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ScienceDirect
Field Crops Research 99 (2006) 147–157
Field Crops Research
www.elsevier.com/locate/fcr

Statistical analysis of outcrossing between adjacent maize grain production fields

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Received 15 December 2005; received in revised form 11 April 2006; accepted 15 April 2006

Abstract

The presence of transgenes in conventional maize, *Zea mays* L., crops is a serious concern when the genetic purity affects the value of the harvested product (i.e., specialty markets, organic products, crops with value-added traits, and hybrid seed production). Gene flow from a central transgenic source plot into a conventional grain production field was quantified using a combination of three marker genes: the *y1* seed color gene, the *Bt-Cry1Ab* gene derived from the soil bacterium *Bacillus thuringiensis* (*Bt*) and the Roundup Ready® (*RR*) gene. Two fields of approximately 36 ha were planted with a nontransgenic, white-seeded corn hybrid in Ankeny, IA, in 2003 and 2004. In the center of each field, a 1 ha plot of yellow-seeded, *Bt/RR* hybrid corn was planted as an adventitious pollen source. Detailed measurements of flowering dynamics confirmed the white- and yellow-seeded hybrids flowered synchronously both years. Grain samples were collected at 1, 10, 35, 100, 150, 200, and 250 m from the transgenic pollen source along eight transects (north [N], northeast [NE], east [E], southeast [SE], south [S], southwest [SW], west [W], and northwest [NW]) and were analyzed for number of *Bt-/RR-/y1-* kernels. The statistical model describes the proportion of outcrossed kernels to decrease exponentially with distance from the yellow pollen source and linearly with the wind speed and direction during silking of the white hybrid. On average, outcrossing at 35 m was 0.4% in both years. At 100 m and beyond, the average level of outcrossing decreased to 0.05% or less. A few *Bt-/RR-/y1-* kernels, however, were detected in the white corn field even at 250 m from the source plot. A single empirical model captured the field-scale patterns of outcrossing from the source plot for both years. These results indicate gene flow from a transgenic pollen source follows a fairly predictable pattern. The results also suggest that extent of outcrossing can be reduced by surrounding the transgenic pollen source with nontransgenic corn producing a high density of local pollen.

Keywords: Maize; Pollen dispersal; Outcross; Statistical models

1. Introduction

Gene flow resulting from cross-pollination is an integral aspect of hybrid seed production and has been fundamental to maize, *Zea mays* L., evolution. Windborne dispersal of pollen grains from one maize field to another, however, has gained renewed interest because transgenic hybrids containing DNA from other species are being planted to large areas in proximity to nontransgenic maize. In 2000, more than 10 million ha of transgenic maize was grown in the United States (James, 2002). The proximity of transgenic and nontransgenic plantings has raised public concerns about food safety, unintended outcrossing with organic (transgene-free) and other nontransgenic maize, and potential increases in pest resistance. Despite the potential for transfer of transgenes to nontransgenic maize (Castillo and Goodman, 1997; Ma et al., 2004; Baltazar et al., 2005), few studies have considered

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doi:10.1016/j.fcr.2006.04.005

management alternatives to minimize its occurrence (Burris, 2001; Stevens et al., 2004; Ireland et al., in press).

The presence of transgenes in conventional maize crops is a serious concern when the genetic purity of the grain affects the value of the harvested product. This concern in the production of genetically pure grains includes producing grain for specialty markets, organic products, crops with value-added traits, and hybrid seed production. Maize exports are negatively impacted by outcrossing between transgenic and nontransgenic maize because several countries have imposed strict standards on the presence of transgenes. For example, the European Union (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; this threshold is 0% transgenes for certified organic products (Messeguer, 2003). These purity standards for export are difficult to achieve for commodity grain produced in the continental United States and nearly impossible to achieve for organic markets, in part because nontransgenic and transgenic hybrids often are produced in adjacent fields.

Limiting the dispersal of pollen is an important factor in preventing cross-pollination between transgenic and nontransgenic maize crops. In most previous studies, the consequences of pollen flow have been quantified indirectly from outcrossing percentages in receiver plots stationed at various distances from the pollen source. Several of these studies used seed color to establish the potential for outcrossing (Paterniani and Stort, 1974; Garcia et al., 1998; Jemison and Vayda, 2001; Ma et al., 2004; Stevens et al., 2004). To our knowledge, only one study (Ma et al., 2004) used transgenic yellow corn to identify the potential for outcrossing because of pollen flow from a source plot. There has been little research to quantify the risk of adventitious presence of transgene flow into nontransgenic maize (Wilkinson et al., 2003), particularly on a large field scale (Chilcutt and Tabashnik, 2004). Unfortunately, the utility of these studies is limited by their small field size, lack of detailed information about flowering dynamics, and the site-specific nature of the results.

The objective of this study was to determine the potential for gene flow from a transgenic field when it is surrounded by a conventional (nontransgenic) grain production field. The level of outcrossing was determined using a combination of genetic markers for seed color, insect resistance, and glyphosate resistance. The occurrence of yellow endosperm [controlled by a single dominant gene (*y1*) (Weber, 1994)] in the surrounding white maize field was taken as the initial indication of an outcross event. Because uncontrolled factors, such as adventitious pollen from surrounding fields, can produce spurious yellow seeds, we also tested for the presence of *RR* and *Bt* genes in the yellow seeds to confirm outcross events caused by pollen from the central source plot. Site-specific environmental effects on outcrossing were assessed by modeling the pattern of outcrossing at different locations and years.

