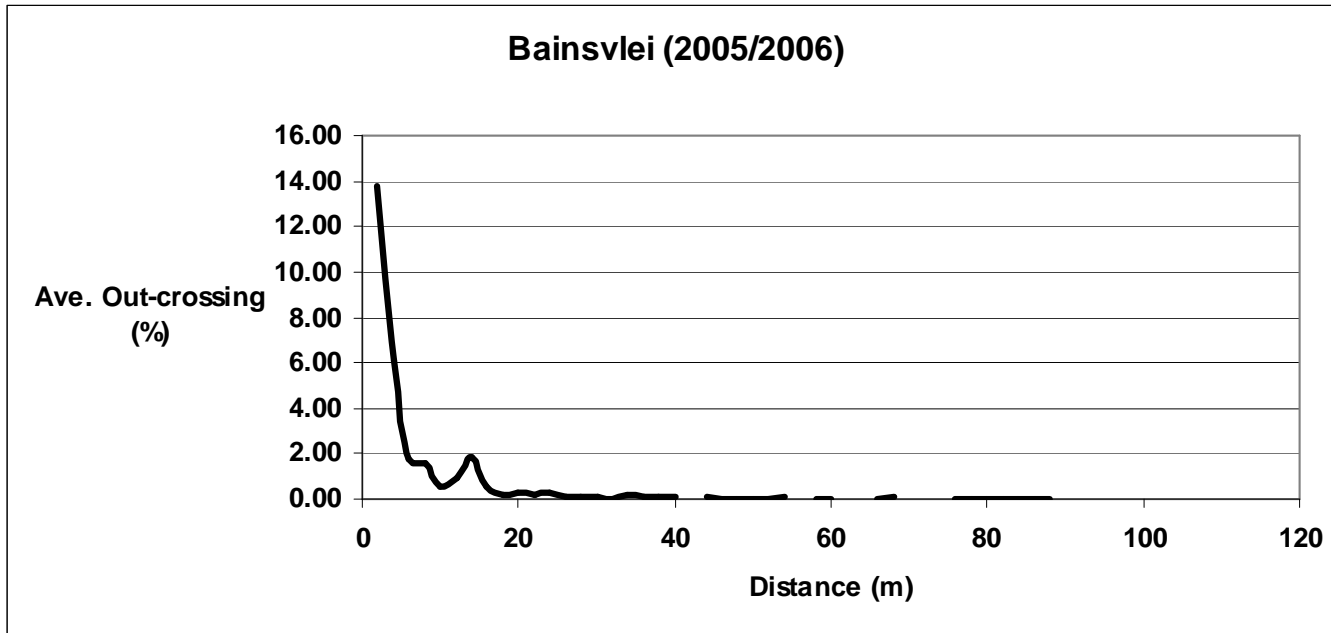
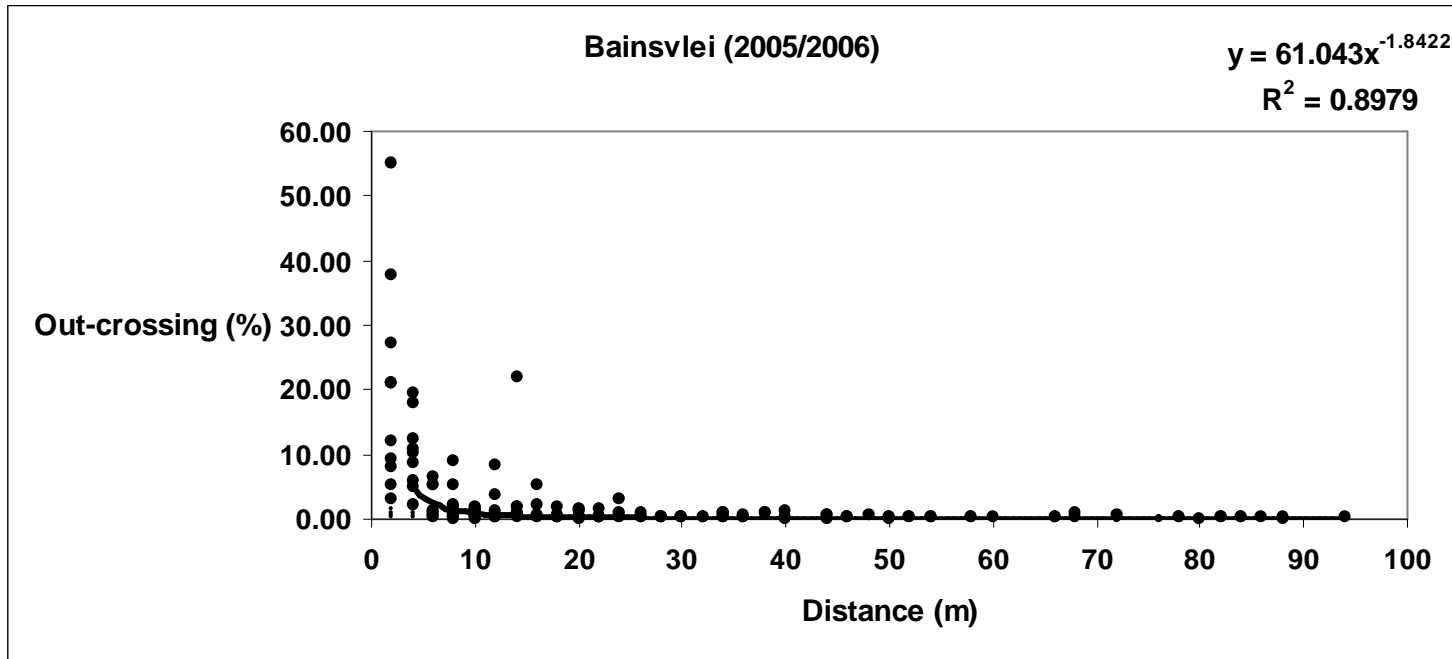Figure 5.2   Average percentage out-crossing over distance for Bainsvlei (2005/2006).

Figure 5.3   Percentage out-crossing for 16 directions over distance in Bainsvlei (2005/2006) with the power

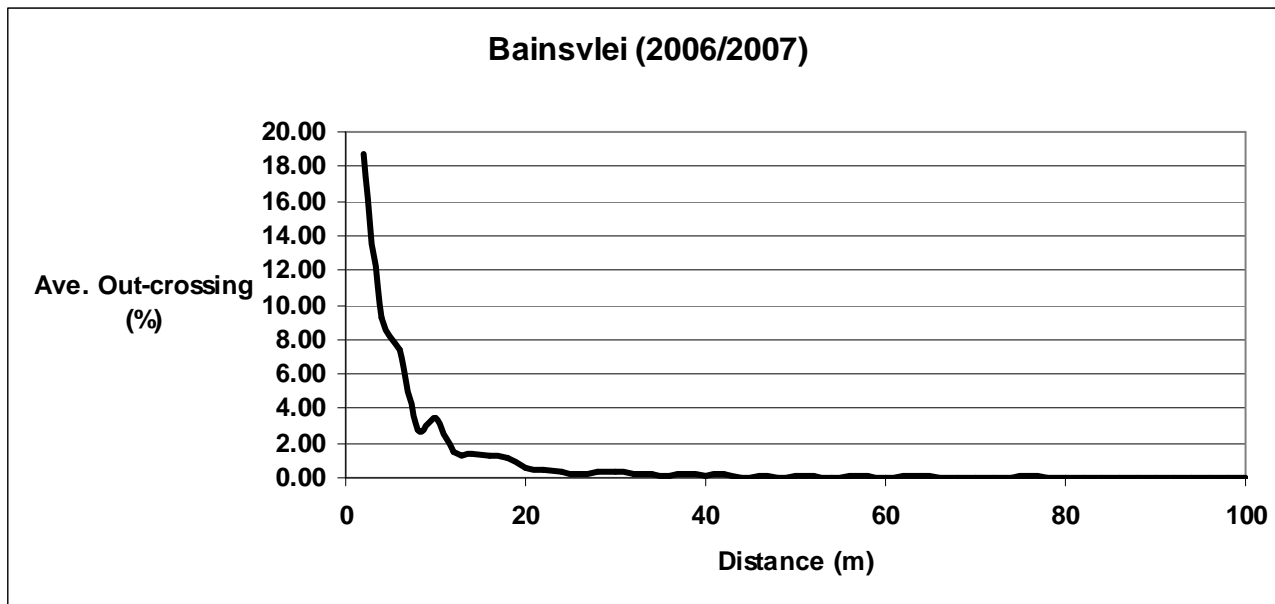trendline and equation.

