Assessing corn pollen flow and outcross in seed and grain production fields

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Assessing corn pollen flow and outcross in seed and grain production fields

by

Higinio Lopez-Sanchez

A dissertation submitted to the graduate faculty in partial
fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Crop Production and Physiology (Seed Science)

Program of Study Committee:
A. Susana Goggi, Major Professor
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For the Major Program
To my family
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ABSTRACT

Pollen movement and the resulting flow of genes between plant populations have been an integral part of corn evolution. However, the adventitious presence of bioengineered genes in conventional varieties due to gene flow is a serious concern when producing grain for specialty markets, organic products, crops with valued added traits, and seed. An additional concern is the release of corn bioengineered to produce pharmaceutical and industrial compounds. The objectives of this study were to detect the presence of transgenes in corn DNA from pollen and to compare the gene flow and outcross in a seed and grain fields using a combination of three markers genes, seed color, Bt, and RR. Chapter 1 contains a pollen DNA extraction protocol for corn and a comparison of the efficiency of 11 primer pairs at detecting the Bt transgene; Chapter 2 presents the study of outcross in large-scale corn fields with different pollen density, grain and seed fields; and Chapter 3 presents the resulting outcross in large-scale corn fields with similar pollen density, grain fields, across environments. The proposed pollen DNA extraction method was very effective in extracting DNA from pollen samples and the primer pair 35S168F/35S317R was the most efficient in identifying the CaMV35S promoter sequence in transgenic varieties. There were significant differences of outcross at different distances and directions from the pollen source associated with wind speed and direction. The differences in outcross distribution between grain and seed fields in 2003 were statistically significant, but inconclusive in grain fields in 2003 and 2004. Our results showed that segregation of transgenic and non-transgenic corn can not be achieved within the 250 m distance because the outcross level seldom reached zero percent.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

Stevens et al. (2004) define gene flow in maize as the fertilization of a population by pollen from another group of genetically different maize plants. This process has been an intricate part of the evolution of maize improvement. The movement of genes from one population to another has been reported between landraces of maize and teosinte in rainfed areas of Mexico (Wilkes, 1970; 1977; Eubanks, 2001; Luna et al., 2001; Doblely, 2004; Baltazar et al., 2005); between improved varieties and landraces in grain production fields (Ortega-Paczka, 1973; Vega Zaragoza, 1973; Castillo Gonzalez and Goodman, 1997; Bellon and Brush, 1994; Bellon and Risopoulos, 2001; Baltazar and Schoper, 2002); and between grain and seed production fields, or among seed production fields, jeopardizing the genetic purity of the seed produced (Burris, 2001). Even though the magnitude of gene flow among plants varies with species, population, genotypes, and environmental conditions (Ellstrand, 2003), it can ultimately modify and define the genetic structure of a plant population. The principal means for gene movement are via pollen flow and, less frequently, by seed dispersal (Ennos, 1994; Dow and Ashley, 1998; Hardy et al., 2004; Kenta et al., 2004). The interruption of the natural flow of genes among plants reduces population variability, causes inbreeding depression and loss of genetic variability in open-pollinated species by promoting self-pollination (Mayr, 1970). Therefore, gene flow has both a positive role enhancing genetic diversity of maize and a negative role compromising the genetic purity of elite germplasm during seed production of improved varieties.
Earlier pollen flow and outcross studies in corn by Bateman (1947a and 1947b), Jones and Newell (1948), Jones and Brooks (1950), Airy (1955), Raynor et al. (1972), Paterniani and Stort (1974), and Murillo Navarrete (1978) show that pollen amounts and outcross percentages declined considerably within the first 10 m away from the pollen source, and that little amount of a long-extending tail of pollen dispersal and outcross persist at distances of 60, 100, 200 or even 250 m. The amount of pollen at the farthest distances is usually less than 1%, thus allowing the seed industry to produce seed with the required standard of 99% of genetic purity (Ingram, 2000; OECD, 2005). Based on these studies new seed production practices are adopted to limit gene flow, such as temporal isolation, greater isolation distances, and border rows. Temporal isolation relies on maintaining the adjoining production fields at different stages of development to ensure sufficient asynchrony of flowering. Standards for temporal isolation vary among plant species, and are influenced by the length of the growing season and the length and synchronicity of the flowering period (Glover, 2002). The most common agricultural practice used to reduce gene flow between plants of wind-pollinated and insect-pollinated crops is isolation distance (Bateman, 1947a). The Association of Official Seed Certifying Agencies requires a minimum separation distance of 201 m from other maize varieties when producing “Certified Seed” (Association of Official Seed Certifying Agencies, 2001). The Organization for Economic Co-operation and Development (OECD) requires a minimum isolation distance, including border rows, of 200 m (Baltazar and Schoper, 2002; OECD, 2005), considered necessary to produce corn seed hybrid of 99% genetic purity. Border rows planted with plants taller than the protected crop is another method used to limit gene flow (Glover, 2002). In wind pollinated crops, the number of border rows necessary to contain the intrusion of foreign pollen depends mainly
on the speed and direction of the predominant wind. Border rows are also used in conjunction with isolation distances to obtain the required purity for seed certification. A separation distance of less than 125 m is acceptable to produce hybrid maize if the protected seed lot is surrounded by border rows of the pollen parent, or even no separation distance is required if 10 rows of the pollen parent surround the seed lot (Association of Official Seed Certifying Agencies, 2001). Border rows have also been effective to reduce the flow of genes beyond a natural barrier; however, at farther distances from the barrier they are not effective (Levin and Kerster, 1974). These standards for seed production remained unchanged for more than 50 years (Burris, 2001).

Even though these agricultural methods have been effective to reduce gene flow they do not eliminate gene flow (Glover, 2002). In maize, for instance, Emberlin (1999) reported outcross levels of 1.9% at 60 m, 1.1% at 200 m and less than 0.7% at 500 m. Although these outcross levels meet the varietal purity requirements for the production of certified seed, they are different from 0% outcross desired when producing certain specialized crops, such as plant-made pharmaceuticals or industrial products.

Today, novel tools of biotechnology and genetic engineering (Potrykus, 1991) make possible the insertion of genes from unrelated species or even from organisms of a different kingdom. The most common traits are herbicide tolerance and insect resistance, and the most important transgenic crops are soybean, corn, cotton and canola (Sweet, 2003). Pollen dispersal and gene flow are now under scrutiny owing to the possibility of transgene flow from engineered hybrids to other non-genetically modified varieties, weeds or wild relatives, and due to the possible effect on the environment (Natural Law Party, 1999) and human
health associated with potential allergenic or toxic effects of DNA and protein (Doering, 2004) international standards.

Weed management in crops has been improved with the transference of genes tolerant to the broad-spectrum herbicides Roundup® (glyphosate) and Liberty® (glufosinate). Those herbicides act on plants disrupting the synthesis of aromatic amino acids (Phe, Trp, and Tyr) and glutamine, respectively. However the success on management can be compromised by the possibility of transgene flow via pollen and seed to the nearby non-GM crops (Downey, 1999; Hall et al., 2000), weeds or wild relatives (Ellstrand, 2001; Ellstrand, 2003; Gepts and Papa, 2003). Herbicide-tolerant weeds and the non-GM crop contaminated with the transgene would affect yield and demand alternative management strategies (Barton and Dracup, 2000).

The Bacillus thuringiensis (Bt) varieties have been modified with a soil-bacterium gene that code for a crystalline (Cry) protein. This protein breaks down in the insect gut releasing a delta-endotoxin that binds to the intestinal lining creating pores. As a result, the insect dies due to ion imbalance and paralysis of the digestive system (Adang, 1991; Byrne et al., 2004). Even though Bt transgenic varieties represent advantages in crop management, there are some concerns about the effect on non-target species such as soil microbes and beneficial insects and the potential impact on the evolution of resistance to the delta-endotoxin in the pests, (Saxena and Stotzky, 2005; St Amand et al., 2002). Insecticides containing Bt (e.g. Dipel, Thuricide, and Vectobac) have been used for more than 30 years (Byrne et al., 2004; Ferre et al., 1991; Tabashnik, 1994). The innovative characteristic of Bt plant species is that crystal protein genes have been cloned and transferred to their genomes. To safeguard the effectiveness of these Bt plants as bio-insecticides it is necessary to delay
the development of resistance in the target insects. Resistance development to Bt Cry proteins has been studied in the laboratory (Yong-Biao et al., 1999), in greenhouse (Tang et al., 2001), in the field (Shelton et al., 2000), and using simulation models (Caprio, 2001). They emphasize the importance of the separate refuge strategy to delay the development of resistance of target pests to Bt toxins (Gould, 1998; Hoy, 1998; Tabashnik et al., 2003). In USA corn farmers are required to plant 20% of toxin-free crop refuge near Bt crops to promote survival of susceptible pests. However, considering the high level of outcross in corn, gene flow to the refuge from the Bt corn increases the possibility of Bt toxin production in grain of the refuge corn, decreasing the effectiveness of the refuges.

Additionally to crop management, contamination among crops by pollen drift and gene flow has become a major concern in the co-existence of genetically modified (GM) crops and non-GM crops, such as conventional varieties, organic crops, and those with a value-added trait (Brookes and Barfoot, 2004; Hayenga and Kalaitzandonakes, 1999; Messeguer, 2003). Pollen drift is a concern even for transgenic-hybrid growers who must avoid contamination of transgenes of corn varieties that have not been approved for global marketing (Nielsen, 2003) as Herculex™ I, YieldGard+Liberty Link™, or YG Plus™. Even though a labeling threshold of 1% GMO content in conventional food products and food ingredients is allowed by EU marketing, their presence is rejected in organic farming (Messeguer, 2003). The cost of avoiding cross pollination in organic grain production is associated with the increased land use for the required border rows and spatial isolation, and for incentives given to other farmers not to grow GM corn in nearby fields (Bullock and Desquilbet, 2002). Thus, the current standards for seed purity need to be reviewed in detail in
order to ensure the minimum isolation distance required in the segregation of transgenic from non-transgenic varieties.

An additional concern is the risk to human health derived from pharmaceutical proteins, industrial enzymes, and vaccines produced by future generations of transgenic corn varieties, entering the food supply (Stewart and Knight, 2005). The production of these new transgenic varieties will need stricter segregation from food crops since a zero level of outcross in the field has not been achieved and even an insignificant level of contamination would be unacceptable. Scientists have been considering new technologies to obtain zero levels of outcross, by minimizing pollen transfer using biological tools such as apomixis (Grimanelli et al., 2001); cleistogamy (Takahashi et al., 2001), and male sterility (Animal and Plant Health Inspection Service, 2001); by plastid transformation (Bock, 2001); incompatible genomes (Gressel, 1999), and manipulations of the pollen coat (Robert and Gleddie, 1999); and by reducing hybrid survival using the “terminator” technology, recoverable block of function (Kuvshinov et al., 2001), transgenic mitigation (Gressel, 1999), and excision technology (Keenan and Stemmer, 2002). However, most of these technologies are in the early stage of development (Glover, 2002). Considering that the area planted with transgenic varieties is increasing constantly, studies to characterize the process of outcross in maize must continue.

Recent studies of transgenic and non-transgenic pollen flow in corn (Ortiz Torres, 1993; Garcia et al., 1998; Burris, 2001; Jemison and Vayda, 2001; Luna et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Baltazar and Schoper, 2002; Henry et al., 2003; Jarosz et al., 2003; Chilcutt and Tabashnik, 2004; Ma et al., 2004; and Stevens et al., 2004) found that outcross in corn decreases within the first 10 or 20 m from the pollen source, and
that small amounts of pollen and outcross are present at distances no longer than 200 m, with
the exception of Stevens et al. (2004) who found 0.02% outcross at 300 m, and Emberlin
(1999) who reported less than 0.7% of outcross levels at 500 m. However, with the
exception of Burris (2001) all studies used small pollen source field (50 x 50 m), small-size
receiving plots, and non-continuous source-receiving field design.

The development of corn varieties as factories of pharmaceutical and industrial
compounds has generated renewed interest in pollen dispersal and gene flow. It is essential to
re-evaluate the minimum distance required to isolate conventional hybrids from these
transgenic varieties to avoid the adventitious presence of proteins and other compounds in
the food supply (Doering, 2004). Large-scale studies are needed to assess how local pollen
density, continuous source and receiver fields, and multi-directional gene flow affect
outcross in order to understand and achieve stricter control of pollen flow and successfully
co-existence of these transgenic crops in the environment. The objectives of this study were
to compare the gene flow and outcross in seed and grain fields in adjacent source-receiver
maize fields under varied environmental conditions.