2. Materials and Methods

2.1. Experimental fields

A nontransgenic white corn hybrid, RX792W, was planted to approximately 36 ha on 20 May 2003 and 4 May 2004 at the ISU Experiment Farm in Ankeny, IA. The population density was 69,187 plants/ha in 2003 and 71,630 plants/ha in 2004. A 1 ha plot of DKC69-71 yellow corn was planted in the center of these fields on 21 May 2003 and 5 May 2004 as a transgenic pollen source. The yellow hybrid was planted at a density of 84,013 and 86,450 plants/ha in 2003 and 2004, respectively. These population densities are typical for maize grain production fields in Iowa (Duvick, 1997). DKC69-71 is a stacked transgenic hybrid carrying the *Bt-Cry1Ab* gene (derived from the soil bacterium *Bacillus thuringiensis*) and *EPSPS* gene conferring resistance to glyphosate herbicide, hereafter referred to as the Roundup Ready® (*RR*) gene. The area planted to DKC69-71 was sprayed with Roundup® after emergence to remove nontransgenic plants. Fields were managed under normal production practices of cultivation and insect and soil fertility management. Soils were silty clay loam and loam of the series Nicollet, Webster and Clarion (USDA–NRCS, 2000).

2.2. Flowering synchrony and weather data

The synchrony between silk exertion and pollen shed was determined by counting plants with silks exposed and tassels shedding pollen from 21 July 2003 to 14 August 2003 and from 13 to 23 July 2004. Approximately 400 plants within the transgenic hybrid plot and 2,200 plants along the N, E, S, and W transects of the nontransgenic hybrid field were monitored for floral development.

During pollination, a portable weather station was placed in each field adjacent to the transgenic pollen source plot. Wind speed and direction were measured at 3.17 m from the ground by an R.M. Young 30001 Wind Sentry Wind Set (Campbell Scientific, Logan, UT) with an accuracy of $\pm$ 0.5 m s^{-1}. The wind data were recorded through the pollen shed period as 15-min average values by using a Campbell Scientific model CR10 data logger (Campbell Scientific, Logan, UT).

2.3. Grain samples

Ears were harvested at locations along the eight transects within the white corn field (N, NE, E, SE, S, SW, W, and NW) when grain moisture was approximately 120 g kg^{-1}. Sampling locations were recorded using a global positioning system. Twenty five ears (approximately 17,000 seeds) were collected at distances of 1, 10, and 35 m from the center transgenic plot; 100 ears (approximately 70,000 seeds) were collected at each sampling point at 100, 150, 200, and 250 m. These samples sizes were varied to ensure we could

detect a minimum of 0.005% outcrossing at $p = 0.05$ (Remund et al., 2001) at each sample location. Ears were shelled in a model LS91 seed sheller (Custom Seed Equipment, Altoona, IA), and seed were sorted by color in a 20-channel ESM ScanMaster, model SM-200DE (SATAKE, Stafford, TX). Sorted seed samples were inspected by hand to ensure all yellow seed were identified. After sorting, the number of seeds in 454 grams was counted using a seed counter model EB00-D (FMC Syntron®, Homer City, PA). The total number of yellow or white seeds in a sample was estimated by multiplying the number of seed per 454 grams by the total weight of the sample. When the total weight of the yellow seeds in a sample was less than 454 g, all seeds were counted by hand.

2.4. RoundUp Ready® gene determination

The yellow seed in each sample were screened for the presence of the *RR* gene by using the preemergence method (Goggi and Stahr, 1997; AOSA, 2003). Groups of 200 seeds were imbibed for 48 h between paper towels moistened with a 3% a.i. (30 g kg^{-1}) solution of glyphosate (Roundup® Ultra). The herbicide-imbibed seeds were germinated on moistened crepe cellulose paper (Kimpak™) on top of plastic trays at 25 °C for 7 days. Seedlings were classified as normal tolerant, normal nontolerant, abnormal tolerant, abnormal nontolerant, and dead following AOSA (2003) classifications. Additionally, 200 seeds from the white-seeded, nontransgenic grain, and 200 seeds from the samples collected at the central plot were tested to assess the potential for segregation and linkage between the yellow and *RR* gene. The proportion of seeds with the *RR* gene was obtained as $P_{RR} = 2\ (N_n + N_a)/N$, where N_n is the number of normal tolerant seedlings, N_a is the number of abnormal tolerant (if any) seedlings, and N is the sum of the number of yellow and white seed in the sample. The factor of 2 is used to calculate the total possible number of outcrosses because the RR trait segregates as a hemizygote. Consequently, only one-half of the gametes (pollen grains) produced by the source plot are expected to carry the *RR* gene.

2.5. Bt-Cry1Ab gene expression

Seed samples also were analyzed for the presence of the Bt-Cry1Ab (Bt) protein. An enzyme-linked immunosorbent assay (ELISA) Bt kit (Agdia Inc., Elkhart, IN) was used to detect the presence of the Bt protein. The test is a double-antibody sandwich (DAS)-ELISA, which detects the protein using a polyclonal antibody. Test protocols followed manufacturer's recommendations. In seed samples collected at 1 and 35 m from the transgenic source, 360 seed (180 RR and 180 non-RR seedling) were examined. For samples collected more than 35 m from the source, all RR-seedlings and 180 seedlings of the RR test susceptible seedlings were tested. If fewer than 180 non-RR yellow seedlings were found in the sample, all seedlings were tested.

The Bt protein was extracted from a small piece of leaf tissue or the tip of the coleoptile taken from each germinating seedling in 300 μL of phospate-buffered saline/Tween 20 (PBST) (ICI Americas, Inc., Wilmington, DE) extraction buffer followed by sonication in a model FS30 sonicator (Fisher Scientific, Hanover Park, IL) for 30 min. A 100-μL aliquot of the extract was transferred into an antibody-coated well of a DAS-ELISA 96-well microplate. The microplates were incubated for at least 2 h at room temperature (23 ± 1 °C) or overnight in the refrigerator at 4 °C at high relative humidity. After incubation, microplates were rinsed with PBST buffer to remove unbound enzyme conjugate, and a TMB peroxidase substrate solution was added to each test well. The reaction with the substrate produces a blue color within 5–15 min, signifying the presence of Bt protein. The amount of protein was quantified by optical density with a plate reader model ELx800 (Bio-Tek Instruments,Winooski, VT) at 650 nm. Samples with an optical density reading greater than the average of the four negative control wells were considered positive for the presence of the Bt protein.