Bainsvlei (2006/2007)

Figure 5.4   Average percentage out-crossing over distance for Bainsvlei (2006/2007).
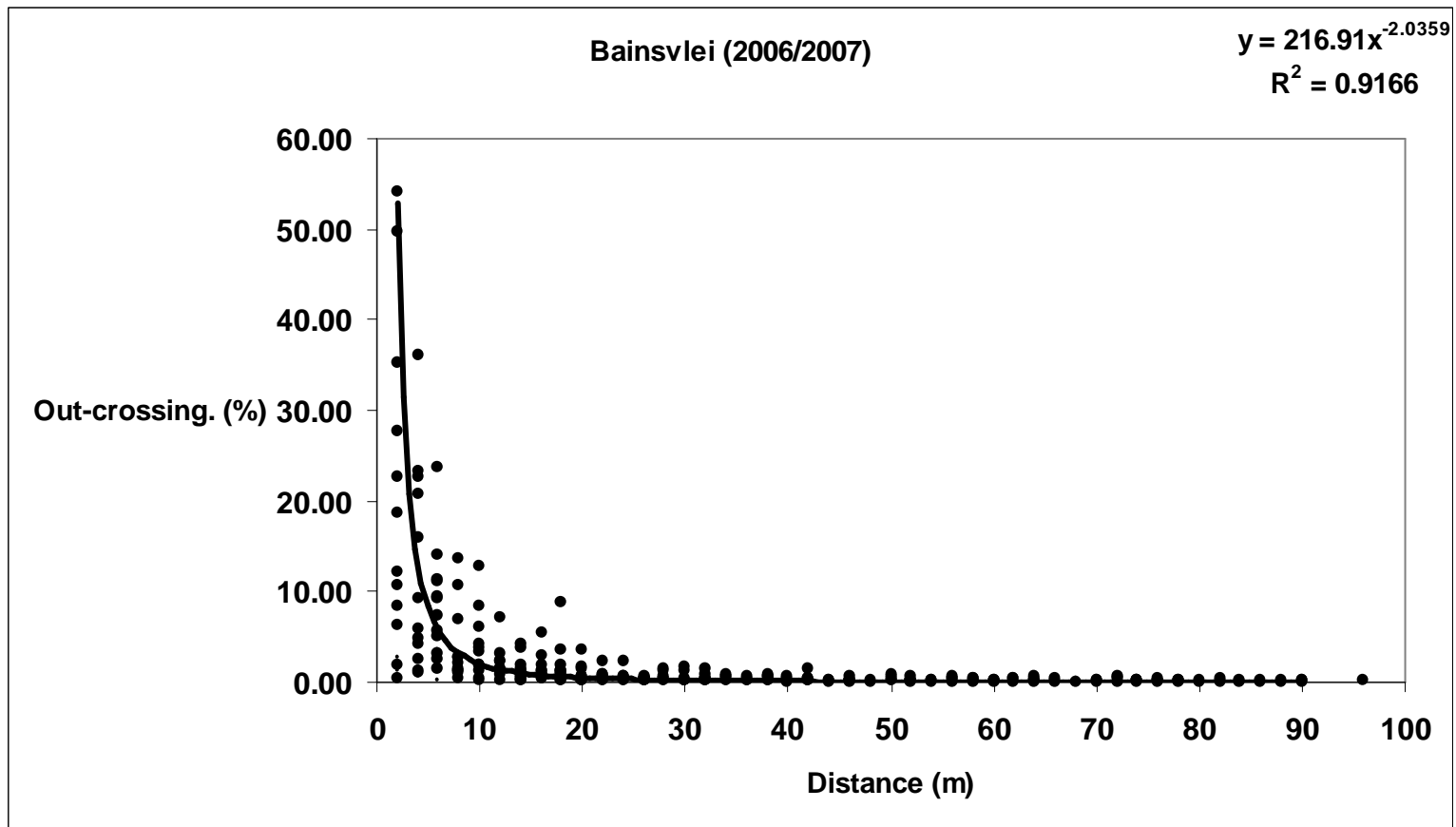
Figure 5.5   Percentage out-crossing for 16 directions over distance in Bainsvlei (2006/2007) with the power
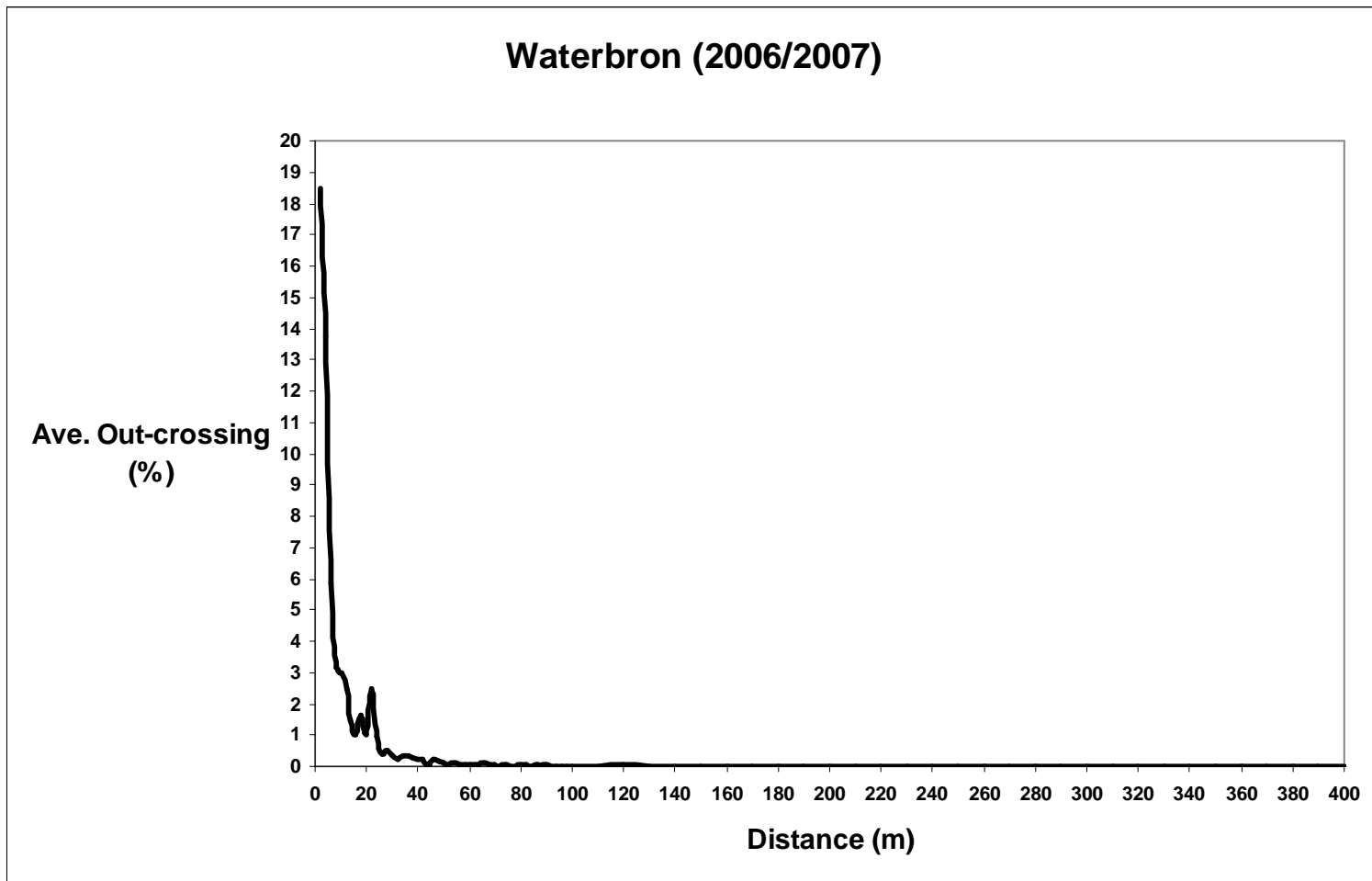trendline and equation.