Dissertation Organization

This dissertation consists of an abstract, a general introduction, three papers, and general
conclusions. The dissertation is divided in two parts. Part I contains Chapter 1 or first paper,
Part II contain Chapter 2 and Chapter 3 or second and last paper. The first paper will be
submitted for publication to the Seed Technology journal, and the second and third
manuscripts will be submitted to the Crop Science journal.
References


Jones, M.D. and L.C. Newell. 1948. Longevity of pollen and stigmas of grasses:


PART I. LABORATORY EXPERIMENT
CHAPTER 1. DETECTION OF THE CAMV-35S PROMOTER SEQUENCE IN MAIZE POLLEN AND SEED

Abstract

During maize transformation, the CaMV35S promoter sequence is inserted with the gene of interest. There are several protocols in the literature for the extraction of seed DNA and the detection of the CaMV35S promoter sequence. However, there is little information on DNA extraction methods for pollen and comparative evaluation of primer efficiency. This study developed an extraction protocol for pollen DNA in corn, and screened current and new primers designed to detect the CaMV35S promoter in corn pollen and seed. Bt transgenic and non-transgenic corn hybrids were used to obtain the seed and pollen DNA. Polymerase Chain Reaction (PCR) and gel electrophoresis were used to evaluate the efficacy of the pollen DNA extraction protocol, and to test the efficiency of 11 primer pairs in detecting the CaMV35S promoter sequence. The DNA extraction method was very successful in releasing the DNA from pollen grains, as determined by the intensity of the 18 bands of genomic DNA samples amplified with the HMG-AF1/HMG-AR1 corn-specific primers. The strong intensity of the bands formed by primers P35S1/P35S2, P35SA/P35SB, and P35S-aflu/P35S-ar1 showed these primers were the most efficient in amplifying transgenic pollen DNA; whereas, primers P35S1/P35S2 generated the strongest band intensity in seed DNA. The new primers 35S168F/35S317R showed higher sensitivity in detecting the CaMV35S promoter than any other primer included in this experiment. The proposed pollen DNA extraction method and the primer 35S168F/35S317R were very effective in extracting DNA from pollen samples and identifying the CaMV35S promoter sequence in transgenic varieties.
Introduction

Important crops have been improved by the introduction of novel genes. These genes are artificially inserted, using genetic engineering techniques. Genes can be cloned from a related or unrelated species (Potrykus, 1991). Some examples are traits for tolerance to herbicide and insect resistance. More recently, transgenic crops have been transformed to produce plant-made vaccines and therapeutic proteins. While the new crops represent many advantages to crop productivity and profitability, many scientists have expressed concern about the potential movement of these genes into conventional crops by pollen flow and their possible impact on the environment (Nordlee et al., 1996; Quist and Chapela, 2001; Dale et al., 2002). Testing of conventional seed and grain for the presence of adventitious biotech traits is mandatory in the European Community (European Commission, 2004). Detection of transgenes can be accomplished by DNA, RNA, or protein isolation techniques. DNA methods are preferred because of stability of DNA during extraction, purification, PCR amplification, sensitivity, and capability to detect a wider range of constructs (Giovanini and Concilio, 2002).

Holst-Jensen (2001) has classified the transgenic DNA detection methods into four categories—screening for 35S and NOS specific sequences that can detect a wide range of transgenic traits, gene specific detection for identification of a transgene, construct specific detection for the identification of a gene construct used in the transformation process, and event specific detection methods that identify the insertion site of the transgenic trait in the genome. Some examples of the use of these methods are detection of the CaMV-35S promoter (Lipp et al., 2001), identification of the bar gene (Kota et al., 1999) and Cry1Ab gene (Studer et al., 1998), junction identification of the P-35S-heat shock protein 70 intron I
in Mon810 maize (Zimmermann et al., 1998), and junction detection of the host plant genome-integrated recombinant DNA in Bt11 maize (Zimmermann et al., 2000).

Several studies have been conducted to develop a 35S promoter specific screening method for biotech traits using DNA from grains or processed food (Vollenhoffer et al., 1999; Tozzini et al., 2000; Lipp et al., 2001). Some of the genetic markers sited in the literature for the identification of transgenic events in corn are considered unreliable and result in a high number of false positives and negatives during PCR amplification (Christou, 2002). Although there is a reliable protocol for the extraction of DNA from single corn seed in the literature (Sangtong et al., 2001), there is little published information on the extraction of DNA from pollen samples. Therefore, the objectives of this study were to develop a protocol to extract DNA from corn pollen, to screen molecular markers available in the literature, and to design new markers for detecting the CaMV-35S promoter in transgenic corn pollen and seed.

Materials and methods

Plant material and DNA extraction and quantification.

Seed DNA extraction. Ten mg of corn meal from transgenic hybrids of FR1064xLH185Bt, LH245xLH185Bt, SGi928xHC50Bt, HCSOBTxSGI905, TR7322xMBS1236Bt, DKC69-71, 4-NK7070Bt, and Asgrow RX792 (non-transgenic hybrid) were used to extract DNA by the extraction method described by Sangtong et al. (2001). Corn meal was extracted from the endosperm of 10 seeds of each hybrid, using a Craftsman rotary drill. Plant Tissue Protocol from PUREGENE Genomic DNA Purification Kit of Gentra Systems (Minneapolis, MN, USA) was used to extract DNA from the corn
meal. An abbreviated description of the DNA extraction steps includes adding 300 μl of Cell Lysis Solution to the corn meal and mixing by vortexing (Genie 2™, Fisher Scientific, Bohemia, N.Y., USA) the tube for 3 seconds. Cell lysate was incubated (Dry bath incubator, Boeckel Scientific, Pennsylvania, PA, USA) at 65 °C for 60 min. After incubation, 1.5 μL of RNAase A solution was added to the cell lysate. Next, samples were incubated in an oven (Thelco, GCA/Precision Scientific, Chicago, IL, USA) at 37 °C for 40 min. After cooling the samples to room temperature, 100 μL of the Protein Precipitation Solution were added to the cell lysate. The solution was mixed by inverting the tube several times and then incubated for 20 min in an ice bath. The protein precipitation solution was mixed by vortexing the tube for 20 seconds. Next, the tube was centrifuged (Centrifuge 5417C, Eppendorf) at 14,000 rpm for 3 min. The supernatant containing the DNA was transferred to a clean 1.5 ml centrifuge tube containing 300 μl 100% isopropanol. After mixing gently by inverting the tube several times, the tube was centrifuged at 14,000 rpm for 2 min. The supernatant was poured off and the tube was drained on clean absorbent paper. The DNA pellet remained in the tube and was washed by adding 300 μL of 70% ethanol and gently mixing. Next, the tube was centrifuged at 14,000 rpm for 2 min and the ethanol was poured off. The tube with the DNA pellet was inverted and drained on clean absorbent paper and air-dried for 15 min. The DNA was rehydrated by adding 50 μL of DNA hydration solution and incubating the sample for 1 hour at 65 °C.

**Pollen DNA extraction.** Pollen DNA was extracted from three corn varieties—Asgrow RX792 (a non-transgenic hybrid), DKC69-71 (a transgenic hybrid), and an experimental HiII transgenic corn variety kindly provided by Dr. Kang Wang, Iowa State
University Plant Transformation Facility (Armstrong et al., 1991). The commercial and the experimental hybrids were grown in the field and in a greenhouse, respectively. DNA was extracted using a modified pollen DNA extraction protocol kindly provided by Dr. Paul Scott, USDA-ARS at Iowa State University. Some additional steps were added, following recommendations in the PUREGENE DNA purification kit. Pollen was collected from the field and greenhouse-grown corn plants. Field samples were collected into a 50 ml centrifuge tube taped to a plastic funnel of 18 cm in diameter. The funnels were supported with wire to a garden stake and placed at the height equivalent to the ear in the corn plant. Tubes were replaced daily. Collected samples were immediately frozen at -80 °C in the lab until used for DNA extraction.

A procedure was used to break the pollen grains and release the DNA. The DNA extraction procedure consisted of mixing 10 mg of pollen grains with 400 μl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Two steel beads were added to grind the pollen grains. The tubes were shaken for one minute with an experimental DNA sample grinder developed by Drs. Yuh-Yuan Shyy and Rai Satish at the Seed Science Center, Iowa State University. The cell lysate was transferred to a new tube for incubation at 65 °C for 60 min. After incubation, the samples were cooled at room temperature and mixed with 400 μL of saturated phenol. Next the tubes were placed on ice for 30 min and centrifuged for 10 min at 12,000 rpm. The aqueous phase was pipetted into a clean 1.5 mL centrifuge tube and mixed with 400 μL of chloroform. The tubes were centrifuged for 10 min at 12,000 rpm. Then, the aqueous phase was pipetted into a clean 1.5 mL centrifuge tube and mixed with 400 μL of iced-cold isopropanol. The tubes were left on a bench at room temperature for 15 min and centrifuged for 10 min at 14,000 rpm. The
isopropanol was discarded, and the tube was inverted and drained on clean absorbent paper. The DNA pellet was washed with 300 μL of 70% ethanol. The tube was centrifuged for 2 min at 14,000 rpm, the ethanol was poured off, the tube was inverted and drained on clean absorbent paper, and the samples air-dried for 10 min. The DNA pellet was re-suspended in 50 μL of distilled deionized water. The amount of DNA in each tube was determined by using a spectrophotometer (SmartSpec™ 3000, BIO-RAD, Richmond, CA, USA).

**Amplification of DNA**

**Primer sequences.** Twelve primer pair sequences were used specifically for detecting the CaMV35S promoter (Table 1). Primer pairs 1 to 7 and 12 were taken from the literature, while primer pairs 8, 9, 10, and 11 were designed, using the sequence of the CaMV35S promoter and the Oligo™ version 5.0 Primer Analysis Program

**PCR analysis.** The amplification of DNA was carried out in a final volume of 20 μL composed of 2 μL of 10x NH₄ buffer (1x), 0.6 μL of 50mM MgCl₂ solution (1.5mM), 0.4 μL of 10mM dNTP mix (0.2mM), 0.2 μL of DNA Polymerase (Biolase™, Bioline, Randolph, MA, USA) (1 unit/reaction), 0.5 μL of forward and reverse primer (0.25μM), 3 μL of DNA sample (10-50ng/μL), and 12.8 μL of distilled deionized H₂O. Two control samples were used each time, a negative control containing water to corroborate that PCR reactions were free of contamination and a positive control containing plasmid DNA (pT102 construct) containing CaMV35S promoter used in the transformation of maize embryos (Frame et al., 2002), kindly provided by Dr. Kan Wang (Agronomy, Plant Transformation Facility, ISU). DNA amplification was performed on a PTC-100™ programmable Thermal Controller cycler (MJ Research, Inc., Watertown, MA, USA). The quantity and quality of DNA were
measured using a spectrophotometer. PCR amplification was carried out using 5.0 μl of
genomic DNA sample and the HMG-AF1/HMG-AR1 primer (high mobility group, corn
specific primer). The steps are listed in Table 2.

Gel electrophoresis

Ten μL of PCR products with 1 μL of 10x DNA loading buffer (Eppendorf) were
separated in a 3 % plus ethidium bromide precasted readyagarose™ gels (BIO-RAD,
Richmond, VA, USA), in 1x TAE buffer during 90 min, using 75 v (power pac 300, and
Mini-Sub® cell GT, BIO-RAD). Ten μL of HypperLadder V (Bioline, Randolph, MA, USA)
was used as the molecular size marker. The results were evaluated visually from the pictures
of gels taken in a UV camera (BIO-RAD), analyzed in Quantity One® Program (BIO-RAD),
and printed with a video copy processor (Mitsubishi P91, Nagakakyo-City, Kyoto, Japan).

Results and Discussion

DNA extraction from pollen and seed

Figures 1 and 2 are gel images of the PCR-amplified pollen DNA. The DNA
extraction method used in this study was very successful at releasing the DNA from the
pollen grains, as determined by the intensity of the bands. Figure 1 shows the genomic DNA
from 18 pollen samples amplified using the primers HMG-AF1/HMG-AR1, a corn specific
primer (Hernandez et al., 2004). The size of the amplicon was 175 base pairs. Most bands
showed a high intensity, which also indicated that the primers have a high sensitivity for the
detection of the pollen DNA. The high intensity of the bands is associated with the high
specificity of the HMG primers for corn DNA. Rychlik and Rhoades (1989) reported that
high specificity of the primers to the target DNA decreases mispriming and increases the
number of copies of the amplicon. There was no amplification in lane 13, because it contained plasmid DNA which lacked corn-like sequences. These results confirm the specificity of primers to corn DNA.

There are several methods available for DNA extraction and each method can have significant differences in the amount and quality of DNA. The extraction method is a critical factor, since one method may work with one type of tissue or sample, but may be inappropriate for other types of samples. Generally, the DNA extraction from pollen samples collected from greenhouse or growth chambers is easier, compared to the sample collected from fields. Field samples lose more moisture and can become contaminated by fungal DNA or dust. A wrong choice of extraction method can yield poor quality DNA; thus translating into higher rates of false positive and negative results and lower detection limits for PCR amplification (Pinero and Poppin, 2003). The results from this study reveal that the DNA extraction method used was equally effective for pollen samples collected from the field and the greenhouse (Figures 1, 2, and 4). The extraction resulted in sufficient quantity of DNA for PCR amplification and PCR inhibitory substances were removed efficiently during the extraction process.