The proportion of seedling expressing the Bt protein was calculated as $P_{Bt} = 2N_{Bt}/N$, where N_{Bt} is the number of seedlings with Bt protein, and N is the total number of seedlings tested. Because the expression of the *Bt* gene is hemizygous, only one-half of the pollen grains will have the gene.

2.6. Data analysis

Analysis of the outcrossing data was carried out using the statistical software R (R Project for Statistical Computing, 2004). The goals were to develop a model to describe the pattern of outcross detected at various distances from the transgenic source plot and to determine whether the patterns of outcrossing were consistent across years. The scope of the statistical analysis was to model in parallel the outcross data collected for similar grain production fields evaluated in 2003 and 2004 at the same relative field locations. The model estimates the percentage of outcrossing, defined as the percentage of transgenic yellow seeds in a given sample collected at each field location, as a function of distance from the source plot, wind speed, and direction during pollen shed. Estimation of model parameters 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). The difference between theoretical and observed *RR* and *Bt* gene frequencies was calculated using Pearson's Chi square test.

3. Results and discussion

3.1. Flowering synchrony and resulting outcrossing

The progress of pollen shed and silking for the white and yellow hybrids is presented in Fig. 1. In 2003, the RR/Bt

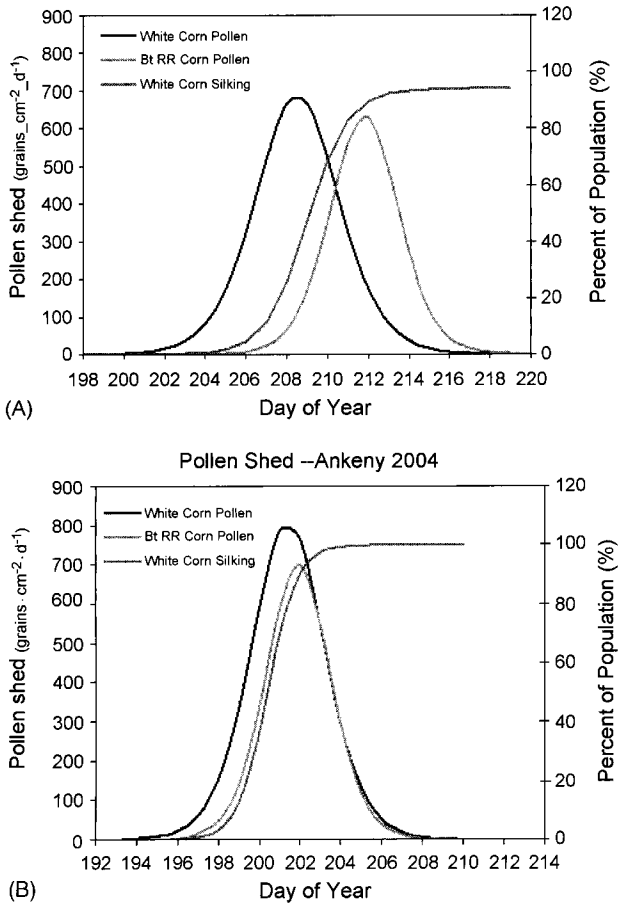

Fig. 1. Flowering synchrony—silk exertion of the white-seeded RX792W in percentage and pollen production of both hybrids in grains cm^{-2} d^{-1}. **A**: 2003; **B**: 2004.

yellow corn source plot reached anthesis (50% of plants shedding pollen) on day of the year (DOY) 210 (29 July); pollen shed peaked 2 days later on DOY 212. The surrounding white corn field reached anthesis on DOY 206 (25 July), and 50% silk exertion on DOY 209 (28 July). In 2004, both the yellow and white hybrids reached anthesis on DOY 200 (18 July) and peak pollen shed occurred on DOY 202 (20 July). Thus, in both years pollen shed within the transgenic source plot peaked at the same time the surrounding nontransgenic field was in the midst of silk exsertion. This synchrony between pollen release from a transgenic hybrid and appearance of receptive silks on a nontransgenic hybrid nearby represents worst-case conditions for unintended gene flow in corn.

Table 1 shows precipitation, maximum and minimum temperatures, and growing degree-days (GDD) data for 2003 and 2004. There was a period of drought in August 2003 that coincided with seed development and maturation. Seed size and weight were affected by the drought but not seed number (data not shown). Weather conditions in 2004 were normal for Iowa in the summer compared with historic climatological weather data.

Fig. 2A and B show the wind rose summaries for Ankeny recorded for DOY 200-215 (19 July–3 August) 2003, and for DOY 199-211 (17–29 July) 2004, coincident with peak pollen shed. In 2003, winds during pollen shed and silk exertion were most commonly from the N to NW quadrant with a secondary maximum from the S quadrant. This weather pattern was an unusual wind pattern compared with climatological average summer winds in central Iowa, which are from the S quadrant. In 2004, a bimodal NNE-SSW wind pattern was observed. The maximum 15-min average wind speed in 2003 was 4.7 m s^{-1}, recorded on DOY 207 (26 July) at 1115 h; in 2004 peak wind was 2.6 m s^{-1} recorded on DOY 210 (28 July) at 1100 h. On both occasions, the winds were from the south-southwest.

All seeds harvested in the source plot were yellow, indicating there was no segregation for seed color, and thus both alleles were dominant yellow. Segregation of the *RR* and *Bt* genes also was determined in seed samples harvested within the yellow source plot. The *RR* gene segregated with a

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

Parameter	Mo						Total
	May	June	July	Aug.	Sept.	Oct.	
2003							
Precipitation (mm)[a]	95.3	60.1	89.0	21.8	87.4	20.6	374.2
Temp. max. (°C)[b]	20.4	24.8	27.9	28.8	22.1	18.7	
Temp. min. (°C)[c]	8.8	13.6	16.9	16.9	8.6	4.4	
GDD_{10}[d]	171	295	387	399	215	143	
2004							
Precipitation (mm)[a]	180.8	80.2	42.9	115.3	29.7	36.3	485.2
Temp. max. (°C)[b]	21.9	23.9	26.2	24.1	24.8	16.5	
Temp. min. (°C)[c]	10.3	13.2	16.0	13.1	11.7	5.3	
GDD_{10}[d]	220	283	344	274	276	105	

[a] Precip. (mm)—precipitation in millimeters.
[b] Temp. max. (°C)—average maximum temperature in °C.
[c] Temp. min. (°C)—average minimum temperature in °C.
[d] GDD_{10}—growing degree days in degree Celsius = [(minimum temperature + maximum temperature)/2] − 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.