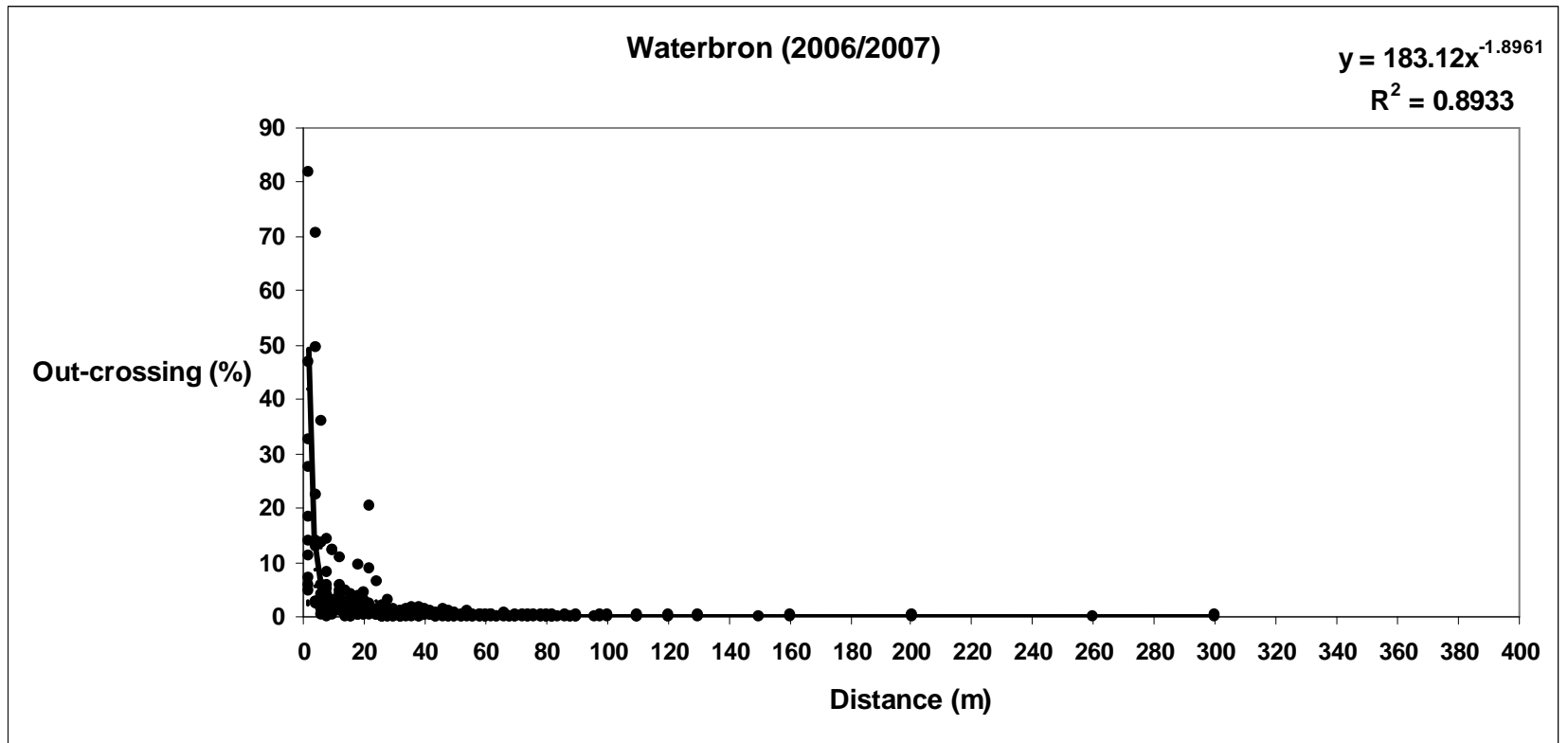Figure 5.6   Average percentage out-crossing over distance for Waterbron (2006/2007).