Detection of the transgene in pollen and seed DNA

The efficiency of the DNA extraction method and the efficiency of the primers to amplify DNA are related to the intensity of the bands in the respective gels. A set of primers reported in the literature and four new primers designed, based on the 35S sequences (Table 1), were screened for their efficiency to amplify DNA extracted from pollen of transgenic plants containing the 35S-CaMV promoter (Fig. 2). DNA bands showed more intensity in the experimental HiII transgenic corn variety (Fig. 2A) than the transgenic hybrid (Fig. 2B).
We can speculate on differences in the transformation efficiency of both varieties. Based on the intensity of bands, primers P35S1/P35S2, P35SA/P35SB and P35S-aflu/P35S-ar1 were more efficient in amplifying transgenic pollen DNA, and the primers P35S-cfr/P35S-cr4 were less efficient (Figs. 2A and 2B).

To test the efficiency of the different primers, seed DNA was extracted from eight transgenic hybrids and one non-transgenic hybrid. Figure 3 shows results from two of the seven primers. There were differences in the amplification of DNA in all hybrids, which may indicate differences in concentration of the transgene in the hybrids. The primer pair P35S1/P35S2 produced DNA fragment of higher intensity (Fig. 3A) than the fragment amplified by P35S-aflu/P35S-ar1 (Fig. 3B).

Four additional primers were designed in our lab to compare their efficiency with those reported in the literature. All primers were used in the amplification of DNA from transgenic pollen and seed. The most intense bands were generated by the 35S168F/35S317R primer combination in both transgenic pollen and seed DNA (Fig. 4), even though intensity was higher in the pollen samples. The size of the amplicon (150 base pairs) was smaller than those of the primers reported in the literature (Table 1), with the exception of the amplicon of the primers P35S-cfr/P35S-cr4. The new primers 35S168F/35S317R showed higher sensitivity to detect the 35S-CaMV promoter than primers reported in the literature.

There are several characteristics associated with the efficiency of the primers (Vanichanon et al., 1999). The length of primers should be at least 18-20 bases, an overall GC content of 40-60%. Higher amounts of GC may result in mispriming (Innis and Gelfand, 1990; Dieffenbach et al., 1993). Also the self-complementary and intraprimer- dimer and mainly interprimer- dimer formation reduces the annealing of the primers to the target
sequence, decreasing yield and signal of the amplicon (Richlyk and Rhoads, 1989; Watson, 1989; Brownie et al., 1997; Vanichanon et al., 1999). Internal stability of the primer, given by a balanced representation of all four bases and by GC content toward the 5’ or 3’ end, should be low at the 3’ end, because it may result in false priming due to a base pairing with non target sequences, which results in a background smear of bands (Richlik, 1995).

Additionally to the internal stability at the 3’, a smear of bands may be caused by nonspecific amplification of target, due to differences in the melting temperatures ($T_m$) of the primers (Kim and Smithies, 1988; Dieffenbach et al., 1993). In this study, all primers have differences in the length in the overall GC content and in the trends of GC content toward 5’ or 3’ end (Table 2).

Beasley et al. (1999) found that shorter primer length, higher primer GC content, and an increasing AT to GC trend toward the 3’ end of the primers have strong effects on the sequence-tagged site failure. In the present study, the best designed primer which generated a 150 base pairs band (Fig. 4), is 24 bases in length, has 50% GC content, and a good balance in the trend of GC content toward 5’ and 3’ end (Table 2).

**Conclusions**

The proposed pollen DNA extraction method was very effective in eliminating the PCR inhibitors from field samples and yielded sufficient quantity of DNA from pollen samples of transgenic and non-transgenic corn varieties. The amplification of pollen DNA using corn specific primers yielded an amplicon of 175 base pairs, indicating that DNA extracted from field samples was from the corn pollen. There were differences in the efficiency of DNA amplification by the primers reported in the literature. Bands produced by
the new primers 35S168F/35S317R were more intense than bands from primers published in the literature, especially in the pollen samples.

References


Christou, P. 2002. No credible scientific evidence is presented to support claims that transgenic DNA was introgressed into traditional maize landraces in Oaxaca, Mexico. Transgenic Research 11:iii-v.


http://www.entransfood.com/workinggroups/GMOdetectionmethodsusingPCR


Table 1. Name, sequence, and amplicon length of primers.

<table>
<thead>
<tr>
<th>Primer Num.</th>
<th>Primer Name</th>
<th>Sense sequence</th>
<th>Antisense sequence</th>
<th>Amplicon size (bp)</th>
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<td>1</td>
<td>P35S-1</td>
<td>5'-GCTCCTACAAATGCCATCA-3'</td>
<td>5'-GATAGTGGGGATTGTGCTC-3'</td>
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<td>Lipp et al., 1999; Tozzini et al., 2000; Windels et al., 2001</td>
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<tr>
<td>2</td>
<td>P35S-A</td>
<td>5'-AAGGGTCTTGCAGAGATAG-3'</td>
<td>5'-AGTGGAAAGGAGGATGCT-3'</td>
<td>226</td>
<td>Lipp et al., 2001</td>
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<tr>
<td>3</td>
<td>P35S-cr3</td>
<td>5'-CCACGTCTTCAAAGCAAGTG-3'</td>
<td>5'-TCTCCTCCTGGAAATTGAAGCTCTC-3'</td>
<td>123</td>
<td>Lipp et al., 2001</td>
</tr>
<tr>
<td>4</td>
<td>P35S-aflu</td>
<td>5'-CCTACAAATGCCATCATTGCG-3'</td>
<td>5'-GCTTAAAGGAAAGGCCATCGTTGAAG-3'</td>
<td>205</td>
<td>Pietsch et al., 1997; Lipp et al., 2001</td>
</tr>
<tr>
<td>5</td>
<td>Cm01</td>
<td>5'-CACTACACAGCCATGCACTTCCGA-3'</td>
<td>5'-CNTCTTCTCAGATGCACTTCCGA-3'</td>
<td>220</td>
<td>Quist and Chapela, 2001</td>
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<td>6</td>
<td>Mp3</td>
<td>5'-CTGCTCCTTACGTCAGTGGAGATAT-3'</td>
<td>5'-GATAAAGGAAAGGCCATCGTTGAAG-3'</td>
<td>155</td>
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<td>7</td>
<td>P35S-VoA</td>
<td>5'-CCGACATGGCTGAAAGATGGAG-3'</td>
<td>5'-TTATAGAAAGGTGCTTGTTAAGG-3'</td>
<td>168</td>
<td>Vollenhoffer et al., 1999; Ovesna et al., 2002</td>
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<td>175</td>
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Table 2. Conditions of thermocycler for DNA amplification of CaMV 35S promoter and corn genomic DNA.

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<td>Denaturation</td>
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<td>Annealing</td>
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<tr>
<td>Extension</td>
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<td>45 s/72 °C</td>
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<tr>
<td>Cycles (number)</td>
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<td>40</td>
<td>40</td>
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<tr>
<td>Final extension</td>
<td>10 min/72 °C</td>
<td>5 min/72 °C</td>
<td>5 min/72 °C</td>
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Table 3. Name, length, and GC content of primers.

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<td>Mp4</td>
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<td>35S215F</td>
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<tr>
<td>HMG-AR1</td>
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<td>1.40</td>
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Figure 1. DNA amplification of 18 non-transgenic corn pollen samples using the HMG-AF1/HMG-AR1 corn-specific primers. In the gel, Lane No. 1 represents the DNA Ladder, Lanes No. 2-12 and Lanes No. 14-20 contain the DNA from the corn pollen samples, and Lane No. 13 contains plasmid DNA with the 35S-CaMV promoter.
Figure 2. DNA amplification of two transgenic corn pollen samples. A: DNA from the experimental HiII transgenic corn variety and B: DNA from DKC69-71 transgenic hybrid. Lane No.1: ladder, Lane No. 2: primers P35S1/P35S2, Lane No. 3: primers P35SA/P35SB, Lane No. 4: primers P35S-cfr/P35S-cr4, Lane No. 5: primers P35S-aflu/P35S-ar1, Lane No. 6: primers Cm01/Cm02, Lane No. 7: primers Mp3/Mp4, and Lane No. 8: primers P35S-VoA/P35S-VoB.
**Figure 3.** DNA amplification of eight transgenic corn hybrid seed samples. A: primers P35S1/P35S2 and B: primers P35S-aflu/P35S-arl. Lane No.1 contains the DNA ladder, Lane No.2: hybrid FR1064xLH185Bt, Lane No.3: hybrid LH245xLH185Bt, Lane No.4: hybrid SGI928xHC50Bt, Lane No.5: hybrid HCSOBtxSGI905, Lane No.6: hybrid TR7322xMBS1236Bt, Lane No. 7: DKC69-71 hybrid, Lane No. 8: 4-NK7070Bt, Lane No. 9: Asgrow RX792 (non Bt-hybrid), Lane No.10: double deionized H₂O, and Lane No.11: plasmid DNA (pT102 construct)
Figure 4. DNA amplification of seed and pollen of transgenic corn with primers 35S168F/35S317R. Lane No.1: ladder, Lane No.2: seed of hybrid FR1064xLH185Bt, Lane No.3: double deionized (dd) H2O, Lane No.4: seed of hybrid LH245xLH185Bt, Lane No.5: dd H2O, Lane No.6: seed of hybrid DKC69-71, Lane No.7: dd H2O, Lane No.8: pollen of hybrid DKC69-71, Lane No.9: dd H2O, and Lane No.10: plasmid DNA (pT102 construct)
PART II. FIELD EXPERIMENTS
CHAPTER 2. ADVENTITIOUS PRESENCE OF TRANSGENIC CORN IN CONTINUOUS FIELDS WITH DIFFERENT LOCAL POLLEN DENSITY

Abstract

Pollen movement and the resulting flow of gene between populations have been an integral part of corn evolution. The development of corn varieties as factories of pharmaceutical and industrial compounds however, has renewed interest in pollen dispersal and gene flow. The objectives of this study were to compare the gene flow and outcross in a seed and a grain fields in adjacent source-receiver maize fields under the same environmental conditions. Two fields of approximately 36 hectares were planted with a non-transgenic, white corn hybrid, in Ankeny, IA. In the center of both fields, a 1 hectare plot of a yellow-seeded stacked RR/Bt transgenic hybrid was planted as an adventitious pollen source. Before flowering, the white receiver corn of one field was mechanically detasseled in a 4:1 ratio to reduce the local pollen density to approximately 40% of the high pollen density field. The hybrids had synchrony of flowering and the yellow corn pollen flowed freely into the white seed corn. The average percentage of outcross in the seed field was 42.15% at 1 m, 6.31% at 10 m and 1.29% at 35 m, while the percentage of outcross in the grain field was 30.13%, 2.65%, and 0.39% respectively. At greater distances from the source field (100 m or greater) the outcross frequency decreased. The statistical analysis of the maize pollen dispersal was approached through model comparison. When the local pollen density was low, the percentage of adventitious RR yellow seed presence was significantly higher and outcross levels fluctuated widely.
Introduction
Stevens et al. (2004) define gene flow in maize as the fertilization of a population by pollen from another group of genetically different maize plants. This process has been an intricate part of the evolution of maize improvement. The movement of genes from one population to another has been reported between landraces of maize and teosinte in rainfed areas of Mexico (Wilkes, 1970; 1977; Eubanks, 2001; Luna et al., 2001; Dobley, 2004; Baltazar et al., 2005); between improved varieties and landraces in grain production fields (Ortega-Paczka, 1973; Vega Zaragoza, 1973; Castillo Gonzalez and Goodman, 1997; Bellon and Brush, 1994; Bellon and Risopoulos, 2001; Baltazar and Schoper, 2002); and between grain and seed production fields, or among seed production fields, jeopardizing the genetic purity of the seed produced (Burris, 2001). Even though the magnitude of gene flow among plants varies with species, population, genotypes, and environmental conditions (Ellstrand, 2003), it can ultimately modify and define the genetic structure of a plant population. The principal means for gene movement are via pollen flow and, less frequently, by seed dispersal (Dow and Ashley, 1998; Ennos 1994, Hardy et al., 2004; Kenta et al., 2004). The interruption of the natural flow of genes among plants reduces population variability, causes inbreeding depression and loss of genetic variability in open-pollinated species by promoting self-pollination (Mayr, 1971). Therefore, gene flow has both a positive role enhancing genetic diversity of maize and a negative role compromising the genetic purity of elite germplasm during seed production of improved varieties.

Earlier corn pollen flow and outcross studies by Jones and Newell (1948), Bateman (1947a and 1947b), Jones and Brooks (1950), Airy (1955), Raynor et al. (1972), Paterniani and Stort (1974), and Murillo Navarrete (1978) show that pollen amounts and outcross
percentages decline considerably within the first 10 m away from the pollen source, and that a long-extending tail of pollen dispersal and outcross persist at distances of 60, 100, 200 or even 250 m. The amount of outcross at the farthest distances is usually less than 1%, thus allowing the seed industry to produce seed with the required standard of 99% of genetic purity (Ingram, 2000). The Organization for Economic Co-operation and Development (OECD) requirement for genetic purity of corn hybrid seed is 99% and the minimum isolation distance required, including border rows, is 200 m (OECD, 2005; Baltazar and Schoper, 2002). These standards for seed production have remained unchanged for more than 50 years (Burris, 2001).