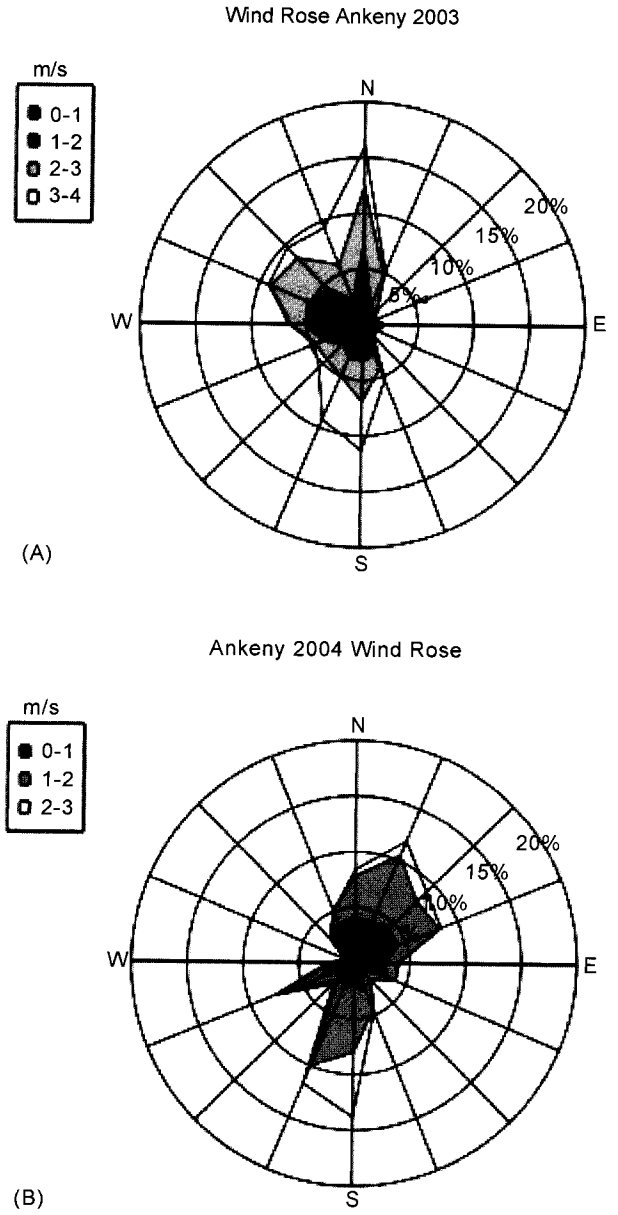

Fig. 2. Wind rose for Ankeny, IA. A: 2003; B: 2004. Wind speed is in meters per second, and concentric circles represent the percentage of the day that the wind came from that direction and that intensity.

frequency of 70% *RR*:30% non-*RR* in 2003 and 73% *RR*:27% non-*RR* in 2004, as determined by the *RR* biological test. These values were not significantly different ($P = 0.64$) from the expected ratio of 75% *RR*:25% non-*RR* (Chilcutt and Tabashnik, 2004).

The *Bt* gene segregated with a frequency of 77% *Bt*:23% non-*Bt* in 2003 and 88% *Bt*:12% non-*Bt* in 2004 as determined by the ELISA test. The observed frequencies were not significantly different from the theoretical 75% *Bt*:25% non-*Bt*. in 2003 ($p = 0.64$), but they were significantly different in 2004 ($P = 0.003$). *Bt* gene segregation followed the theoretical frequency at proximity to the central source plot but not at 100–250 m in 2003 and 2004. The anomalous results at the farthest distances from the source led us to suspect contamination by a Bt pollen source from surrounding fields. Although fields in proximity to the experimental site were reported to be non-Bt hybrids, low levels of Bt protein were found in leaf samples collected from these fields (data not shown). Thus, results presented for gene flow are based on the presence of two genes, yellow seed color and RR, unless otherwise stated. We also analyzed the data by using only the number of yellow seed in the sample, which did not alter the general conclusions drawn from the data.

As expected, wind direction affected the percentage of outcross downwind (Ma et al., 2004; Stevens et al., 2004). The percentage of outcross was greatest near to the source plot and in the downwind direction from it. At 1 m from the source, the average percentages 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 47.0% outcross at the SE, 43.5% at the S, and at the 45.1% SW sampling sites (Table 3). In 2004, the highest percentages of outcross were also at the sampling locations towards the south; outcross levels were 21.2% in the SE, 30.6% in the S, and 15.4% in the SW (Table 3). These results coincide with the predominant wind directions 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 (30–31 July 2003 and 19–21 July 2004; Figs. 1 and 3). These results emphasize the importance of wind direction and intensity during the peaks of flowering synchrony as primary determinants of outcrossing patterns (Jones and Newell, 1946; Jones and Brooks, 1950; Burris, 2001; Ireland et al., in press). The average percentage of outcross at 35 m was 0.4% in 2003 and 2004 (Table 2). These results are in agreement with Ma et al. (2004) who reported very low levels of outcross at 37 m from the pollen source. The values of outcross at 35 m towards the SE were 0.9 and 0.7% in 2003 and 2004, but they were lower in all other sampling directions (Table 3). These levels of outcross at a distance of 35 m from the pollen source are also in agreement with those reported from other studies (Foueillassar and Benetrix, 2003; Jarosz et al., 2003; Ma et al., 2004; Stevens et al., 2004). At 100 m from the source plot and beyond, the levels