Figure 5.7   Percentage out-crossing for 16 directions over distance in Waterbron (2006/2007) with the power trendline and equation.
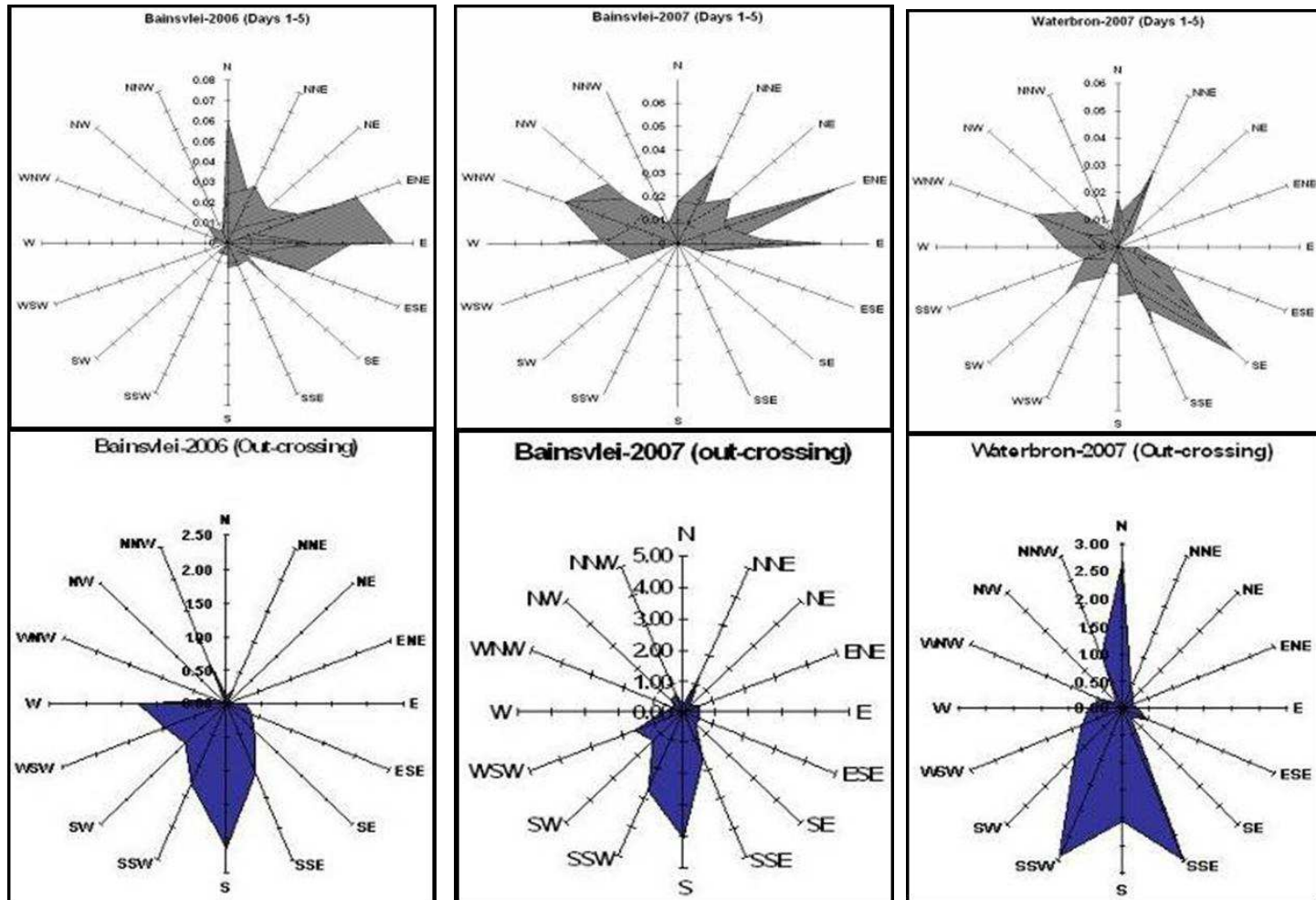
Figure 5.8   Out-crossing (■) observed in Bainsvlei (2005/2006), Bainsvlei (2006/2007) and Waterbron

(2006/2007) with the corresponding wind roses (■).

Figure 5.9   Temperature for five flowering days in Bainsvlei (2005/2006).

Figure 5.10   Relative humidity for five flowering days in Bainsvlei (2005/2006).

Figure 5.11   Temperature for five flowering days in Bainsvlei (2006/2007).

Figure 5.12   Relative humidity for five flowering days in Bainsvlei (2006/2007).