Recent studies (Ortiz Torres, 1993; Garcia et al., 1998; Burris, 2001; Jemison and Vayda, 2001; Luna et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Baltazar and Schoper, 2002; Henry et al., 2003; Jarosz et al., 2003; Chilcutt and Tabashnik, 2004; Ma et al., 2004; and Stevens et al., 2004) found that pollen flow and outcross in corn decreases within the first 10 or 20 m from the pollen source, and that small amounts of pollen and outcross are present at distances not longer than 200 m, with the exception of Stevens et al. (2004) who found 0.02% outcross at 300 m.

Most of the studies are characterized by use of small pollen source field (50 x 50 m), small-size receiving plots, and non-continuous source-receiving field design. The development of corn varieties as factories of pharmaceutical and industrial compounds has generated renewed interest in pollen dispersal and gene flow. It is essential to re-evaluate the minimum distance required to isolate conventional hybrids from these transgenic varieties to avoid the adventitious presence of proteins and other compounds in the food supply (Doering, 2004). Large-scale studies are needed to assess how local pollen density,
continuous source and receiver fields, and multi-directional gene flow affect outcross in order to understand and achieve strict control of pollen flow and successful co-existence of these transgenic crops in the environment. The objectives of this study were to compare the gene flow and outcross in a seed and a grain fields in adjacent source-receiver maize fields under the same environmental conditions.

Materials and Methods

Experimental fields

Two fields of approximately 36 hectares were planted with a non-transgenic, white corn hybrid, RX792W in Ankeny, IA. In the center of both fields, 1 hectare plot of DKC69-71 yellow corn was planted as an adventitious pollen source. This yellow-seeded hybrid is a stacked transgenic, Roundup Ready™ (RR) /Bt hybrid. Planting dates were 20 May 2003 for the white seeded hybrid and 21 May 2003 for the yellow seeded, transgenic hybrid. The population for the transgenic hybrid was 84,013 plants/ha and 69,187 plants/ha for the non-transgenic variety. Seed and grain fields were managed under normal production practices of cultivation, insect and soil fertility management. Soils were silty clay loam to loam, predominantly of the series Nicollet, Webster, Clarion and Harps (USDA - Natural Resources Conservation Service, 2000). Before flowering, the white receiver corn of one field was mechanically detasseled in a 4:1 ratio to reduce the local pollen density, as in a typical seed production field. The hybrids had synchrony of flowering and the yellow corn pollen flowed freely into the white seed corn. Sampling in each experimental field was conducted along eight transects (N, NE, E, SE, S, SW, W and NW) relative to the source field to cover all possible wind directions. Samples were collected at approximately 1, 10,
35, 100, 150, 200 and 250 m away from the source field (Figure 1). Sampling locations were recorded using a global positioning system. At harvest maturity, samples of 25 ears were collected at 1, 10 and 35 m and 100 ears were collected at 100, 150, 200 and 250 m away from the adventitious yellow corn source. Because the level of outcross decreases as distance from the source field increases, sample size increased at the locations farthest from the center of the field, according to calculations using Seedcalc6 (Remund et al., 2001). Ears were dried to storage moisture (120 mg of water per g fresh weight) with forced, 40°C heated air seed driers at the Agronomy Farm, Iowa State University, Ames. Samples were shelled (Custom Seed Equipment, model LS91) and sorted with a 20-channel SATAKE ESM ScanMaster color-sorter, model SM-200DE at Seed Science Center, Iowa State University. After sorting, seed samples were weighed and the number of seeds in 454 grams was counted using a seed counter (FMC Syntron®, model EB00-D). The total number of seed in a sample was obtained by extrapolating the number of seed per 454 g by the total weight of the sample. When the weight of the adventitious seeds was below 454 g, yellow seeds in the sample were counted by hand.

**Flowering synchrony**

Silk and pollen shed synchrony was quantified by counting the numbers of plants with silks exposed and tassels shedding pollen during 21 July 2003 to 14 August 2003. The synchrony is presented as the average of 400 plants in the transgenic hybrid and the average of 2,200 plants in the non-transgenic hybrid.

**Meteorological data**

During pollination, a portable weather station was placed by the center field. R.M. Young 3001 Wind Sentry Set with an accuracy of ± 0.5 ms⁻¹ recorded local wind speed and
direction at a height of 3.17 m from the ground. Data was averaged and stored every 15 minutes during the flowering period using a Campbell Scientific CR10 data logger.

**Screening for the RoundUp Ready™ gene**

After color sorting, the remaining seeds mixed in the yellow and white seed fractions in the samples were separated by hand. The yellow seeds, separated from the white seed, were screened for the presence of the RR gene using the pre-emergence method established by Goggi and Stahr (1997) and AOSA (2003). The yellow seeds were imbibed for 48 hours in groups of 200 seeds between papers towels moistened with a 3% solution of glyphosate (Roundup™ Ultra). Two hundred seeds from the white seeded, non-transgenic seed in the sample, and 200 seeds from samples of the yellow-seeded, pollen source field were also tested to assess the segregation and linkage between the yellow and RR gene. The herbicide-imbibed seeds were planted on moistened crepe cellulose paper (Kimpak™) on top of plastic trays. Trays were placed inside a cart and moved into a constant 25 °C germination chamber for seven days. The number of normal, abnormal, and dead seeds seedling were evaluated following the AOSA (Association of Official Seed Analysts) Rules for Testing Seeds (AOSA, 2003). Briefly, the non-tolerant seedlings showed a short and thickened primary root with a light brown coloration, and the secondary roots, when present, were very short and stubby. In general, these herbicide-susceptible seedlings showed little or no growth due to the effect of the glyphosate herbicide. Abnormal seedlings and dead seeds were considered non-RR seeds. The percentage of outcross in yellow seeds using the information from the RoundUp Ready™ gene was obtained from the equation:

\[
\text{Outcross} (%) = \left( \frac{\text{Number of normal tolerant seedlings}}{\text{Total number of seeds}} \right) \times 100 \times 2
\]

where, outcross in percentage is:
Number of normal tolerant seedlings = number of yellow seed that produced a normal seedling after the RR seed pre-emergence glyphosate-imbibition test;
Total number of seeds = number of yellow + white seed in the sample;
The resulting number is multiplied by 100 in order to express results in percentage;
This number is multiplied by 2, because the RR gene was segregating as a true hemizygote, indicating that only half of the gametes (pollen grains) produced by the source field carried the RR gene.

**Screening for the Bt-Cry1Ab gene**

Normal and abnormal seedlings from the RR pre-emergence glyphosate-imbibition test were also analyzed for the presence of the Bt-Cry1Ab gene. Control samples collected from the yellow-seeded source field and from the white-seeded receiver field were also analyzed. In samples collected at 1 m, 10 m, and 35 m from the source, 360 seed (180 RR and 180 non-RR seedlings) were tested. In samples collected at greater distances from the source, all RR-seedlings and 180 seedlings of the non-RR seedlings were tested. If fewer than 180 non-RR yellow seedlings were found in the sample, all seedlings were tested. An enzyme-linked immunosorbent assay (ELISA) Bt kit (Agdia Incorporated) was used to detect the Bt protein. The test is a double-antibody sandwich (DAS) where the protein is captured and detected using a polyclonal antibody. The procedure used is described in detail in the ELISA kit. The protein was extracted by placing a small piece of leaf of the RR-tolerant seedlings or the tip of the coleoptile of the non-RR seedlings into a single well of the 96-wells plates and adding 300 µl of the PBST protein extraction buffer. The protein was extracted from the tissue using an ultrasonic cleaner (Fisher Scientific, model FS30) for 30 minutes. An aliquot (100 µl) of the extracted protein was transferred into the antibody-
coated, DAS-ELISA microplate well. If Bt-Cry1Ab protein is present in the sample, some will be bound by the antibodies and captured. The enzyme conjugate (100 μl), consisting of an antibody chemically linked to an enzyme was added to detect any captured Bt-Cry1Ab protein. Four wells were filled with a negative control (PBST protein extraction buffer) and two wells with the positive control. The microplate was incubated inside a humid box at room temperature (23 ± 1 °C) for at least 2 hours or overnight in the refrigerator at 4° C. After incubation, microplates were washed five times with PBST buffer to remove any unbound conjugate. After washing the microplate, 100 μl of a blue substrate (TMB) for the peroxidase solution was added to each test well. The reaction with the substrate produces a blue color within 5 to 15 minutes, signifying that the Bt-Cry1Ab protein is present. The intensity of the color is related with the amount of protein and can be quantified by optical density with a plate reader (BIO-TEK Instruments, Inc., model ELx800) at 650 nm. Seedlings lacking the Bt-Cry1Ab protein showed a clear or light blue color in the test wells. Data from the plate reader were used to determine the number of seedlings expressing the protein. The average of the data for the four negative control wells multiplied by two was used as the base number to determine the wells considered positive and negative for the presence of the protein. Samples in test wells with an optical density reading equal or lower than the average of the negative control wells multiplied by two were considered non-Bt samples. The percentage of Bt-Cry1Ab outcross was obtained with the equation:

\[
\text{Outcross (\%)} = \left( \frac{\text{Number of seedlings with the Bt-Cry1Ab protein}}{\text{Total number of seedlings tested}} \right) \times 100
\]

where, Number of seedlings with the Bt-Cry1Ab protein = number of wells in the test with optical density readings higher than the average control;
Total number of seedlings = total number of seedlings tested;
Results are multiplied by 100 to obtain percentage values.
The segregation for the Bt gene was 77% Bt and 23% non-Bt. It was accepted as segregation
of a hemizygote according to the $X^2$ test.

Statistical analysis

The statistical analysis to determine if the seed and the grain fields had significantly
different distribution of yellow seed outcross at the different distances from the source field
was carried out using the open source statistical software R ("The R Project for Statistical
Computing", 2004). Parameter estimation was performed using the maximum likelihood
estimation technique. Numerical optimizations of the log-likelihood function were
performed using a Newton-Raphson algorithm (Press et al., 1986).

Results and Discussion

Silk and pollen shed synchrony between the yellow and white corn is shown in Figure
2. The RR/Bt yellow corn source field reached an average of 50% pollen shed by 26 July
2003 and 50% silk exertion by 28 July 2003. The white corn receiver field reached an
average of 50% pollen shed by 26 July 2003 and 50% silk exertion by 27 July 2003. This
flowering synchrony was ideal for our study of the worst-case-scenario for chances of
adventitious presence in corn.

Table 1 shows precipitation, maximum and minimum temperatures, and GDD data
from Ankeny, IA (National Climatic Data Center). Figure 3 shows the wind rose for Ankeny,
IA from 19 July 2003 to 3 August 2003, which coincided with pollen shed. Predominant
winds during pollen shed and silk exertion were from the N and the NW (Figure 3). The
maximum 15 minutes average wind speed in 2003 was 4.7 ms\(^{-1}\), recorded on 26 July 2003 at 11:15 am, from the south-southwest. There was a late season drought in 2003, where rainfall for the month of August was 21.8 mm.

Several studies have used yellow seed to trace outcrossing (Paterniani and Start, 1974; Garcia et al., 1998; Jemison and Vayda, 2001; Stevens et al., 2004). The yellow or white color in the corn endosperm is owed to a single gene (\(y_1\)), with yellow being dominant over white (Weber, 1994). We expect all progeny of the yellow pollen source to bear a yellow seed. However, external contamination from surrounding fields can also produce yellow seed. In our study two other genes, the RoundUp Ready\textsuperscript{TM} (RR) gene and the Bt-Cry1Ab gene, were used to identify the seed resulting from an outcross with pollen from our source field. This allowed us to monitor the adventitious seed using these three genes. The segregation of the RR and Bt gene were determined in seed samples harvested in the yellow source field. The RR gene was segregating with a frequency of 70% RR and 30% non-RR as determined by the RR biological test, rather than the expected 75% RR: 25% non-RR (Chilcutt and Tabashnik, 2004). The Bt gene was segregating with a frequency of 77% Bt and 23% non-BT as determined by the ELISA test, rather than the expected 75% Bt: 25% non-Bt. All seeds harvested in the source field were yellow, indicating there was no segregation for seed color, and thus both alleles were dominant yellow. We applied these observed gene frequencies to the yellow seed in the samples to determine the number of seeds crossed by incoming pollen from the center source field. Because of the simplicity and low cost of the RR biological test, all yellow seeds were tested for the presence of the RR gene. For practical reason of cost and time, only a portion of the seed could be tested for the presence of the Bt gene. For this reason, results presented are based on the presence of two
genes, yellow seed color and RR, unless otherwise stated. To corroborate the accuracy of this assumption, we also analyzed the data using yellow seed in the sample which did not alter the general conclusions drawn from the data.