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

Distance from yellow pollen source (m)	Outcrossing (%)	
	2003	2004
1	29.9	17.0
10	2.5	1.5
35	0.4	0.4
100	0.03	0.05
150	0.01	0.03
200	0.007	0.03
250	0.002	0.03

Table 3
Average percentage of yellow, RR grain in the adjacent production field in 2003 and 2004

Year	Distance (m)	Transect							
		NW	N	NE	E	SE	S	SW	W
		Outcrossing (%)							
2003	1	20.7	26.1	20.9	25.7	47.1	43.5	45.1	9.8
	10	3.0	1.8	2.4	3.1	3.9	3.1	2.9	0.2
	35	0.2	0.2	0.2	0.9	0.9	0.4	0.6	0.0
	100	0.003	0.05	0.05	0.01	0.05	0.06	0.04	0.005
	150	0.001	0.02	0.03	0.002	0.03	0.04	0.004	0.0
	200	0.004	0.005	–	0.006	0.01	0.01	0.011	0.0
	250	0.000	–[a]	–	–	0.003	–	0.002	0.003
2004	1	8.9	9.9	19.4	18.4	21.2	30.6	15.4	12.3
	10	2.1	4.0	2.9	1.8	4.4	2.1	1.2	1.0
	35	0.5	0.5	0.5	0.3	0.7	0.4	0.4	0.2
	100	0.05	0.06	0.05	0.01	0.09	0.03	0.05	0.03
	150	0.04	0.03	0.06	0.06	0.01	0.02	0.02	0.01
	200	0.01	0.01	0.04	0.00	0.01	0.01	0.01	0.12
	250	0.08	0.01	–	–	0.00	0.02	0.04	–

Samples were collected in each of the eight transects at increasing distances from the yellow-seeded, RR pollen source following eight cardinal directions.
[a] –, no data collected.

of outcross decreased to less than 0.1%. The outcross at 200 m W was 0.12%. This slightly higher level of outcross was probably the result of local outcross with a few yellow seeded corn volunteer plants observed in the proximity of this location and recorded during field sampling. These outliers were eliminated from the data set. The average percentage of outcross never reached 0% within the 250 m where samples were collected. Only a few samples collected at 250 m from the source plot (NW and SW in 2003; SE and E in 2004) had 0% outcrossing (Table 3).

Although very low levels of outcrossing were observed at distances greater than 100 m from the source plot, a few yellow kernels (out of the 70,000 + kernels sampled) were detected in nearly all cases. Limiting outcrossing by the Bt/RR corn to less than 0.1% was readily accomplished by isolating the source plot by 100 m of non-Bt/RR corn, regardless of the relative direction from the Bt/RR pollen source. It was not possible, however, to ensure 0% outcrossing by isolating the source plot even with 250 m of intervening nontransgenic plants. This result is especially important for organic growers desiring to produce 100% pure nontransgenic seed in areas of transgenic corn production such as the Midwest.

3.2. Statistical analysis of outcross data in 2003 and 2004

It was evident from the flowering data (Fig. 1), wind data (Figs. 2 and 3), and weather patterns (Table 1) that year-to-year variation in flowering dynamics could impact the patterns of pollen dispersal and the resulting extent of outcrossing observed. Therefore, we analyzed the outcrossing patterns in the two fields to determine year effects on outcrossing levels. The scope of the statistical analysis was to model in parallel the outcross data collected for similar grain production fields evaluated in 2003 and 2004 at the same relative field locations, as a function of distance from the source plot, wind speed, and direction during pollen shed. This approach assumes observed outcrossing is a fair representation of actual pattern of pollen dispersal (Lisazo et al., 2003; Westgate et al., 2003).

A potential complication in modeling the response variable (proportion of yellow kernels) was the possibility for cross-pollination of the white hybrid by yellow corn pollen originating from the surrounding fields. A spot check of corn seedlings from the surrounding fields showed low levels of Bt protein, but it did not show the *RR* gene. Thus, we used resistance to glyphosate herbicide to segregate yellow seeds produced by pollen from neighboring fields from those yellow seed resulting from fertilization by pollen dispersed from the central Bt/RR source plot. Segregating seeds in this way did not lead to any loss of information in the statistical analysis, and the parameter estimates retained their desirable statistical properties (unbiased, minimum variance).

Because identifying outcrosses from other sources was of particular importance to our model, we estimated the number of yellow RR seeds ($\hat{Y}$) originating from the source plot at each field location i as

$$\hat{Y} = R_i p^{-1}$$

where R_i is the number of RR seeds at location i, and p is the probability that a seed originating from the source plot is RR. Because p is not known exactly, we estimate it based on the assumption that yellow seeds collected at locations near the source plot (no farther than 35 m) originated from the RR source plot. Thus, the unbiased estimator of p is the proportion of resistant yellow seeds collected at locations 1–35 m from the source plot.