Figure 5.13   Temperature for five flowering days in Waterbron (2006/2007).
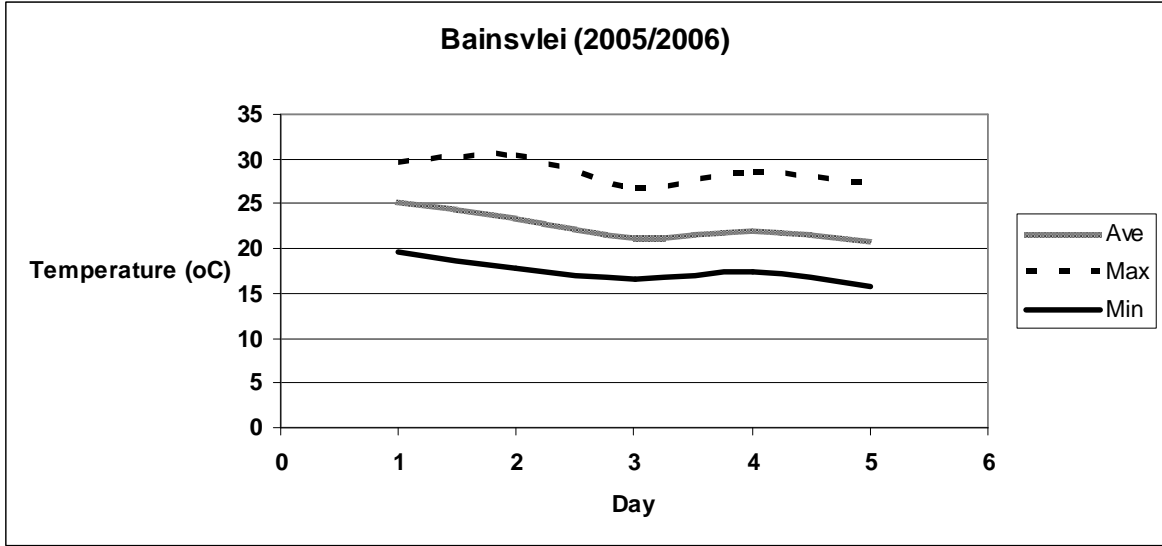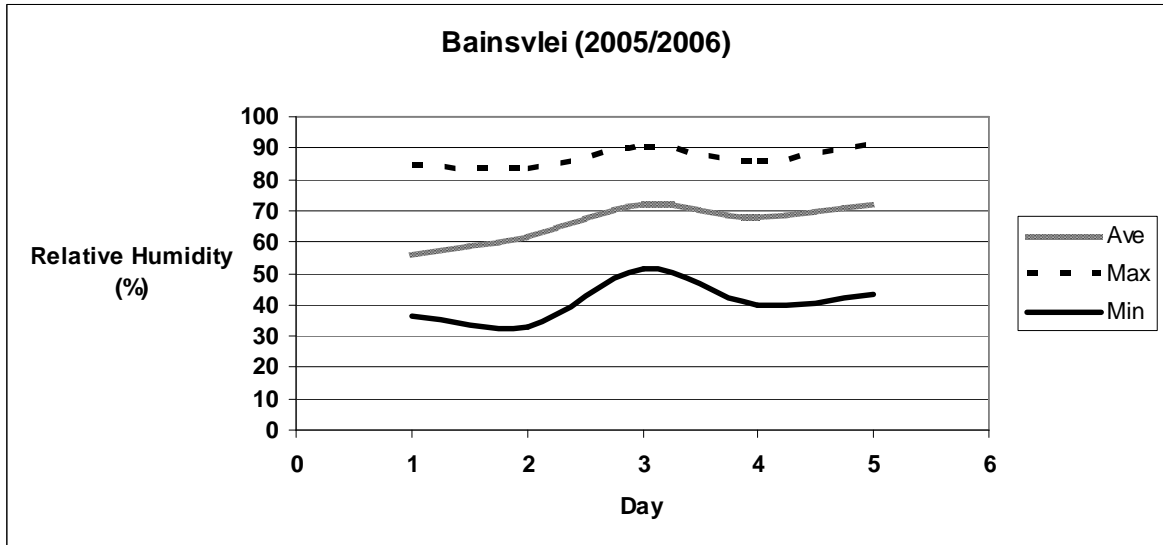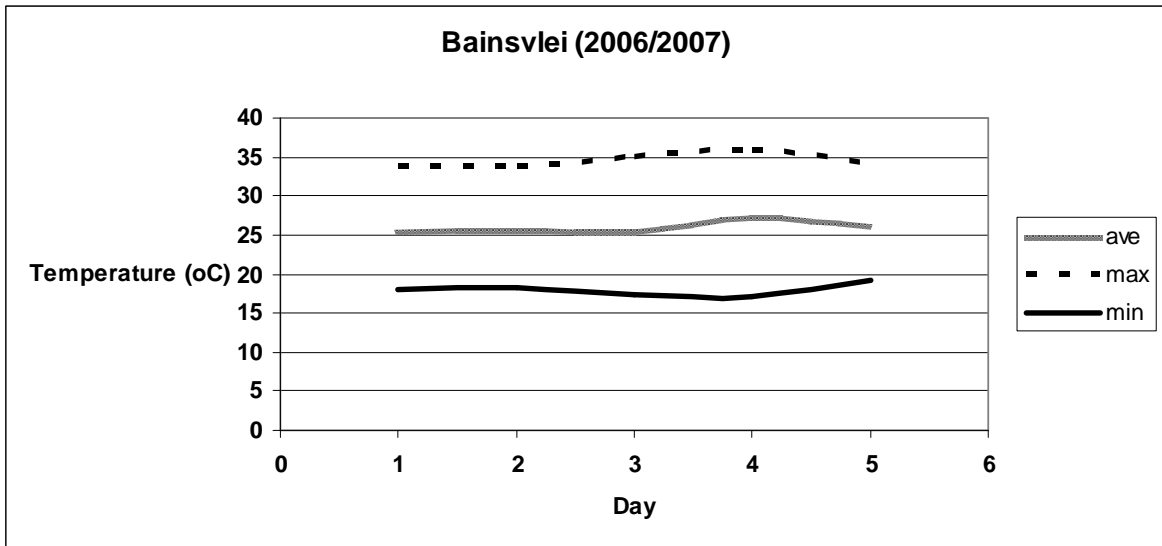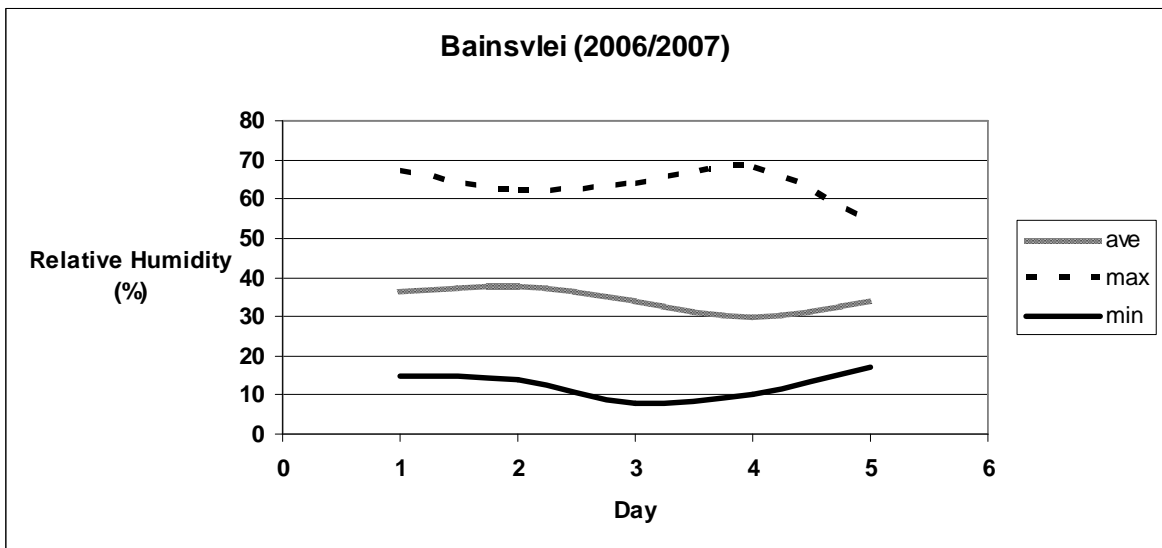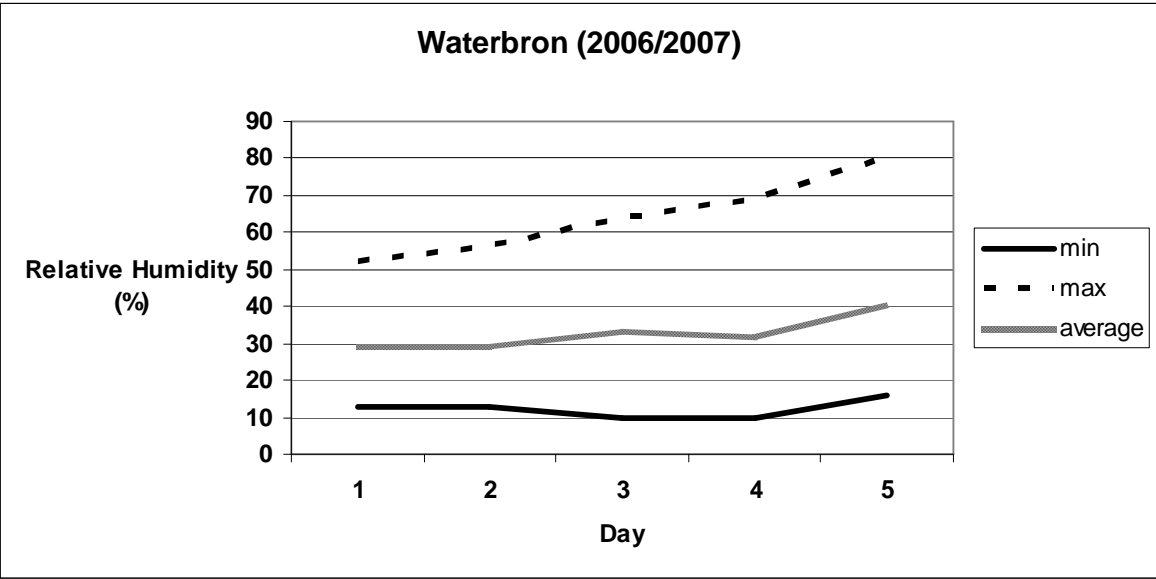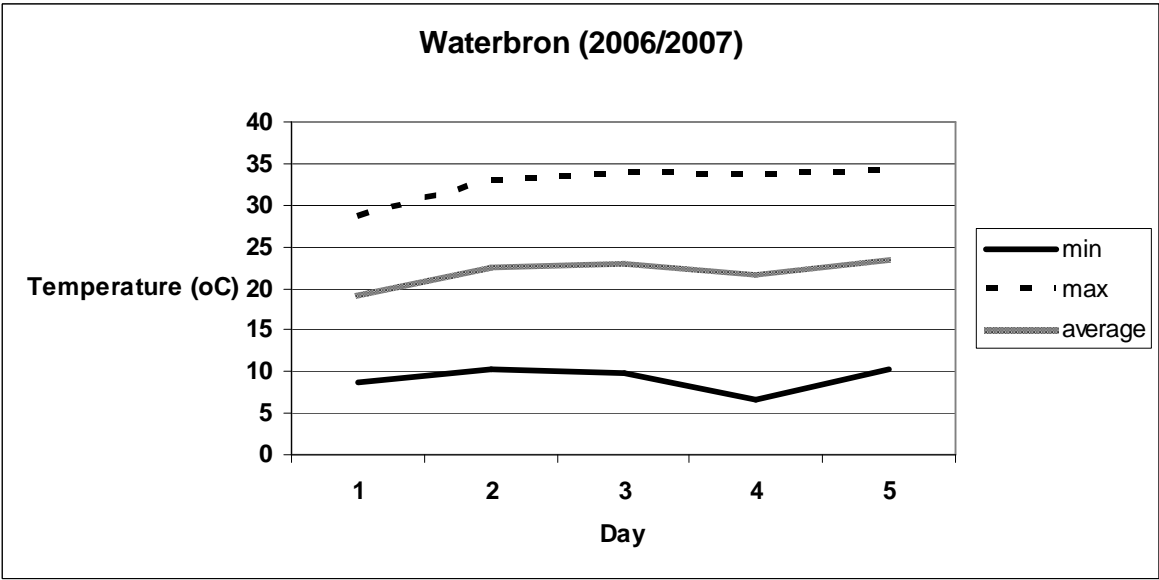