The outcross frequency was high at the closest sampling locations from the source field in both fields (Table 2). The average percentage of outcross in the seed field was 42.15% at 1 m, 6.31% at 10 m and 1.29% at 35 m, while the percentage of outcross in the grain field was 29.85%, 2.53%, and 0.42% at 1, 10, and 35 m from the pollen source, respectively. However; at greater distances from the source field (100 m or greater) the outcross frequency decreased further. At 100 m the percentage of outcross was 0.11% in the seed field and 0.033% in the grain field. Although at 250 m from the source the percentage of outcross decreased to 0.012% in the seed field and 0.003% in the grain field, it never reached zero for the distances evaluated in this experiment. These results were in agreement experiments by other authors (Ma et al., 2004; Stevens et al., 2004; Luna et al., 2001), where frequencies were as low as 0.01% at 200 m from the source, but never 0%. Table 2 shows that surrounding a transgenic corn with a continuous corn field is effective to keep gene flow close to the pollen source.

Wind had a strong influence on the percentage of outcross. The predominant wind direction was unusual since, historically, the predominant summer wind directions in Iowa are south, southwest to north, northeast. The outcross was higher in the down-wind directions. Considering that the predominant winds came from north and northwest (Figure 3), the highest outcross values were in the south, southeast, and southwest transects in both the seed and grain fields (Table 3). The outcross values in the cardinal directions were higher in the seed field, with the exception of the northeast transect. There was an exponential
decline in the percentage of outcross away from the pollen source (Tables 4 and 5). At 35 m from the source field the percentage of outcross was below 1% in both fields and all cardinal directions, with the exception of the down-wind directions in the seed field. When local pollen density was lower, as in the seed field, the percentage of outcross at 35 m downwind was 1.98% at south transect and 3.26% at southeast transect. Outcross data in the grain field or high local pollen density were smaller in all distances and cardinal directions with the exceptions of NW at 10 and 35 m, NE at 10 m and 150 m, and N at 100 m.

However, gene flow confinement in a continuous field and local pollen density is affected by wind direction, but only at the closest distances to the pollen source. Figure 4 and 5 show the outcross in a down-wind (S) and an up-wind (N) transect in both seed and grain fields. The average percentage of outcross of both fields (Table 2) at 1 m, 10 m, and 35 m, was 36%, 4.4% and 0.86%, respectively. The outcross was higher in the down-wind direction than the average at 1 m in both seed and grain field and at 10 m in the seed field (Figure 4). The outcross in the up-wind direction was lower than the average close to the pollen source (Figure 5). Predominant wind direction had no effect at farther distances in both the down-wind and the up-wind direction in both seed and grain field.

To facilitate visual interpretation of the data we also present the results as contour plots of logarithmic values of the observed outcross in figures 6 and 7. Values are expressed logarithmically to minimize the numerical difference between data points close to the source field (1 m) with mean outcross values of 30% in the grain field and close to 43% in the seed field, to data points at 200 m with a mean of 0.007% in the grain field and 0.03% in the seed field. The percentage of outcross downwind was greater than upwind in both fields. This increase in percentage of outcross was more pronounced, however, when the local pollen
density was low. Outcross levels in the seed field were generally higher, especially downwind. Outcross at 200 m in the seed field were as high as 0.63% at the SE while in the grain field the highest value at 200 m was 0.014% at the SE transect.

Comparison of outcross between grain and seed fields

The statistical analysis of the maize pollen dispersal between a grain and a seed field was approached through model comparison. Since the data consisted of observations about the outcome of the outcross between the pollen of the yellow corn and the surrounding white corn, we modeled the distribution of the yellow seeds originating from the source field. Based on the Roundup Ready™ (RR), yellow seeds, we estimated the number of yellow seeds originating from the source field \( \hat{Y}_i \) at each observed location \( i \) as

\[
\hat{Y}_i = R_i p^{-1}
\]

where \( R_i \) is the number of yellow, RR seeds at location \( i \), and \( p \) is the probability that a seed originating from the source field is resistant. Since \( p \) is not known exactly, we estimated it based on the assumption that yellow seeds collected at locations near the source field (no further than 35 m from the field) have originated from the source field. Thus, we estimate \( p \) by the proportion of RR yellow seeds collected at locations near the source field.

Exploratory data analysis suggested that the response variable \( Y \), which is defined as the number of adventitious yellow seeds from the pollen source, follows a Poisson distribution (Neter et al., 1996).

\[
Y \sim \text{Poisson} (\lambda)
\]

Several statistical models based on the distance from the source field, recorded wind direction and speed were considered. Statistical diagnostics showed that the model that best fits the data is,
\[
\lambda_i = \exp(\beta_0 + \beta_1 d_i) + \beta_2 W_i
\]

In this equation, \(d_i\) is the distance of a given location \(i\) from the source field, and \(W_i\) is a composite covariate, based on the hourly wind speed and direction during the peak shed period (July 28 – August 1st) and recorded distance from the source field:

\[
W_i = \sum_{h=1}^{H} \frac{Z_{ih}}{d_i} \mathbb{1}(i \in T(\text{direction}_h))
\]

where, \(Z_{ih}\) denoted the hourly wind speed at location \(i\), for our \(h\), with \(H\) being the total number of hours for which the wind speed and direction were recorded during the pollination period. \(T(\text{direction}_h)\) denoted the tolerance region for wind direction recorded during the \(h\)-th hour. The tolerance region for the wind was constructed based on general pollen dispersal behavior, as described by meteorologists. The basic idea behind the tolerance region was to include the down-wind concept in the statistical model: if a location is down-wind from the source field for a given hour it will receive more pollen than a location that is not.

The above model was fitted to data from both, the seed and the grain field. A graph of the resulting fitted values (for each field) superimposed on a scatterplot of the observed number of RR yellow seeds by distance from the field is presented in Figure 8. Based on a likelihood ratio test, there was very strong evidence that the two fields were significantly different (observed \(p\)-value is practically 0). Results indicated that when local pollen is low, the incoming pollen has a competitive advantage and the level of outcross is significantly greater than when the local pollen is abundant.

Residual plots and model diagnostics suggested that, although the proposed model captured the main behavior of the process, it didn't fit very well the data collected at the
farthest locations away from the source field. Based on these results and on the reality that the pollen dispersal pattern was different for locations situated near the source field and locations far away, we performed a second data analysis only for the locations situated farther from the field. Data suggested, however, that the same model fits best. Figure 9 displays the fitted values based on the model for each of the two fields, superimposed on the scatter plot of the observed number of RR yellow seeds at increasing distance from the source field. Likelihood ratio tests reinforced the hypothesis that there were significant differences between the two fields (p-value is practically 0). One interesting observation related to Figure 9 was that the number of RR yellow seeds from the source field in the seed field exhibited a much noisier behavior than the ones for the grain field, with about 10% of the observations having extremely high values. If the local pollen density was high, the percentage of adventitious RR yellow grain decreased rapidly and remained low as the yellow pollen moved away from the source. If the local pollen density was low (40% of the high pollen density field), the percentage of adventitious RR yellow seed presence was significantly higher as pollen moved away from the source field and levels fluctuated widely.

Conclusions

Maize gene flow away from a central pollen source field into surrounding maize moves differently depending on local pollen density. If the local pollen density is low, as in a seed production field, the chances for outcross are higher. These chances remain higher through longer distances. The model explained appropriately data from both fields, capturing the large-scale behavior of the physical process of pollen dispersion. The outcross of both fields was significantly different, according to the model comparison. These results suggest
that surrounding a maize field with a dense crop of the same height could minimize the chances of outcross into neighboring fields located beyond 200 m. More research is needed to validate this assumption.

References


Jones, M.D. and L.C. Newell. 1948. Longevity of pollen and stigmas of grasses:


Organization for Economic Co-operation and Development (OECD) Seed Schemes 2005:
Rules and Directions. 2005. Maize and Sorghum. 2nd Part
Rules and Directions By Seed Scheme (Annexes VI to XII to the Decision). OECD
May, 2005. Available at: http://www.oecd.org/dataoecd/41/0Z15290084.PDF


Table 1. Precipitation, maximum and minimum temperatures, and growing degree days (GDD) in 2003.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>May</th>
<th>June</th>
<th>July</th>
<th>August</th>
<th>September</th>
<th>October</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitation (mm)</td>
<td>95.3</td>
<td>60.1</td>
<td>89.0</td>
<td>21.8</td>
<td>87.4</td>
<td>20.6</td>
<td>374.2</td>
</tr>
<tr>
<td>GDD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>171</td>
<td>295</td>
<td>387</td>
<td>399</td>
<td>215</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Temp. Max. (°C)</td>
<td>20.4</td>
<td>24.8</td>
<td>27.9</td>
<td>28.8</td>
<td>22.1</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>Temp. Min. (°C)</td>
<td>8.8</td>
<td>13.6</td>
<td>16.9</td>
<td>16.9</td>
<td>8.6</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Precip. (mm) – precipitation in millimeters

<sup>b</sup>GDD<sub>10</sub>– growing degree days in degree Celsius = [(minimum temperature + maximum temperature) \times 2<sup>1/3</sup>] - 10 °C accumulated per days.

If maximum temperature is > 30 °C, then maximum temperature = 30 °C

If minimum temperature is < 10 °C, then minimum temperature = 10 °C

<sup>c</sup>Temp. Max. (°C) – Average maximum temperature in °C

<sup>d</sup>Temp. Min. (°C) – Average minimum temperature in °C
Table 2. Average percentage of yellow-RR grain in the samples collected at increasing distances from the yellow-seeded-RR pollen source following eight cardinal directions in the seed and grain fields.

<table>
<thead>
<tr>
<th>Distance (m) from the source</th>
<th>Seed field</th>
<th>Grain field</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42.151</td>
<td>29.850</td>
</tr>
<tr>
<td>10</td>
<td>6.308</td>
<td>2.531</td>
</tr>
<tr>
<td>35</td>
<td>1.289</td>
<td>0.425</td>
</tr>
<tr>
<td>100</td>
<td>0.111</td>
<td>0.033</td>
</tr>
<tr>
<td>150</td>
<td>0.048</td>
<td>0.014</td>
</tr>
<tr>
<td>200</td>
<td>0.026</td>
<td>0.007</td>
</tr>
<tr>
<td>250</td>
<td>0.012</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Table 3. Average percentage of yellow-RR grain in the samples collected at the eight cardinal directions and increasing distances from the yellow-seeded-RR pollen source in the seed and grain fields.

<table>
<thead>
<tr>
<th>Cardinal direction</th>
<th>Field</th>
<th>Seed field</th>
<th>Grain field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Outcross (%)</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>15.156</td>
<td>8.259</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>12.663</td>
<td>7.079</td>
<td></td>
</tr>
<tr>
<td>SW</td>
<td>11.961</td>
<td>6.839</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>8.659</td>
<td>4.423</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>6.017</td>
<td>4.342</td>
<td></td>
</tr>
<tr>
<td>NW</td>
<td>4.780</td>
<td>3.350</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>4.441</td>
<td>2.421</td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>4.298</td>
<td>5.161</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Average percentage of outcross in the seed field. Samples were collected in each of the eight cardinal directions at increasing distances from the yellow-seeded-RR pollen source.

<table>
<thead>
<tr>
<th>Transect</th>
<th>NW</th>
<th>N</th>
<th>NE</th>
<th>E</th>
<th>SE</th>
<th>S</th>
<th>SW</th>
<th>W</th>
<th>Outcross (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.386</td>
<td>26.864</td>
<td>26.127</td>
<td>41.814</td>
<td>58.820</td>
<td>64.201</td>
<td>55.735</td>
<td>26.193</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.084</td>
<td>4.334</td>
<td>1.436</td>
<td>5.680</td>
<td>10.496</td>
<td>12.084</td>
<td>8.460</td>
<td>1.011</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0.122</td>
<td>0.422</td>
<td>0.769</td>
<td>0.921</td>
<td>3.257</td>
<td>1.984</td>
<td>0.920</td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>100*</td>
<td>0.033</td>
<td>0.048</td>
<td>0.065</td>
<td>0.067</td>
<td>0.403</td>
<td>0.220</td>
<td>0.041</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>150*</td>
<td>0.021</td>
<td>0.060</td>
<td>0.022</td>
<td>0.028</td>
<td>0.091</td>
<td>0.137</td>
<td>0.011</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>200*</td>
<td>0.028</td>
<td>†</td>
<td>0.013</td>
<td>0.011</td>
<td>0.063</td>
<td>†</td>
<td>0.021</td>
<td>0.021</td>
<td></td>
</tr>
<tr>
<td>250*</td>
<td>†</td>
<td>†</td>
<td>0.013</td>
<td>0.011</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td></td>
</tr>
</tbody>
</table>

*: Data is the average of two samples.
†: Sampling location not available because of the shape of the field.
Table 5. Average percentage of outcross in the grain field. Samples were collected in each of the eight cardinal directions at increasing distances from the yellow-seeded-RR pollen source.