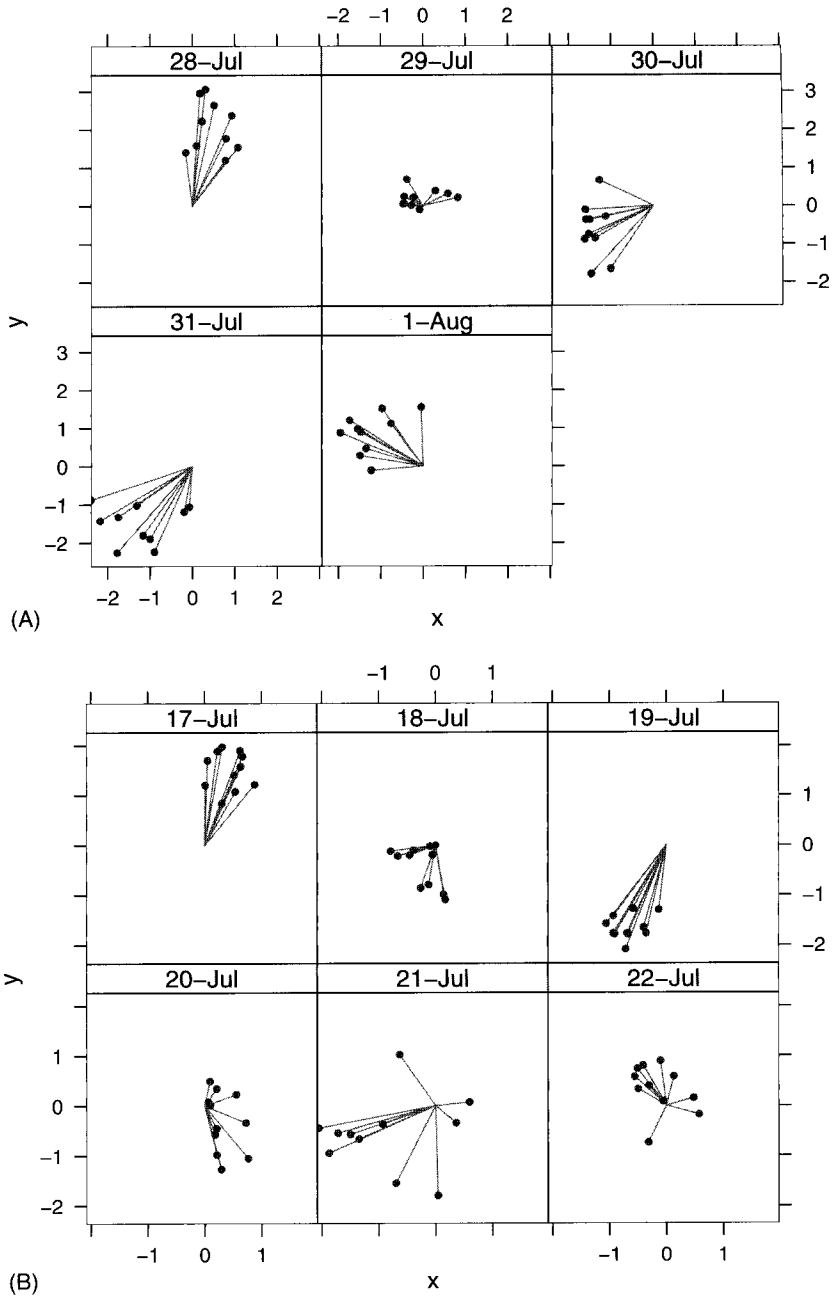

Fig. 3. Wind speed and direction for Ankeny, IA. Data correspond to peak pollen shedding for the yellow RR/Bt center source plot. A: from 28 July to 1 Aug. 2003. B: from 17 to 22 July 2004. Direction of the hourly wind is from the solid circles to the center of the graph, and the hourly wind speed (in meters per second) is represented by the distance between the solid circles and the center of the graph.

Initial data analysis indicated that the estimated yellow counts $\hat{Y}_i$ were distributed as Poisson random variables (Neter et al., 1996) with mean μ_i:

$$Y \sim \text{Poisson}(\mu_i)$$

As such, we reparametrized their means μ_i as functions of the percentage of outcross, denoted here by λ_i (i.e. $\mu_i = \lambda_i t_i$). After careful consideration of several statistical models incorporating distance from the source plot and several covariates based on wind direction and intensity, the model that best fit the data based on Akaike's Information criteria (AIC) (Neter et al., 1996) was given by

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

where d_i denotes the distance from the source plot and W_i is a constructed covariate that incorporates wind speed, wind direction, and proximity to the source plot. Specifically,

$$W_i = \sum_{h=1}^{H} \frac{Zih}{di} I\,(i \in T(\text{direction } h))$$

where Z_{ih} denotes the hourly wind speed at location i, for hour h, with H being the total number of hours for which the wind speed and direction were recorded during the peak shed period. **I** is the indicator function that determines the locations that are included in the covariate W_i for a given wind speed and direction. Specifically, **I** = 1 if a location lies in the tolerance region $T(\text{direction}_h)$, and **I** = 0 if the location is not in the tolerance region. T (direction$_h$) denotes the tolerance region for wind direction recorded during the h-th hour. For a tolerance region having a width of zero, only locations directly downwind from the source are included in W_i; the wider the tolerance region, the more locations are included in W_i. The tolerance region was constructed based on standard dispersal statistics for moderately unstable stratification (USEPA, 1995).

The model parameters β_0, β_1, and β_2 were estimated by maximizing the log-likelihood function, by using the Newton-Raphson algorithm as the numerical optimization procedure. The resulting values for the two data sets are presented in Table 4. The estimated values for β_1 are negative, which implies that the amount of outcross decreases exponentially away from the source plot, whereas the estimated values for β_2 are positive, indicating the quantity of pollen increases as the wind speed increases in a given direction. Although the estimates ($\hat{\beta}_0, \hat{\beta}_1$, and $\hat{\beta}_2$) of the model parameters (β_0, β_1, and β_2) for 2003 and 2004 were not identical, the model indicates that outcrossing in each year can be attributed to similar fundamental processes. Fig. 4 presents the proportion of RR/Bt yellow seeds for each of the 2 year plotted against the distance from the source plot as well as the fitted models (indicated by the lines drawn on the scatterplot). The model captured the overall trend of outcrossing with distance from the source plot, suggesting that the model accounts for the role of pollen dispersion in producing outcrosses. A number of pollen flow models provide mathematical descriptions of observed outcrossing data with distance (Jarosz et al., 2003; Ma et al., 2004; Kawashima et al., 2004; Yamamura, 2004). Our model, however, also has prediction capabilities because it incorporates wind speed and direction as well as distance from the pollen source.