Figure 5.14   Relative humidity for five flowering days in Waterbron (2006/2007).
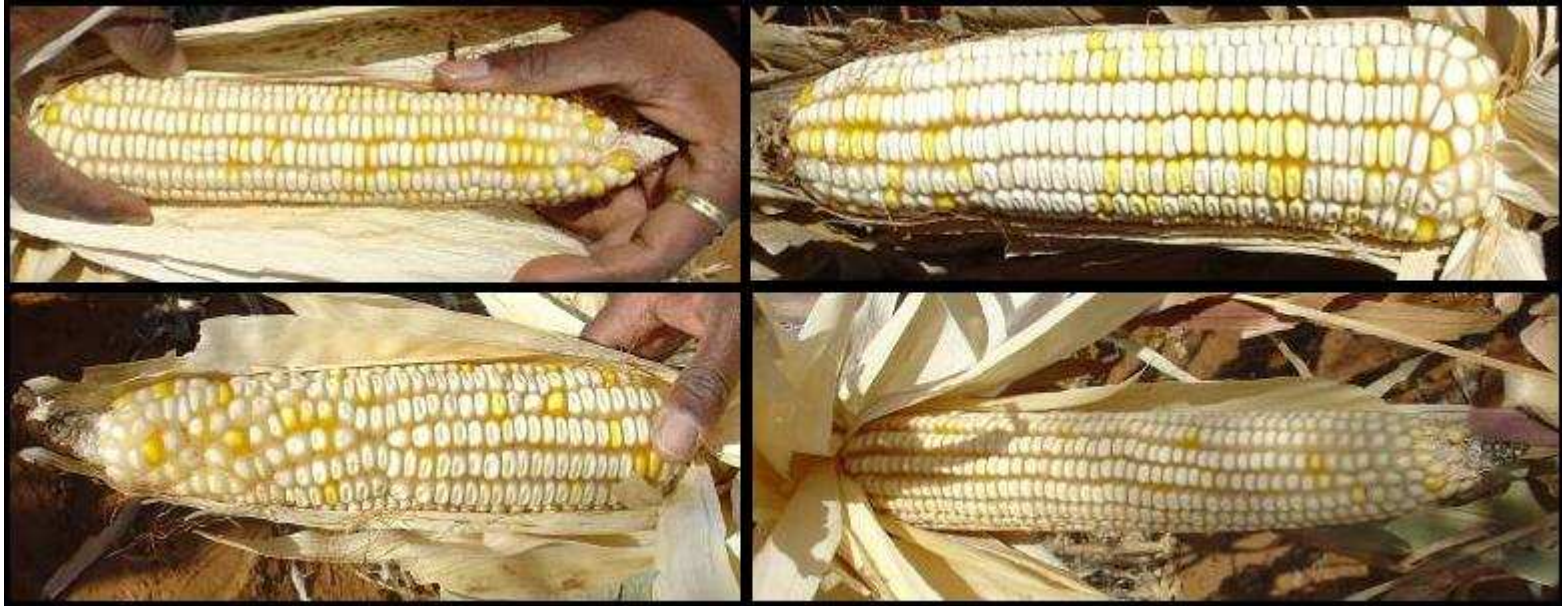
Figure 5.15   Out-crossing observed during the duration of the study.

CHAPTER 6: CONCLUSION

6.1 Making Biotech crops work for Africa requires effective management

When GM crops were introduced, one of the promised benefits was increased food security, more cost effective agricultural production with a positive impact on the environment (Carvalho, 2006). Thus, in comparison to conventional agricultural practices, GM crop production is intended to provide a more environmentally sound yet sustainable alternative. It is difficult to currently ascertain the true impact of genetic engineering as so many aspects require consideration: human health, socio-economics, the environment and pesticide and herbicide use, non-target organisms in the case of insecticidal toxins (Bt) as well as gene flow to wild relatives and landraces. It has been suggested that in order to truly determine the impact of GM one must compare the collective positive and negative impacts to current conventional farming practice. Although this is true, the reality is that we are only beginning to understand what those potential positive and negative impacts are. What we do know is that GM technology impacts various aspects of our environment and society.

One unanticipated aspect that cuts across various spheres of impact in terms of the environment and society is gene flow. Gene flow can lead to adventitious comingling of GM with non-GM or organic crops and result in a loss of market

value and it can also lead to a violation of patent rights with a requirement to pay royalties for the unintended presence of a transgene (Demont and Devos, 2008; Lee, 2008). GM gene flow could also impact on the biodiversity of land races and wild relatives. While this is often compared to the potential for gene flow from conventional crops, which it is, the reality is that most genes in conventional crops come from wild relatives and are not patented. The genes used in GM technology are not from wild relatives and all carry patents. Thus introducing a new gene into the gene pool of an organism could have devastating, primary as well as secondary impacts. Primary impacts could include selection benefits for Bt toxin producing plants due to less insect damage or weediness as a result of herbicide tolerance as has been seen with Bentgrass in the US (Reichman *et al.*, 2006). However, there are also secondary environmental impacts such as the development of weediness due to increased exposure to herbicides as is the case with Johnson grass (O'Kennedy *et al.*, 2006) or the development of resistance against the Bt toxin in target insects as has been recoded in South Africa (Van Rensburg, 2007). Chilcutt and Tabashnik (2004) suggested that out-crossing of insect resistance to refugia could contribute to the development of target insect resistance. We hypothesize that out-crossing of the Bt gene to land races could result in sub optimal exposure of target insects to the toxin and thus facilitate the development of resistance.

In this study, the extent of pollen mediated GM gene flow was investigated in soybeans and maize under environmental conditions typical of commercial production regions for these crops in South Africa. Molecular technology was

combined with field trials to study potential pollen mediated gene flow (PPMGF) by PCR detection of GM pollen. Pollen mediated gene flow (PMGF) was investigated though phenotypic and genotypic detection of out-crossing.