<table>
<thead>
<tr>
<th>Distance</th>
<th>NW</th>
<th>N</th>
<th>NE</th>
<th>E</th>
<th>SE</th>
<th>S</th>
<th>SW</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.674</td>
<td>26.079</td>
<td>20.881</td>
<td>25.706</td>
<td>47.055</td>
<td>43.479</td>
<td>45.107</td>
<td>9.820</td>
</tr>
<tr>
<td>10</td>
<td>3.015</td>
<td>1.782</td>
<td>2.351</td>
<td>3.079</td>
<td>3.857</td>
<td>3.142</td>
<td>2.869</td>
<td>0.151</td>
</tr>
<tr>
<td>35</td>
<td>0.221</td>
<td>0.194</td>
<td>0.166</td>
<td>0.854</td>
<td>0.936</td>
<td>0.431</td>
<td>0.598</td>
<td>0.000</td>
</tr>
<tr>
<td>100*</td>
<td>0.003</td>
<td>0.050</td>
<td>0.051</td>
<td>0.011</td>
<td>0.046</td>
<td>0.057</td>
<td>0.041</td>
<td>0.005</td>
</tr>
<tr>
<td>150*</td>
<td>0.001</td>
<td>0.016</td>
<td>0.027</td>
<td>0.002</td>
<td>0.025</td>
<td>0.034</td>
<td>0.004</td>
<td>0.000</td>
</tr>
<tr>
<td>200*</td>
<td>0.004</td>
<td>0.005</td>
<td>†</td>
<td>0.006</td>
<td>0.014</td>
<td>0.012</td>
<td>0.011</td>
<td>0.000</td>
</tr>
<tr>
<td>250*</td>
<td>0.000</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>0.003</td>
<td>†</td>
<td>0.002</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*: Data is the average of two samples.
†: Sampling location not available because of the shape of the field.
Figure 1. Sampling locations at increasing distances away from the source field. Samples were collected at 1 m, 10 m, 35 m, 100 m, 150 m, 200 m, and 250 m following the N, E, S, W, NE, SE, SW, and NW cardinal points. Additional samples were taken at 100 m, 150 m, 200 m, and 250 m to minimize separation among sampling locations at the farthest distances from the source field.
Figure 2. Flowering synchrony - pollen shed vs. silk exertion – in percentage of plants. A: between the plants in the Bt/RR yellow source field and the white receiver field; B: among the plants in the white receiver field.
Figure 3. Wind rose for Ankeny, Iowa from 19 July to 3 August 2003. Wind speed is in ms$^{-1}$ and concentric circles represent the percentage of the day that the wind came from that direction and that intensity. Data was collected on-site using a R.M. Young 3001 Wind Sentry Set in a portable weather station.
Figure 4. Percent of adventitious RR-yellow seed in a down-wind direction (south transect) from the transgenic hybrid in the seed and grain fields. **A:** Distances from 1 to 200 m. **B:** Close up of distances from 100 to 200 m from the source field.
Figure 5. Percent of adventitious RR-yellow seed in an up-wind direction (north transect) from the transgenic hybrid in the seed and grain fields. A: Distances from 1 to 200 m. B: Close up of distances from 100 to 200 m from the source field.
Figure 6. Distribution of adventitious RR-yellow seed in a detasseled seed field (low local pollen density) in Ankeny, Iowa, 2003, based on a logarithmic scale.
Figure 7. Distribution of adventitious RR yellow grain in a non-detasseled grain field (high local pollen density) in Ankeny, Iowa, 2003, based on a logarithmic scale.
Figure 8. Graphic representation of the model-fitted values of outcross for the seed and grain field superimposed on a scatterplot of the number of observed adventitious RR yellow seeds by distance from the field.

+ represents the observed RR-yellow outcross values in the seed field
• represents the observed RR-yellow outcross values in the grain field
Figure 9. Graphic representation of the model-fitted values of outcross for the seed and grain fields at selected locations farthest away from the source field, and the superimposed scatterplot of the number of observed adventitious RR yellow seeds.

+ represents the observed RR-yellow outcross values in the seed field
● represents the observed RR-yellow outcross values in the grain field
CHAPTER 3. ADVENTITIOUS PRESENCE OF TRANSGENIC CORN IN CONTINUOUS FIELDS FOR GRAIN PRODUCTION IN DIFFERENT ENVIRONMENT

Abstract

The adventitious presence of transgenes in conventional varieties due to gene flow is a serious concern when producing grain for specialty markets, organic products, crops with value added traits, and seed. Controlling maize pollen dispersal is an important factor in transgenic and non-transgenic coexistence. The objectives of this study were to determine the gene flow from a central transgenic source field into a conventional grain production field, and to quantify the level of outcross by using a combination of three marker genes, seed color, Bt and RR. Two fields of approximately 36 hectares were planted with a non-transgenic, white-seeded corn hybrid in Ankeny, IA in 2003 and 2004. In the center of both fields, a 1 hectare plot of yellow-seeded, Bt/RR staked genes hybrid corn was planted as an adventitious pollen source. Samples were collected at 1, 10, 35, 100, 150, 200 and 250 m away from the adventitious pollen source following the eight cardinal transects (N, NE, E, SE, S, SW, W, NW). The average percentage of outcross over all transects at 35 m was 0.4% in both years. At 100 m distance and beyond, the average level of outcross decreased even further but never reached 0% within the 250 m sampled. The statistical analysis of the maize pollen dispersal between two grain production fields was approached through model comparison. The model explained appropriately data from both years, capturing the large-scale behavior of the physical process of pollen dispersion.
Introduction

For centuries, plants have cross-pollinated through several means, including wind dispersion, insects and human intervention. Gene flow derived from cross-pollination of distinct populations has been an integral part of maize evolution. A monoecious plant with separated male and female flowers, the maize tassel can produce $4.5 \times 10^6$ pollen grains per plant and shed the pollen for 5 days or longer (Westgate et al., 2003). Among species with wind dispersed pollen, maize produces one of the largest and heaviest pollen grains, being about 90 μm in diameter (Di-Giovanni et al., 1995).

The wind frequently carries pollen grains from one maize crop to another, causing cross-pollination. This factor has gained renewed interest now that transgenic maize crops that contain novel DNA from different species are planted in a larger area in co-existence with non-transgenic maize. Since the first transgenic maize variety was released in 1996, the area planted with transgenic crops has increased from 1.7 million ha in 1996 to 44 million ha in 2000, of which 30 million ha of transgenic crops were planted in USA (James, 2002). In 2000, over 10 million ha of transgenic maize was being grown in the USA (James, 2002).

Food safety, organic and other non-transgenic maize, and pest resistance have become public concerns as there is little data about the transfer of transgenic maize by cross-pollinating with non-transgenic maize, especially when the area planted to transgenic varieties is constantly increasing. The possibility of adventitious presence of transgenes in conventional varieties is a serious concern when producing grain for specialty markets, organic products, crops with value added traits, or in seed production, where the genetic purity of product is very important. The maize exports have been negatively impacted because several countries have imposed strict standards that are difficult to achieve in the US.
where non-transgenic and transgenic varieties are being produced commercially in nearby areas. For example, the EU market (European Commission, 2004) has a maximum labeling threshold of 0.5% genetically modified organism (GMO) content in conventional food products and food ingredients, but this threshold is 0% in certified organic products (Messeguer, 2003).

Controlling maize pollen dispersal is an important factor in preventing or limiting cross-pollination between transgenic and non-transgenic crops. When introducing plant-made pharmaceuticals and industrial products into conventional cropping systems it is essential to avoid adventitious presence of these compounds in the food supply (Stewart and Knight, 2005). To prevent the natural crop-to-crop gene flow, a combination of five containment strategies (physical, biological, mechanical, spatial and temporal barrier) must be used (Lamkey, 2002). Other containment techniques include maternal inheritance of the transgenic trait, male sterility, and seed sterility (Daniell, 2002). In his comprehensive review of containment strategies, Daniell (2002) suggests the evaluation of alternative mechanisms, such as apomixis (asexual seed formation), cleistogamy (self-fertilization before flower opening), chemical induction and deletion of the transgene, and genome incompatibility, among others.

Several studies have used seed color to trace outcrossing in maize (Paterniani and Start, 1974; Garcia et al., 1998; Jemison and Vayda, 2001; Stevens et al., 2004). The yellow or white color in the corn endosperm is owed to a single gene (y1), with yellow being dominant over white (Weber, 1994). Little research has been done to determine the adventitious presence of transgenes in non-transgenic crops (Wilkinson, 2003), and to assess the gene flow into large-scale fields (Chilcutt and Tabashnik, 2004). The objectives of this
study were to determine the gene flow from a central transgenic source field into a conventional grain production field, and to quantify the level of outcross by using a combination of three marker genes, seed color, Bt and RR.

**Materials and Methods**

**Experimental fields**

Two fields of approximately 36 hectares were planted with a non-transgenic, white corn hybrid, RX792W in Ankeny, IA, in 2003 and 2004. In the center of both fields, 1 hectare plot of DKC69-71 yellow corn was planted as an adventitious pollen source. This yellow-seeded hybrid is a stacked transgenic, Roundup Ready™ (RR) /Bt hybrid. Planting dates for the white seeded hybrid and the yellow seeded, transgenic hybrid were May 20th and May 21st in 2003; and May 4th and May 5th in 2004. In 2003 the population for the transgenic hybrid was 84,013 plants/ha and 69,187 plants/ha for the non-transgenic, white-seeded hybrid. Plant populations in 2004 were 86,450 plants/ha for the transgenic hybrid and 71,630 plants/ha of the non-transgenic hybrid. Fields were managed under normal production practices of cultivation, insect and soil fertility management. Soils were silty clay loam and loam of the series Nicollet, Webster and Clarion (USDA - Natural Resources Conservation Service, 2000). The sampling was conducted along eight transects (N, NE, E, SE, S, SW, W and NW) relative to the source field. Samples were collected at 1, 10, 35, 100, 150, 200 and 250 m away from the source field (Figure 1). Sampling locations were recorded using a global positioning system. The hybrids had synchrony of flowering and the yellow corn pollen flowed freely into the white seed corn (Figure 2, A and B). At harvest maturity, samples of 25 ears were collected at 1, 10 and 35 m and 100 ears were collected at 100, 150,
200 and 250 m away from the adventitious yellow corn source following the eight directions. Sample size increased at the locations farthest from the center of the field to compensate for decreasing numbers of outcross at the farthest locations, according to calculations using Seedcalc6 (Remund et al., 2001). Ears were dried to storage moisture (120 mg of water per g$^{-1}$ fresh weight) with forced, 40° C heated air seed driers at the Agronomy Farm, Iowa State University, Ames. Samples were shelled (Custom Seed Equipment, model LS91) and sorted with a 20-channel SATAKE ESM ScanMaster color-sorter, model SM-200DE at Seed Science Center, Iowa State University. After sorting, seed samples where weigh and the number of seeds in 454 grams was counted using a seed counter (FMC Syntron®, model EB00-D). The total number of seed in a sample was obtained by extrapolating the number of seed per 454 grams by the total weight of the sample. When the weight of the adventitious seeds was below 454 g, yellow seeds in the sample were counted by hand.

**Flowering synchrony**

Silk and pollen shed synchrony was quantified by counting the numbers of plants with silks exposed and tassels shedding pollen during 21 July 2003 to 14 August 2003 and during 13 to 23 July 2004. The synchrony is presented as the average of 400 plants in the transgenic hybrid and the average of 2200 plants in the nontransgenic hybrid.

**Meteorological data**

During pollination, a portable weather station was placed by the center field. R.M. Young 3001 Wind Sentry Set with an accuracy of ± 0.5 ms$^{-1}$ recorded local wind speed and direction at a height of 3.17 m from the ground. Data was averaged and stored every 15 minutes during the flowering period using a Campbell Scientific CR10 data logger.
Screening for the RoundUp Ready™ gene

After color-sorting, the remaining seeds mixed in the yellow and white fractions were separated by hand. The yellow seed were screened for the presence of the RR gene using the pre-emergence method established by Goggi and Stahr (1997) and AOSA (2003). The yellow seeds were imbibed for 48 hours in groups of 200 seeds between paper towels moistened with a 3% solution of glyphosate (Roundup™ Ultra). Two hundred seeds from the white seeded, non-transgenic seed in the sample, and 200 seeds from samples of the yellow-seeded, pollen source field were also tested to assess the segregation and linkage between the yellow and RR gene. The herbicide-imbibed seeds were planted on moistened crepe cellulose paper (Kimpak™) on top of plastic trays. Trays were placed inside a cart and moved into a constant 25 °C germination chamber for seven days. The number of normal, abnormal, and dead seedlings was evaluated following the AOSA (Association of Official Seed Analysts) Rules for Testing Seeds (AOSA, 2003). Briefly, the non-tolerant seedlings showed a short and thickened primary root with a light brown coloration, and the secondary roots, when present, were very short and stubby. In general, these herbicide-susceptible seedlings showed little or no growth due to the effect of the glyphosate herbicide. Abnormal seedlings and dead seeds were considered non-RR seeds. The percentage of outcross in yellow seeds using the information from the RoundUp Ready™ gene was obtained from the equation:

\[
\text{Outcross} \, \% = \left[ \frac{\text{Number of normal tolerant seedlings}}{\text{Total number of seeds}} \right] \times 100 \times 2
\]

where, outcross in percentage is:

Number of normal tolerant seedlings = number of yellow seed that produced a normal seedling after the RR seed pre-emergence glyphosate imbibition test;

Total number of seeds = number of yellow + white seed in the sample;
The resulting number is multiplied by 100 in order to express results in percentage; this number is multiplied by 2, because the RR gene was segregating as a true hemizygote, indicating that only half of the gametes (pollen grains) produced by the source field carried the RR gene.