Table 4
Parameter estimates and their corresponding standard errors for the model fitted to data collected at all locations (1–250 m) and only at locations situated father than 70 m away from the source plot (70–250 m)

Distance from the source plot	Year	Parameter		
		β_0	β_1	β_2
1–250 m	2003			
	Estimates	−0.76	−0.54	0.003
	SE	(0.010)	(0.010)	(0.000007)
	2004			
	Estimates	−1.39	−0.44	0.003
	SE	(0.010)	(0.007)	(0.000008)
70–250 m	2003			
	Estimates	5.90	−0.03	0.0009
	SE	(0.467)	(0.005)	(0.00009)
	2004			
	Estimates	7.56	−0.0009	0.0006
	SE	(0.179)	(0.001)	(0.0001)

Outlying observations from the data collected in 2004 were removed.

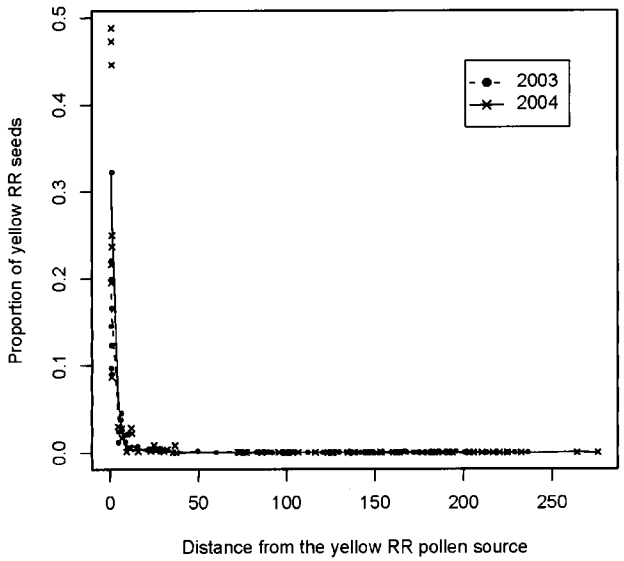

Fig. 4. Graphic representation of the model-fitted values of percent outcross for the 2003 and 2004 fields superimposed on a scatterplot of the proportion of observed RR yellow seeds in the sample by distance from the source plot.

Although the models for 2003 and 2004 fitted to the entire data set captured the large scale behavior of the outcross process, they consistently underestimated outcrossing at locations farther than 70 m from the source plot. Because we are mostly concerned with the possibility of outcrossing at greater distances from a transgenic source, we performed a separate analysis of the data collected at sites farther than 70 m from the center RR/Bt plot. The same modeling principles were followed for this segment of the data. Again, based on model comparisons and diagnostics used for the entire data set (AIC; Neter et al., 1996), the same specification of the statistical model provided the best fit to the measured field values at distances beyond 70 m. Other specifications, such as the power law specification suggested by Aylor et al. (2003) did not fit these data.

Table 4 displays the estimates for the three parameters β_0, β_1, and β_2. The two parameters of interest, β_1 and β_2, bear the same practical interpretation as their counterparts derived from the whole data set, although their values are now significantly smaller (see Table 4 for estimates $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2$ and their standard errors). An illustration of the model fit is given in Fig. 5, where observed outcross percentages for the 2 year are plotted against the distance from the field, and the lines superimposed on the scatterplot represent the fitted values.

The analysis of the data from 2004 was rather challenging, mainly because of three unusual observations collected to the West of the source plot. Some of the largest observed values are located the farthest from the field on the

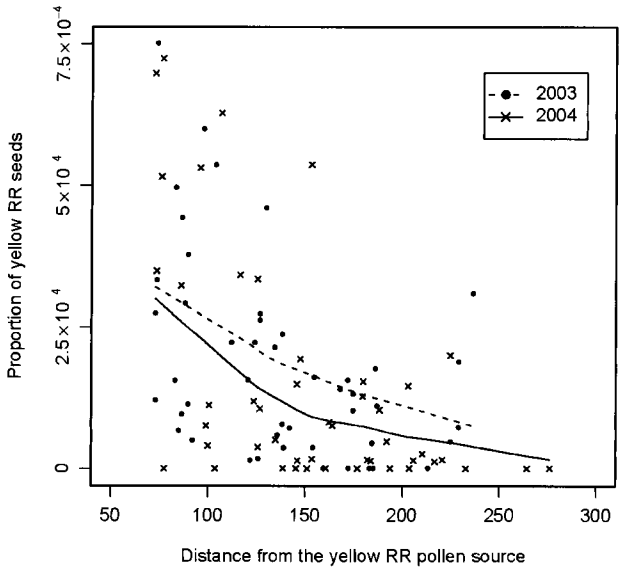

Fig. 5. Graphic representation of the model-fitted values of percentage of outcross for the 2003 and 2004 fields at locations farther than 70 m from the source plot, and the superimposed scatterplot of the proportion of observed RR yellow seeds by distance from the source plot.

west edge or in the NW corner, which was counterintuitive. If wind speed and direction were the main drivers for transport of pollen, the number of yellow seeds should at worst remain fairly constant with increasing distance from the source plot. Further, there was no evidence of strong wind from the E or SE (Fig. 3B). When harvesting the samples of the 2004 field, however, we observed the presence of yellow seeded corn plants in proximity to these three sampling locations, which had higher level of outcross. We suspected a few volunteer plants from the previous year provided an unintended source of yellow pollen at these locations. Thus, data for these three field locations were discarded from the statistical analysis.

