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Original Paper

# Effect of a special screened greenhouse covered by fine mesh on maize outcrossing

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**Abstract** Risk assessment and management of gene flow via pollen dispersal from genetically modified (GM) plants is critical. To minimize the likelihood of undesirable outcrossing, it is important to better understand the relationship between pollen dispersal distance and outcrossing rate in objective materials under physical containment. This study examined how a special screened greenhouse for Type-2 use, covered by a fine, 1-mm mesh reduced pollen dispersal and affected the outcrossing rate of non-GM yellow maize in the greenhouse and silver maize outside the greenhouse. Results suggest that the mesh-covered greenhouse was effective in reducing the outcrossing rate as a whole, although it may be less effective in reducing the longest distance of pollen dispersal. Further, the isolation distance threshold for the screened greenhouse is discussed in relation to risk assessment.

**Key words:** Outcrossing, special screened greenhouse, Type-2 use, *Zea mays*.

Gene contamination by genetically modified (GM) plants may harm biological diversity and introduce herbicide or pesticide resistance into related plants or weeds (National Academy of Science [NAS] 2002). The main concern is that widespread cultivation of some transgenic crops could accelerate the evolution of undesirable and more invasive weeds, thereby leading to ecosystem disequilibrium effects or the erosion of biodiversity (Tiedje et al. 1989, Snow and Morán Palma 1997).

Because of such concerns, risk assessment by a domestic institute is required for Type-2 use of GM plants in Japan. GM plants are first developed and checked for biosafety in a laboratory, then checked again in a special screened greenhouse covered by 1-mm fine mesh (as specified by biosafety regulations of the Japanese government for Type-2 use) in an attempt to reduce gene flow via pollen dispersal from GM plants into the surrounding environment. Gene flow is a natural process of gene movement between individual organisms (Glover 2002). Among plants, this flow occurs mainly by pollen from one plant successfully cross pollinating a flower from another plant and producing viable seed, a process known as outcrossing. During risk assessment, it is important to determine the possibility of gene flow via uncontrollable pollen escape from the screened greenhouse.

Maize (*Zea mays*) is not only one of the most important crops in the world, but also one of the crops most at risk for gene leakage via pollen flow into the environment. Among GM crop species worldwide, maize is the second largest by cultivated area (21.2 million hectares; 24% of global GM crop area; James 2005). Maize is a monoecious species with separate male and female flowers at different locations on the same plant (Poethig 1982). Pollen is produced in the anthers in the tassel (male flower) at the top of the plant and typically has volume-equivalent diameters of 90–100 $\mu$m. The silks are the female part of the flower. One silk directs the germ tube of a germinating pollen grain to one ovule, which must be fertilized for a kernel to develop. Silks emerge from the top the ear and continue to grow until fertilized, some reaching lengths of approximately 15 cm or more. Maize is predominantly a wind-pollinated species, with limited evidence of insect pollination (Eastham and Sweet 2002). For this reason, insect-borne pollen transport leading to outcrossing can be neglected, making maize an ideal plant for studying and modeling pollen dispersal driven by wind-borne pollen transport (Loos et al. 2003). Moreover, maize has shown the xenia effect, namely pollen from the male parent genetically affects the fruit development or seed color. Thus, it is possible to research the outcrossing rate between two maize types with different kernel colors without using GM maize.

Although field measurements of outcrossing levels at various distances from a pollen source are the most

This article can be found at http://www.jspcmb.jp/

direct way of assessing effective pollen dispersal, the results depend on local conditions, such as topography and weather (e.g., rainfall, wind speed, and direction; Aylor et al. 2003). Such site-specific parameters limit the ability to generalize results and predict pollen dispersal, but model simulations can fill in differences in field experiments. However, no previous studies have examined outcrossing under physical containment designed to restrict gene flow via pollen dispersal under practically applicable conditions. Physical containment is very significant for countries with narrow land orientations, such as Japan, where it is realistically impossible to create sufficient isolation distances to avoid genetic contamination by pollen dispersal during production of certified seed, even for risk-assessment purposes. In most cases, recommended isolation distances for maize require fields of 2 ha or more to be 200 m apart to maintain 99% crop purity and 300 m to maintain 99.5% grain purity (Eastham and Sweet 2002).

The main aim of this study was to estimate how using a fine, 1-mm mesh to cover all openings of a screened greenhouse would reduce pollen dispersal of non-GM maize. This paper presents results from a pollen-dispersal experiment performed with two varieties of non-GM maize. We counted kernels that received pollen from different varieties and determined effective pollen dispersal via outcrossing.