**Screening for the Bt-Cry1Ab gene**

Normal and abnormal seedlings from the RR pre-emergence glyphosate-imbibition test were also analyzed for the presence of the Bt-Cry1Ab gene. Control samples collected from the yellow-seeded source field and from the white-seeded receiver field were also analyzed. In samples collected at 1 m and 35 m from the source, 360 seed (180 RR and 180 non-RR seedling) were tested. In samples collected at greater distances from the source, all RR-seedlings and 180 seedlings of the non-RR seedlings were tested. If fewer than 180 non-RR yellow seedlings were found in the sample, all seedlings were tested. An enzyme-linked immunosorbent assay (ELISA) Bt kit (Agdia Incorporated) was used to detect the Bt protein. The test is a double-antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) where the protein is captured and detected using a polyclonal antibody. The procedure used is described in detail in the ELISA kit. The protein was extracted by placing a small piece of leaf of the RR-tolerant seedlings or the tip of the coleoptile of the RR-non-tolerant seedlings into a single well of the 96-well plates and adding 300 µl of the PBST protein extraction buffer. The protein was extracted from the tissue using an ultrasonic cleaner (Fisher Scientific, model FS30) for 30 minutes. An aliquot (100 µl) of the extracted protein was transferred into the antibody-coated, DAS-ELISA microplate well. If Bt-Cry1Ab protein was present in the sample, some was bound by the antibodies and captured. The enzyme conjugate (100 µl), consisting of an antibody chemically linked to an enzyme was added to
detect any captured Bt-Cry1Ab protein. Four wells were filled with a negative control (PBST protein extraction buffer) and two wells with the positive control. The microplate was incubated inside a humid box at room temperature (23 ± 1 °C) for at least 2 hours or overnight in the refrigerator at 4° C. After incubation, microplates were washed five times with PBST buffer to remove any unbound conjugate. After washing the microplate, 100 μl of a blue substrate (TMB) for the peroxidase solution was added to each test well. The reaction with the substrate produces a blue color within 5 to 15 minutes, signifying that the Bt-Cry1Ab protein is present. The intensity of the color is related with the amount of protein and can be quantified by optical density with a plate reader (BIO-TEK Instruments, Inc., model ELx800) at 650 nm. Seedlings lacking the Bt-Cry1Ab protein showed a clear or light blue color in the test wells. Data from the plate reader were used to determine the number of seedlings expressing the protein. The average of the data for the four negative control wells was used as the base number to determine the wells considered positive and negative for the presence of the protein. Samples in test wells with an optical density reading equal or lower than that negative control wells were considered non-Bt samples. The percentage of Bt-Cry1Ab outcross was obtained with the equation:

\[ \text{Outcross (\%)} = \left( \frac{\text{Number of seedlings with the Bt-Cry1Ab protein}}{\text{Total number of seedlings tested}} \right) \times 100 \]

where, number of seedlings with the Bt-Cry1Ab protein = number of wells in the test with optical density readings higher than the average control;

Total number of seedlings = total number of seedlings tested;

Results are multiplied by 100 to obtain percentage values.
The segregation for the Bt gene was not a hemizygote. The progeny of the yellow-seeded source field presented 77% Bt and 23% non-Bt in 2003 and 88% Bt and 12% non-Bt, thus calculations were adjusted accordingly.

**Data analysis**

The statistical analysis to determine if the grain fields in 2003 and 2004 had significantly different distribution of yellow seed outcross at the different distances from the source field was carried on using the open source statistical software R ("The R Project for Statistical Computing", 2004). Parameter estimation was performed using the maximum likelihood estimation technique. Numerical optimizations of the log-likelihood function were performed using a Newton-Raphson algorithm (Press et al., 1986).

**Results and Discussion**

The hybrids had synchrony of flowering and the yellow corn pollen flowed freely into the white seed corn (Figure 2, A and B). In 2003 the RR/Bt yellow corn source field reached 50% pollen shed by 26 July 2003 and 50% silk exertion by 28 July 2003. The white corn receiver field reached 50% pollen shed by 26 July 2003 and 50% silk exertion by 27 July 2003. In 2004 both fields reached 50% anthesis and silk exertion on 18 July. This flowering synchrony was ideal for our study of the worst-case-scenario for chances of adventitious presence in corn.

Table 1 shows precipitation, maximum and minimum temperatures, and GDD data for 2003 and 2004. Figure 3 and 4 show the wind rose for Ankeny, IA from 19 July to 3 August 2003 and from 17 to 29 July 2004. These time periods coincided with peak pollen shed. Predominant winds during pollen shed and silk exertion were from the N and the NE.
This was an unusual wind pattern. Historically, the predominant summer winds in central Iowa are from the south, southwest. In 2004, a more predictable south-southwest wind pattern was observed. The maximum 15 minutes average wind speed in 2003 was 4.7 ms\(^{-1}\), recorded on 26 July at 11:15 am, and 28 July 2004 at 11:00 am the 15 minutes average was 2.6 ms\(^{-1}\). In both occasions the winds were from the south-southwest.

The yellow or white color in the corn endosperm is owed to a single gene (\text{y1}), with yellow being dominant over white (Weber, 1994). We expect all progeny of the yellow pollen source to bear a yellow seed. However, external contamination from surrounding fields can also produce yellow seed. In our study two other genes, the RoundUp Ready\textsuperscript{TM} (RR) gene and the Bt-Cry1Ab gene, were used to identify the seed resulting from an outcross with pollen from our source field. This allowed us to monitor the adventitious seed using these three genes. The segregation of the RR and Bt gene were determined in seed samples harvested in the yellow source field. The RR gene was segregating with a frequency of 70% RR and 30% non-RR in 2003 and 73% RR and 27% non-RR in 2004 as determined by the RR biological test, rather than the expected 75% RR: 25% non-RR (Chilcutt and Tabashnik, 2004). The Bt gene was segregating with a frequency of 77% Bt and 23% non-Bt in 2003 and 88% Bt and 12% non-Bt in 2004 as determined by the ELISA test, rather than the expected 75% Bt: 25% non-Bt. All seeds harvested in the source field were yellow, indicating there was no segregation for seed color, and thus both alleles were dominant yellow. We applied these observed gene frequencies to the yellow seed in the samples to determine the number of seeds crossed by incoming pollen from the center source field. The frequency of RR followed the expected segregation frequency of ½ yellow RR and ½ yellow Bt (expected frequency of ¼ Bt & RR; ¼ Bt; ¼ RR; and ¼ non-Bt or RR). Bt did follow the same
frequency at the distances close to the central source field, but not at the farthest distances (100 – 250 m) in 2003 and 2004. We suspected contamination from surrounding fields. Although fields in close proximity were planted with non-Bt hybrids, low levels of Bt-Cry1Ab were found in samples collected from these fields. Thus, results presented are based on the presence of two genes, yellow seed color and RR, unless otherwise stated. We also analyzed the data using only the number of yellow seed in the sample which did not alter the general conclusions drawn from the data.

The percentage of outcross was highest at the closest distance and downwind from the source field. At one meter from the source, the average percentage of outcross in 2003 and 2004 were 29.9% and 17.0% respectively (Table 2). The highest percentages of outcross in 2003 were towards the south; with 47.1% outcross at the SE, 43.5% at the S and 45.1% at the SW sampling sites (Table 3). In 2004 the highest percentages of outcross were also at the sampling locations towards the south; outcross levels at 1 m were 21.2% in the SE and 30.6% in the S (Table 4). This coincides with the predominate wind direction during the peaks of flowering when 100% of the white-seeded corn silks were exposed and 100% of the tassels of the yellow-seeded, RR/Bt hybrids were shedding pollen (30 to 31 July 2003 and 19 to 21 July 2004) (Figures 2, 5, and 6). These figures also illustrate that, wind intensity was an important factor in the incidence of outcross. Our results emphasized the importance of wind direction and intensity during the peaks of flowering synchrony (Burris, 2001; Jones and Brooks, 1950; Jones and Newell, 1946).

In our experiments, the average percentage of outcross at 35 m was 0.4% in 2003 and 2004 (Table 2). These values were 0.9% and 0.7% towards the SE in 2003 and 2004 but lower in all other sampling directions (Tables 3 and 4). These results are in agreement with
those reported by other authors (Jaroz et al., 2003; Foueillassar and Benetrix, 2003). At 100 m from the source field and beyond, the levels of outcross decreased even further but the average percentage of outcross never reached 0% within the 250 m where samples were collected. Only in a few samples at 250 m (NW in 2003 and SE in 2004) the percentage of outcross reached 0% (Tables 3 and 4). This is not surprising for the NW quadrant in 2003 because most winds came from the NW, but it was unusual to find 0% outcross in 2004 in one of the samples collected at 250 m at the SW quadrant away from the pollen source field (Table 4). On 31 July 2003 when 100% of the population of white- and RR/Bt yellow-seeded plants were at peak silk exertion and pollen shed there was a mild NE wind that could have produced an outcross.

Statistical analysis of the outcross data for grain fields planted in Ankeny, Iowa in 2003 and 2004

The goal of the statistical analysis was to perform a parallel analysis of the outcross data collected for two white grain production fields in experiments conducted in two consecutive years, 2003 and 2004. We attempted to model the outcross resulting from the dispersal process of genetically modified corn pollen from the central source field. The actual data consists of white and yellow corn seeds collected at a number of locations surrounding the source field. We assume that the outcross process is a fair representation of the actual pollen dispersion. One of the first complications that arises in modeling the response variable --number of genetically modified yellow seeds in a given seed sample collected at each location--is that there may have been cross-pollination between the white and yellow corn from the surrounding fields. However, we checked all the neighboring fields and determined they were not planted with genetically modified corn.
Several genetic identification tests have been conducted on the yellow seeds collected establishing their resistance to glyphosate herbicide. This is of particular importance to our model, as we estimate the number of yellow RR seeds \( \hat{Y} \) originating from the source field at each observed location \( i \) as

\[
\hat{Y}_i = R_i p^{-1}
\]

Where \( R_i \) is the number of RoundUp Ready™ (RR) seeds at location \( i \), and \( p \) is the probability that a seed originating from the source field is RR. Since \( p \) is not known exactly, we estimate it based on the assumption that yellow seeds collected at locations near the source field (no farther than 35 m) have originated from the source field. Thus, the unbiased estimator of \( p \) is the proportion of resistant yellow seeds collected at locations near the source field.

We are now in the position to model the outcross process through the response variable defined above. Specifically, we treat the estimated yellow counts \( \hat{Y}_i \) as Poisson random variables (Neter et al., 1996) with means \( \lambda_i \) satisfying the following model:

\[
\lambda_i = \exp(\beta_0 + \beta_1 d_i) + \beta_2 W_i
\]

Where \( d_i \) denoted the distance from the source field and \( W_i \) is a constructed covariate that models wind speed, direction and proximity to the source field (for more details on the construction of \( W_i \) see results and discussion of Chapter 2).

Estimation of the three model parameters \( \beta_0, \beta_1, \) and \( \beta_2 \) is performed by maximizing the log-likelihood function, using a numerical optimization algorithm known as Newton-Raphson. The results of the estimation procedure for the two data sets are presented in Table 5. Although the estimators \( (\beta_0, \beta_1, \beta_2) \) for the two data sets were not identical, we can certainly note that the models described two very similar fundamental processes. Figure 7
presents the number of yellow seeds for each of the two years plotted against the distance from the source field, as well as the fitted models (indicated by the lines drawn on the scatterplot). The model seemed appropriate for both data sets, capturing the large-scale behavior of the physical process of pollen dispersion.

The scale of the graph in Figure 7 made it difficult to note any details of the actual model fit for locations situated far from the source field. In Figure 8 we presented only a portion of the data set, restricting our attention to locations farther than 70 m from the field.

There are several issues worth mentioning regarding this graph. First note that the two models were not very different for the two data sets. Although both models exhibited a decrease of the number of yellow seeds as the distance from the source field increased, they both underestimated the actual behavior of the actual process at distances of 70 to 90 m. Figure 9 displays the number of yellow seeds for data from 2003 and the actual fitted values when the model was fitted to data coming only from locations situated at least 70 m away from the source field.

Although not perfect, the model fitted only to the data from locations situated farther from the source field reflects the characteristics of the fundamental pollen dispersion process with much more precision than the model fit to the entire data set. This is along the lines expressed in the results of Chapter 2 that it is perhaps worth considering the dispersion process as two separate courses, one for locations near the source field and one for the ones farther away.