A likelihood ratio test suggested that the model parameters (β_0, β_1, and β_2) were significantly different for the 2 year for models fitted to the entire data set as well as models fitted to data beyond 70 m. We investigated this difference further through a detailed comparison of outcrossing predictions generated by the 2003 and 2004 models. We used the estimates $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2$ for a given year and calculated the fitted values for that year (i.e., using the explanatory variables sampling distance, wind speed, and direction observed for that year). In addition, we calculated predicted values for the same explanatory variables but using the estimates obtained for 2004. Close correlation between such fitted and predicted values provides evidence that the two models are similar. Fig. 6 illustrates this aspect of the data for models fitted to the whole data set, by using data collected in 2003 as a baseline. The slope of the regression line for these two data sets is 0.582, which confirms the visual impression suggested by Fig. 6 that the two models are significantly different. When the same analysis was performed for the

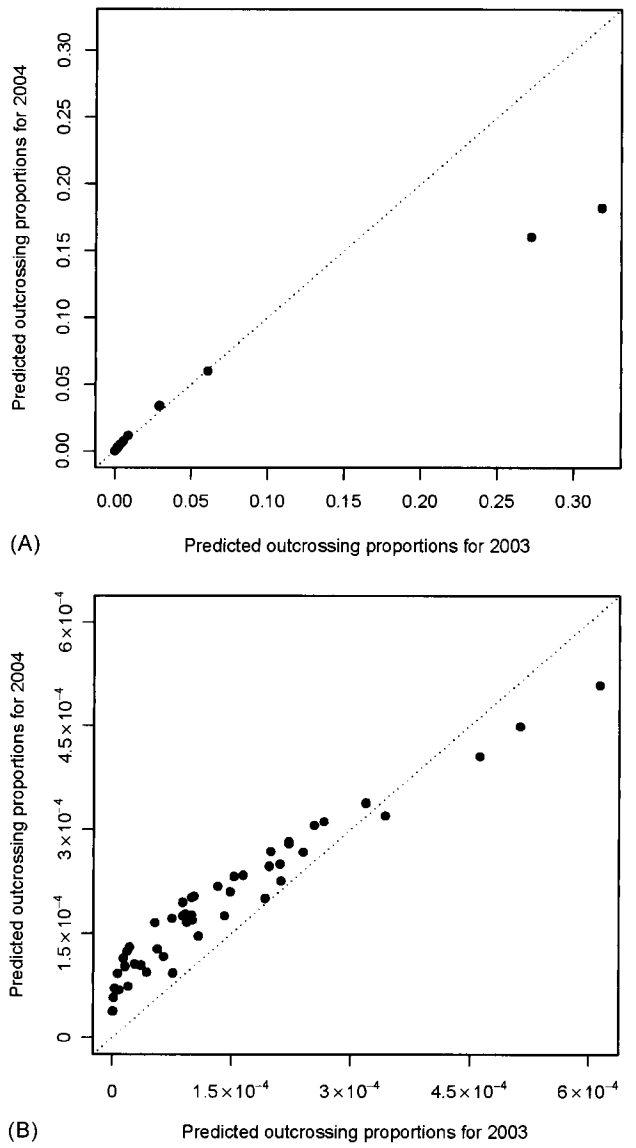

Fig. 6. (A) Scatterplot of model-predicted proportion of RR yellow seeds in the sample for 2003 and 2004 by using the model estimated for 2003 and the covariates observed for 2003 and 2004, respectively; (B) Scatterplot of model-predicted proportion of RR yellow seeds in the sample for 2003 and 2004 at locations farther than 70 m from the source plot by using the model estimated for 2003 and the covariates observed for 2003 and 2004, respectively.

two models fitted to the data collected 70 m or more from the source plot, the slope = 1.05, indicating the models for 2003 and 2004 are very similar. This fact also is depicted by Fig. 6B.

These results suggested that additional explanatory variables were needed to define the observed pattern of outcrossing at proximity to the source plot (i.e., within 70 m) more accurately. At greater distance, these local effects apparently become less important for accurate model prediction. Wind gusts at peak pollen shedding (Halsey et al., 2005) and frequent changes in wind direction (Aylor, 1990) influence outcrossing and add complexity to the

prediction. Our results suggest that the pollen dispersion process (quantified here from the proportion of RR/Bt yellow seed in the sample) should be viewed as scale-dependent, relative to the proximity to a particular source plot. Considering the large settling velocity of maize pollen (about 20 cm s^{-1}), it is likely that far-field transport requires lofting in turbulent boundary layer eddies (similar to the argument of Nathan et al. (2002) regarding the effect of turbulent lofting on seed dispersal). More detailed observations of atmospheric turbulence above maize fields and of the vertical distribution of maize pollen would aid in resolving this issue (Brunet and Irvine, 2000).

4. Conclusion

The percentage of adventitious grain observed in a nontransgenic grain production field was as high as 47% adjacent to the transgenic pollen source. Outcrossing decreased rapidly to an average of 0.5% or less at 35 m and beyond. Based on our results and the information available in the literature, surrounding a field of a transgenic corn hybrid with 35 m of a synchronously flowering corn crop would be an effective means of producing grain suitable for the EU market, meeting the maximum labeling threshold of 0.5% GMO content in conventional nontransgenic grain (European Commission, 2004). Our results indicate that in contiguous cornfields, 100 m distance is sufficient to achieve 0.05% outcrossing consistently.

At 250 m from the source, the frequency of transgenic yellow seed decreased to levels below 0.002% in 2003 and 0.03% in 2004. Very few samples collected at 250 m from the source plot were completely free of outcrosses. As such, the goal of producing 0% transgenic seed in organically grown corn may not be consistently achievable when organic fields are separated by less than 250 m from transgenic corn with no additional temporal isolation. Much more stringent measures, such as those proposed by Halsey et al. (2005) (2 week of temporal isolation and isolation distances of 750 m), may be needed to achieve 0% outcrossing consistently.

Successful coexistence of transgenic and nontransgenic corn hybrids produced via open pollination will likely require acceptance of non-zero thresholds for adventitious presence of transgenes. This non-zero threshold is a particularly important consideration for advancing the production of plant-made pharmaceuticals and industrial products in maize. Differences in outcrossing patterns noted between years in this study also highlight the importance of environmental effects on pollen dispersal and effectiveness of temporal isolation techniques. Plant physiological changes depend on weather conditions (i.e., accumulation of heat units, water availability at critical stages) because they affect time to flower, floral synchrony, duration of pollen shed and silking, seed formation and maturation, and extent of seed abortion.

Acknowledgements

This journal paper of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 3638, was supported by Hatch Act, State of Iowa fund and by a USDA-CSREES BRA Grant.

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