Although soybean is widely acknowledged to be a self-pollinating crop, there was no published data to indicate that GM gene flow can not occur. Commingling of GM soybean has severe impacts in vegetarian food products marketed as non-GM or as a protein supplement in baby foods. It is a popular food crop due to its use as a vegetable oil and protein (Gardener and Payne, 2003; Lu, 2004). Soybean is the leading biotech crop in terms of global production at 51% (58.6 million hectares) of total GM crop production (James, 2007). Soybean has been modified for herbicide tolerance to Roundup Ready. In 2007, approximately 80% of South Africa's soybean production was GM. Future GM soybean traits are expected to include a high oleic acid and insect resistance (Cahoon, 2003; Kinney, 2003; Conner *et al.*, 2004).

In this study, GM out-crossing was detected at 0.9 m from the GM source over two seasons in two locations (Greytown 2005/2006 and Delmas 2006/2007). However, soybean pollen movement was not detected. Therefore, the GM detected was attributed to insect-mediation. The role of insects in contributing to pollen-mediated gene flow was not within the scope of this study and should therefore be investigated further in future soybean gene flow research. Based on data from this study, the isolation distance of 5 m recommended for non-GM soybean production is sufficient to minimize PMGF in the self-pollinating varieties grown in South Africa

(SANS, 2005). However, the greatest impact for the commingling of non-GM with GM soybean is during harvesting, transport and storage. Therefore, management practices to minimise commingling of GM to non-GM soybean should focus on post-harvest processing.

Maize is a staple and therefore an important food crop in Africa including South Africa. Maize has become crop with cultural significance among rural communities where farmers plant traditional varieties.   In 2007, more than half the maize produced was attributed to GM (57%) (James, 2007). Given commercial trends, there is little doubt that GM maize production is set to continue to increase in future with the addition new first, second and third generation GM traits.

There has been a great deal of discussion on the impacts of GM potential pollen-mediated gene flow in maize (Miller, 2007). However, there is very little research to support arguments either dismissing PMGF or underpinning its importance. But there are many challenges in terms of studying PPMGF in maize, not the least of these is its short viability.

In this aspect of the study I made use of a simple pollen trap together with molecular techniques (PCR) to determine that GM maize pollen can be detected up to 400 m from the source. In the maize PPMGF component of this study, it was found that maize pollen was detected at a distance of up to 200 m at Bainsvlei (2006/2007) and 400 m at Waterbron (2006/2007). Nonetheless, the detection of GM pollen was not as extensive as one would have predicted in terms of the extent

of out-crossing observed phenotypically. This is possibly due to a loss of pollen viability resulting in DNA degradation and hence the ability to detect the transgene using PCR. However, this research has implications for the regulatory decision-making process, especially with the introduction of new GM traits such as those with improved nutritional value and pharmaceutical crops. These new crops will require various levels of segregation with third generations GMOs (pharmaceutical and industrial) requiring 100% segregation from the food and feed chain. This approach can also be used as a regulatory tool to monitor PPMGF in GM field trials, especially for second and third generation GMOs.

In contrast to PPMGF that only evaluated pollen movement, the out-crossing component of this study evaluated actual GM gene flow in maize. The furthest distance that out-crossing was observed was 300 m at 0.01% from a GM pollen source. It was found that GM out-crossing declined sharply from 2 m (13 to 18%) up to 25 m (0.1 to 0.3%) from the GM plot. On average, the out-crossing results up to approximately 25 m were similar over different environmental conditions over more than one season. In contrast to this, there were significant differences in the extent of out-crossing after 25 m over different seasons and locations. We thus conclude that impact of the environment on out-crossing is more noticeable after approximately 25 m. For example, based on average out-crossing over distance per location, the theoretical zero was calculated as 1382 m, 1295 m and 2009 m for Bainsvlei (2005/2006), Bainsvlei (2006/2007) and Waterbron (2006/2007), respectively. However, the outcome for out-crossing data per location in a particular wind direction was significantly different. For example, in the ENE

direction in Bainsvlei, the expected distance to achieve 0.01% admixture was calculated as 79 km and 956 m in 2005/2006 and 2006/2007, respectively. Compared to this, a distance of 3.5 km was calculated to achieve a level of commingling of 0.01% at Waterbron (2006/2007). These data exemplify the importance of location specific environmental conditions on gene flow in maize. Thus it would be incorrect to base isolation distances only on average data. The impact of environmental conditions over different seasons should also be taken into consideration.

A further conclusion from these data is that that the recommended isolation distances for maize (50 m up to 800 m) do not guarantee 0% out-crossing under typical maize growing environmental conditions In South Africa (Devos *et al.*, 2008). I suggest that for non-GM production below 1.0% the isolation distance be set to 25 m and for 0.01% 300 m be used. Organic production in SA currently requires 0% GM commingling. I thus suggest that an isolation distance of 1500 m be used for organic agriculture. For GM field trials involving new traits, or GM production of second and third generation GMOs where there is 0% tolerance for GM maize gene flow, it is recommended that a minimum isolation distance of 2000 m be used.

In conclusion, it is important to set differential isolation distances in a tiered approach for field trials and non-GM or organic production based on regulatory and market tolerance levels. This study has highlighted the importance of not assuming dogmatic theories or attempting simplistic extrapolation of gene flow data

across different geographic locations. Although achieving coexistence of GM and non-GM crops is difficult, it is possible given correct management practice supported by location specific data.

In this study I have attempted to provide fundamental data that can be used to inform regulatory and on farm decisions. The development of GM crops has preceded our technical ability to determine their impacts through research and monitoring. Although these data provide guidelines as to the use of isolation distances to minimise or total prevent commingling, there are other aspects in terms of gene flow that require further research. For example, in the soybean component I was not able to study the potential pollen vectors affecting gene flow. In the maize part of this study, I did not investigate the impact of out-crossing on the expression of the Bt toxin – that could effect the development of resistance in target insects. Secondly, it would be important to study the effect of out-crossing on landraces in terms of their fitness and selection pressure and how this impacts resistance developments. Future gene flow studies should also consider the partial introgression of the transgene into the genome and its impact on the stability of the transgene.

Regarding this study, that for soybean PCR detection is used to determine whether potential pollinators carry GM pollen. For maize, I suggest that further work needs to be done on the real-time PCR detection of pollen to be able to correlate gene copy number to GM pollen counts. This would enable a more accurate assessment of GM pollen load.

Finally, based on the data from this study, I suggest that studying gene flow in either maize or soybean is critical in the adoption and management of GMOs in terms of biodiversity and agro-biodiversity. If anything, the promise of second and third generation GM crops should be the necessary encouragement to regulators to insist on region-specific research and monitoring.

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SUMMARY

Over centuries, crop domestication and improvement has led to modern commercial agriculture. Agricultural biotechnology is considered by many a natural step in the course of crop improvement by utilizing genetic engineering. Currently, the global production of biotech crops is approximately 34% of global agriculture. The major biotech crops in terms of production volumes are canola, cotton, maize and soybean.

In Africa, South Africa is the only country to accept and commercially produce genetically modified (GM) crop. The 2007, GM traits per crop with environmental release status in South Africa included insect resistant (IR) and herbicide tolerant (HT) cotton (including the stack for both traits) (90% of total cotton production), IR and HT maize (including the stack for both traits) (57% of total production) and HT soybean (80% of total production).

There are several factors that impact on the application of this technology in terms of commercial as well as small scale farming. These include: intellectual property rights, socio-economics, regulatory frameworks, agriculture, environment, niche markets and cost benefit. Of all of these aspects, gene flow from GM to non-GM or organic products, land races and wild relatives is a critical consideration. In this study, the impact of potential pollen mediated gene flow (PPMGF) and pollen

mediated gene flow (PMGF) was studied in GM soybean and maize, two of the most important GM food crops in terms of production volumes.