In spite of this observation, we encountered serious problems while attempting to fit the same model to data collected in 2004: model parameters were not estimable. This is not
very surprising, as exploratory data analysis suggested there were some anomalies in the data set.

Figure 8 indicated that there were some extremely high numbers of RR yellow seeds collected at locations situated more than 100 m from the field. To better illustrate the outlying cases, we constructed a graph (Figure 10) displaying the magnitude of the number of yellow seeds recorded at locations situated far from the field. The sizes of the points that indicate the location are proportional to the value of the response variable. It is notable that some of the largest observations were located the farthest from the field on the west edge or in the NW corner, which was counterintuitive: if the wind is the main source of transport for the pollen, the number of yellow seeds should at worst remain fairly constant while moving away from the source field, not increase significantly. In addition, there was no evidence of strong wind from the east or south-east, as illustrated by Figure 6. We can think of a few reasons for which we were having difficulties fitting the model for 2004. One possibility is that the construction of the covariate measuring wind speed and direction should be changed to take into account more complicated micro-scale meteorological structures. Exploratory data analysis suggested that simply the hourly wind speed average and its direction were not significantly different from 2003, and therefore should not lead to a more difficult model fitting. Another, more plausible scenario was that the data in 2004 was contaminated: the high observations in the western part of the observed field may actually be associated with a different process than the one we are attempting to represent: that of the pollen dispersion as it relates to meteorological conditions. In fact, the higher percentages of RR yellow seeds in the western edge of the field are likely to be the consequence of improper cleaning of the equipment at planting (Kent Berns, personal communication).
Conclusions

Wind speed and direction influenced the percentage of outcross mainly at 1 and 10 m. Wind speed and direction affected the outcross in both years in all cardinal directions at 1, 10, and 35 m from the pollen source, but at farthest distances the effect was non-significant, regardless of distance and cardinal directions. The outcross in both years was high close to the pollen source, but decreased below 0.5% at 35 m away from the source and below 0.08 at 250 m from the pollen source. The model explained appropriately data from both years, capturing the large-scale behavior of the physical process of pollen dispersion.

References


Table 1. Meteorological data: precipitation, maximum and minimum temperatures, and GDD data for 2003 and 2004.

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<sup>b</sup>Temp. Max. (°C) – Average maximum temperature in °C
<sup>c</sup>Temp. Min. (°C) – Average minimum temperature in °C
<sup>d</sup>GDD<sub>10</sub> – Growing degree days in degree Celsius = [(minimum temperature + maximum temperature) \times 2<sup>-1</sup>] - 10 °C
  
  If maximum temperature is > 30 °C, then maximum temperature = 30 °C
  
  If minimum temperature is < 10 °C, then minimum temperature = 10 °C
Table 2. Average percentage of yellow-RR grain in the samples collected at increasing distances from the yellow-seeded-RR pollen source following eight cardinal directions.

<table>
<thead>
<tr>
<th>Distance (m) from the source</th>
<th>2003</th>
<th>2004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outcross (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>29.850</td>
<td>17.011</td>
</tr>
<tr>
<td>10</td>
<td>2.531</td>
<td>1.507</td>
</tr>
<tr>
<td>35</td>
<td>0.425</td>
<td>0.382</td>
</tr>
<tr>
<td>100</td>
<td>0.033</td>
<td>0.046</td>
</tr>
<tr>
<td>150</td>
<td>0.014</td>
<td>0.028</td>
</tr>
<tr>
<td>200</td>
<td>0.007</td>
<td>0.026</td>
</tr>
<tr>
<td>250</td>
<td>0.002</td>
<td>0.027</td>
</tr>
</tbody>
</table>
**Table 3.** Average percentage of yellow-RR grain of the production field in 2003. Samples were collected in each of the eight transects at increasing distances from the yellow-seeded-RR pollen source following eight cardinal directions.

<table>
<thead>
<tr>
<th>Distance</th>
<th>NW</th>
<th>N</th>
<th>NE</th>
<th>E</th>
<th>SE</th>
<th>S</th>
<th>SW</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.674</td>
<td>26.079</td>
<td>20.881</td>
<td>25.706</td>
<td>47.055</td>
<td>43.479</td>
<td>45.107</td>
<td>9.820</td>
</tr>
<tr>
<td>10</td>
<td>3.015</td>
<td>1.782</td>
<td>2.351</td>
<td>3.079</td>
<td>3.857</td>
<td>3.142</td>
<td>2.869</td>
<td>0.151</td>
</tr>
<tr>
<td>35</td>
<td>0.221</td>
<td>0.194</td>
<td>0.166</td>
<td>0.854</td>
<td>0.936</td>
<td>0.431</td>
<td>0.598</td>
<td>0.000</td>
</tr>
<tr>
<td>100</td>
<td>0.003</td>
<td>0.050</td>
<td>0.051</td>
<td>0.011</td>
<td>0.046</td>
<td>0.057</td>
<td>0.041</td>
<td>0.005</td>
</tr>
<tr>
<td>150</td>
<td>0.001</td>
<td>0.016</td>
<td>0.027</td>
<td>0.002</td>
<td>0.025</td>
<td>0.034</td>
<td>0.004</td>
<td>0.000</td>
</tr>
<tr>
<td>200</td>
<td>0.004</td>
<td>0.005</td>
<td>†</td>
<td>0.006</td>
<td>0.014</td>
<td>0.012</td>
<td>0.011</td>
<td>0.000</td>
</tr>
<tr>
<td>250</td>
<td>0.000</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>0.003</td>
<td>†</td>
<td>0.002</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**: Data is the average of two samples.
†: Sampling location not available because of the shape of the field.
**Table 4.** Average percentage of yellow-RR grain of the production field in 2004. Samples were collected in each of the eight transects at increasing distances from the yellow-seeded-RR pollen source following eight cardinal directions.

<table>
<thead>
<tr>
<th>Transect</th>
<th>NW</th>
<th>N</th>
<th>NE</th>
<th>E</th>
<th>SE</th>
<th>S</th>
<th>SW</th>
<th>W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.108</td>
<td>3.951</td>
<td>2.907</td>
<td>1.798</td>
<td>4.429</td>
<td>2.122</td>
<td>1.242</td>
<td>1.035</td>
</tr>
<tr>
<td>35</td>
<td>0.482</td>
<td>0.511</td>
<td>0.503</td>
<td>0.272</td>
<td>0.652</td>
<td>0.442</td>
<td>0.423</td>
<td>0.147</td>
</tr>
<tr>
<td>100(^\ddagger)</td>
<td>0.054</td>
<td>0.060</td>
<td>0.050</td>
<td>0.007</td>
<td>0.090</td>
<td>0.028</td>
<td>0.046</td>
<td>0.026</td>
</tr>
<tr>
<td>150(^\ddagger)</td>
<td>0.040</td>
<td>0.030</td>
<td>0.055</td>
<td>0.061</td>
<td>0.007</td>
<td>0.018</td>
<td>0.017</td>
<td>0.014</td>
</tr>
<tr>
<td>200(^\ddagger)</td>
<td>0.010</td>
<td>0.014</td>
<td>0.035</td>
<td>0.000</td>
<td>0.012</td>
<td>0.013</td>
<td>0.009</td>
<td>0.120</td>
</tr>
<tr>
<td>250(^\ddagger)</td>
<td>0.081</td>
<td>0.006</td>
<td>†</td>
<td>†</td>
<td>0.000</td>
<td>0.015</td>
<td>0.035</td>
<td>†</td>
</tr>
</tbody>
</table>

\(^\ddagger\) Data is the average of two samples.

† Sampling location not available because of the shape of the field.
Table 5. Parameter estimates and their corresponding standard errors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>$\beta_0$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2003</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>estimates</td>
<td>8.97</td>
<td>-0.67</td>
<td>62.44</td>
</tr>
<tr>
<td>std. errors</td>
<td>(0.012)</td>
<td>(0.012)</td>
<td>(1.421)</td>
</tr>
<tr>
<td><strong>2004</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>estimates</td>
<td>8.45</td>
<td>-0.57</td>
<td>96.95</td>
</tr>
<tr>
<td>std. errors</td>
<td>(0.013)</td>
<td>(0.012)</td>
<td>(1.936)</td>
</tr>
</tbody>
</table>
Figure 1. Sampling locations at increasing distances away from the source field. Samples were collected at 1, 10, 35, 100, 150, 200, and 250 following the N, E, S, W, NE, SE, SW, and NW cardinal points. Additional samples were taken at 100 m, 150 m, 200 m, and 250 m to minimize separation among sapling locations at the farthest distances from the source field.
Figure 2. Flowering synchrony - pollen shed vs. silk exertion in percentage. A: Bt/RR yellow source field pollen shedding and the white receiver field silk exertion for 2003; B: Bt/RR yellow source field pollen shedding and the white receiver field silk exertion for 2004.
Figure 3. Wind rose for Ankeny, Iowa from 19 July to 3 August 2003. Wind speed is in m/s$^{-1}$ and concentric circles represent the percentage of the day that the wind came from that direction and that intensity. Data was collected on-site using a R. M. Young 3001 Wind Sentry Set in a portable weather station.
Figure 4. Wind rose for Ankeny, Iowa from 17 to 29 July 2004. Wind speed is in ms\(^{-1}\) and concentric circles represent the percentage of the day that the wind came from that direction and that intensity. Data was collected on-site using a R. M. Young 3001 Wind Sentry Set in a portable weather station.
Figure 5. Graphical representations of the hourly wind direction and intensity for Ankeny, Iowa, during the dates of peak flowering (28 July to 1 August 2003).
Figure 6. Graphical representation of the hourly wind direction and intensity for Ankeny, Iowa, during the dates of peak flowering (17 to 22 July 2004).
Figure 7. Graphic representation of the model-fitted values of outcross for a grain production field superimposed on a scatterplot of the number of observed adventitious RR yellow seeds by distance from the source field in 2003 and 2004.
Figure 8. Graphic representation of the Model-fitted values of outcross for a grain production field superimposed on a scatterplot of the number of observed adventitious RR yellow seeds at increasing distance from the source field for samples collected at 70 of beyond in 2003 and 2004.
Figure 9. Observed and fitted RR yellow seed values for locations 70 m and beyond from the source field in 2003.
Figure 10. Number of RR yellow seeds for locations 100 m and beyond from the source field in 2004
 GENERAL CONCLUSIONS

Important crops have been improved using genetic engineering techniques. Novel genes have been artificially inserted into the plant genome. However, in open-pollinated crops, pollen flow and the resulting outcross is undesirable when growing varieties for specialty markets, organic products, varieties with valued-added traits, and seed. Movement of transgenes through pollen flow and their detection in non-transgenic plants have gained importance. This research presents a protocol to extract corn pollen DNA and to detect the presence of the transformation-specific sequence of the CaV-35S promoter in Bt maize varieties. This protocol for pollen DNA extraction was used in transgenic and non-transgenic corn grown in the field or the greenhouse. The efficiency of the method was corroborated by Polymerase Chain Reaction (PCR) using primers for the identification of corn-specific sequences and the CaV-35S-promoter sequences. The newly designed primers 35S168F/35S317R were the most effective among the 11 primer pairs tested. The amplification efficiency was determined by the intensity of the bands in gel electrophoresis.

Two sets of experiments are conducted to determine the outcross in maize fields of different local pollen density. Results were recorded at increasing distances and cardinal directions away from the pollen source. In Chapter 3, the outcross levels of two fields with different local pollen densities were considered, low density in a seed field vs. high pollen density in a grain production field in 2003. In Chapter 4, the outcross levels of two grain production fields were evaluated when planted in two growing environments and having similar pollen density. There were significant differences in outcross percentage among distances and cardinal direction at 1, 10 and 35 m from the pollen source; but at 100, 150,
200 and 250 m the outcross level was non-significant regardless of distance and cardinal direction. Wind speed and direction affected the level of outcross among cardinal directions. There were significant differences between fields with different pollen density and between years in fields with similar local pollen density. However, differences between years could be attributed to anomalies in the data set related to experimental variations.

According to the outcross results, zero level of outcross is not achievable at distances of 250 m or closer. Further research should be conducted to explain outcross expectations at greater distances from the source.

This research represents the first large-field study of adventitious presence of transgenic corn in non-transgenic varieties. The gene flow and the resulting outcross in all cardinal directions from the transgenic source was quantified at distances up to 250 m, farther than the seed production standard of 200 m to ensure 99% of seed genetic purity. The important contribution of this research is that outcross in corn was quantified under conditions not considered in other studies, i.e., larger and continuous pollen source field (1 ha), larger and continuous receiver non-transgenic field (36 ha), different local pollen density, different environment, all cardinal directions, and distances farther than the recommended standard. It provided a better understanding of how transgenes from GM corn move into adjacent corn fields. This is very important in organic production, valued-added trait varieties, and corn for food production at commercial scale, where transgenic and conventional varieties are grown in close proximity.