In this study, GM gene flow was found to have occurred up to 0.9 m from a GM source at two locations over two seasons, despite being considered a self-pollinating crop (Greytown 2005/2006 and Delmas 2006/2007, respectively). However, it was also found that GM soybean pollen was not wind borne and we suggest that the gene flow observed was due to insect-mediation. Future studies of PPMGF in South Africa should include a survey of insects present with the potential to act as a pollen vector in soybean.

In the maize component of this study, molecular technology was used to detect GM maize pollen up to 400 m from a GM pollen source. Furthermore, it was found that out-crossing of GM to non-GM maize was possible at a distance of 300 m from the GM field. Based on the statistical analysis of out-crossing data, I have determined that the average theoretical zero (0.0001%) level of out-crossing was between 1.3 km and 2.0 km over different geographic locations. However, what was unexpected is the difference in out-crossing per location for a specific direction. For example, in Bainsvlei (2005/2007) for the ENE direction, the calculated distance to achieve 0.01% out-crossing is 79 km, yet the average is 113 m. Similarly in the second season for the same direction, the calculated distance is 956 m and the average is 135 m.

The implication of these data is that it is not possible to establish a one size fits all isolation distance to minimize or prevent gene flow. Different threshold levels of commingling require different isolations distances and should be determined by the acceptable level of tolerance for commingling. For non-GM production in South Africa, based on the 1.0% threshold applied by the Department of Agriculture, I suggest a minimum isolation distance of between 120 m up to 200 m, assuming that the weather patterns are comparable to those of the current study as well as that the non-GM seed being planted contains 0% GM. However, for more stringent thresholds, the isolation distance would need to be extended.

For organic crop production, at 0% adventitious GM, as well as field trials of second and third generation GMOs, it is suggested that the isolation distance be set at a minimum of 1.5 km and 2.0 km, respectively. In addition, for non-GM seed production (with a mandatory 0% tolerance so as not to contravene patents) I recommend a 1.5 km isolation distance. These suggested isolation distances are based on the absence of time isolation. It is hoped that this study will help to inform regulatory as well as on farm decision making and that it could be used as a blueprint for other GM crops, especially indigenous African crops such as sorghum and cassava.

OPSOMMING

Oor die eeue, het die teling en verbetering van plantgewasse gelei tot die hedendaagse moderne kommersiële verbouing van gewasse. Landbou biotegnologie word deur baie beskou as die voorsetting van plantteling deur gebruik te maak van genetiese ingenieurswese (GI). Tans is die bydra van biotegnologie gewasse ongeveer 34% ten opsigte van totale globale produksie. Die hoof GI gewasse in terme van produksie volume is huidig raapsaad, katoen, mielies en sojaboon.

In Afrika, is Suid-Afrika tans die enigste land wat GI gewasse kommersieel al vrygestel het. In 2007, is die volgende GI gewasse alreeds in Suid-Afrika vrygestel naamlik: insekweerstand (IW) en onkruiddodder tolerante (OT) katoen (as ook die stapel van beide eienskappe) (90% van totale katoen produksie), IW en OT mielies (as ook die stapel van beide eienskappe) (57% van totale mielie produksie) en OT sojabone (57% van totale sojaboon produksie).

Daar is verskeie faktore wat 'n impak het op die toepassing van hierdie tegnologie ten opsigte van kommersiële sowel as klein maat boerdery. Dit sluit in intellektuele eiendoms reg, sosio-ekonomies, regulatoriese stelsels, landbou, omgewing, nismarkte, en koste voordeel. Van al hierdie oorwegings is geen vloei vanaf GI tot nie-GI en organiese gewasse, landrasse en wilde plantfamilies die grootste. In hierdie studie, is die impak van potensiële stuifmeel medieerde geen vloei

(PSMGV) en stuifmeel medieerde geen vloei (SMGV) bestudeer in sojabone en mielies, twee van die belangrikste GI gewasse ten opsigte van produksie volumes.

Ten spyte daarvan dat sojabone beskou word as 'n self bestuiwings gewas, is daar gevind dat GI geen vloei plaasgevind het tot op 0.9 m vanaf die GI bron in twee gebiede en oor twee seisoene (Greytown 2005/2006 en Delmas 2006/2007, respektiewelik). Nietemin was GI stuifmeel nie aangetref in die omgewing en deur die wind gedra nie en dus stel ek voor dat die geen vloei as gevolg is van insek bemiddeling. Toekomstige studies van PSMGV behoort dus 'n opname in te sluit van potensiële stuifmeel draers as vektor vir bestuiwing in sojabone.

In die mieliekomponent van hierdie studie, is molekulêre tegnologie gebruik om GI stuifmeel tot op 'n afstand van 400 m vanaf 'n GI bron aan te dui. Dit was ook gevind dat verbastering van GI tot nie-GI moontlik was tot 'n afstand van 300 m vanaf die GI landery. Gebaseer op statistiese analise, is vasgestel dat die gemiddelde teoretiese nul waarde (0.0001%) van verbastering tussen oor die verskillende geografiese gebiede tussen 1.3 km tot 2.0 km vêr is. Nieteenstaande, is gevind dat daar beduidende verskille is tussen verbastering oor verskillende geografiese gebiede asook wind rigting. Byvoorbeeld, in Bainsvlei (2005/2007) in die rigting van ONO, was die beraamde afstand om 0.01% verbastering te verkry 79 km, ten spyte daarvan dat die gemiddeld 113 m was. Soortgelyks, in die tweede seisoen vir dieselfde rigting, was die beraamde afstand 956 m terwyl die gemiddeld 135 m was.

Hierdie navorsing toon aan dat dit onmoontlik is om 'n een grote norm vas te stel vir isolasie afstande om geen vloei te beperk of voorkom. Dus verg verskillende drempelvlakke om verbastering te voorkom verskillende isolasie afstande en behoort vasgestel te word na gelang van die toleransie vlak van vermenging. Vir die nie-GM produksie van mielies in Suid-Afrika, gebaseer op 'n 1.0% drempel soos toegepas deur die Departement van Landbou, stel ek voor dat 'n minimum isolasie afstand van tussen 120 m tot en met 200 m, gebruik word – met die aanname dat die weerpatrone vergelykbaar is met die van die huidige studie asook dat die geplante saad 0% GI bevat. Nietemin, vir 'n strenger drempel sal die isolasie afstand verder verleng moet word. Ek stel ook voor dat vir organiese gewasproduksie, teen 0% toleransie vir GI, asook veld proewe van 2de en 3de generasie GIs (met 'n 0% verpligte toleransie), moet 'n minimum isolasie afstand van 1.5 km en 2.0 km, onderskeidelik gebruik word. Vir die produksie van nie-GM saad (met 'n verpligte toleransie van 0% sodat patent reg nie oorskry word nie) stel ek voor dat 'n isolasie afstand van 1.5 km gebruik word. Die bogenoemde voorgestelde isolasie afstande is in die afwesigheid van enige tyd isolasie. Dit is my hoop dat hierdie studie 'n bydra sal lewer om die regulatoriese sowel as die op plaas besluitnemings proses in te lig en dat dit as bloudruk gebruik kan word vir ander GI gewasse insluitend inheemse gewasse soos sorghum en kassawe.