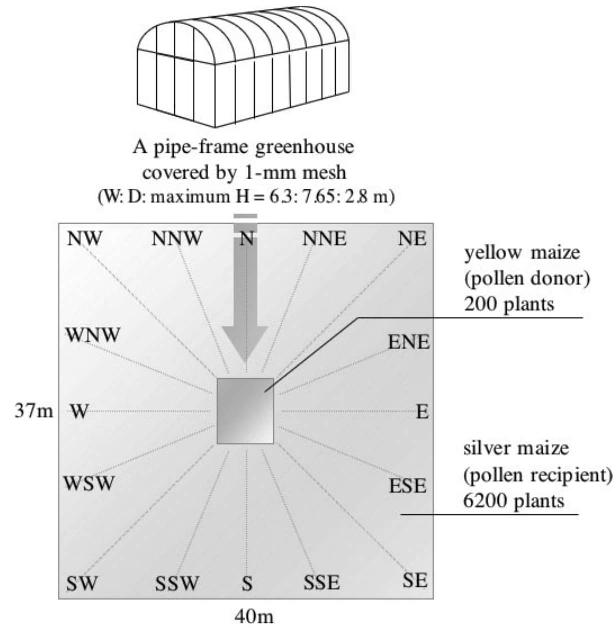


Figure 1. Experimental design of the 40×37-m maize field. In the center, a 6.0×7.6-m plot with 200 yellow maize plants served as the pollen donor population; this plot was located within a field containing about 6200 silver maize pollen recipient plants of the same variety. The radial lines spreading in 16 directions specify sampling locations. A pipe-frame greenhouse was covered with 1-mm mesh just before the flowering period began at the beginning of July 2004.

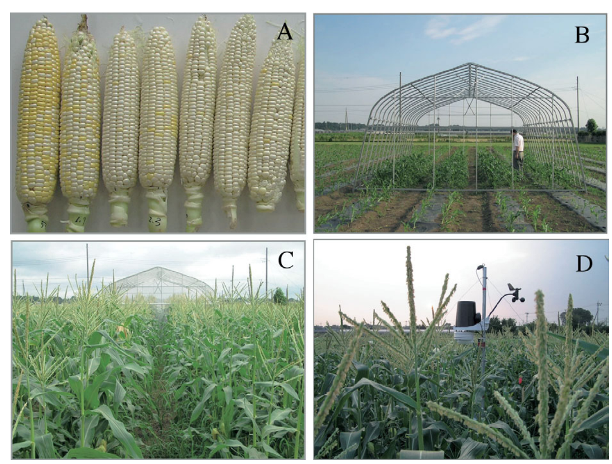


Figure 2. (A) Xenia effect observed on silver maize fruit. (B) Pipe-frame greenhouse covering the yellow maize. (C) The pipe-frame greenhouse covered by 1-mm fine mesh, and the silver maize plants during the flowering period. (D) Meteorological equipment was placed 8 m from the pipe-frame greenhouse in the SW direction to collect meteorological data, including air temperature, luminous intensity, precipitation, and wind speed and direction, in the experimental field.

## Materials and methods

### *Plant materials*

Two commercial sweet corn varieties with different grain colors were selected. We used the xenia phenomenon to determine the percentage of outcrossing, as shown by the presence of yellow grains on the female ears of the recipient. *Zea mays* L. cv. Honey Bantam with yellow grain (yellow maize) was used as the pollen donor, and *Zea mays* L. cv. Silver Honey Bantam with white grain (silver maize) was used as the pollen recipient (Figure 2A).

### *Field design*

The experiment was performed at a 40×37-m experimental field in Tsukuba, Ibaraki Prefecture, Japan. Adequate nutrition, as defined by standard prescriptions, was supplied to the field. To ensure congruence between male pollen release from the yellow maize and silk receptivity on the silver maize, yellow maize was sown on 17, 22, and 27 May 2004 in a 6×7.6-m square at the center of the experimental field (Figure 1).

A pipe-framed greenhouse without a cover (W : D : maximum H=6.3 : 7.65 : 2.8 m) was built on the area where the yellow maize was sown (Figure 2B). The entire greenhouse was covered by 1-mm nylon fine mesh to reduce pollen dispersal from yellow maize, just before the tassels at the top of the plant opened (Figure 2C). The area surrounding the greenhouse was sown with silver maize on 22 May 2004. All yellow and silver maize plants were sown in rows at intervals of approximately 0.75 m, with a distance of approximately 0.3 m between individual plants.

The ratio of pollen donors to pollen recipients can significantly affect the outcrossing ratio in gene-flow research. This study used 200 yellow and 6200 silver maize plants. The 200 plants were considered sufficient for the assessment of biosafety; this was also the maximum number of plants that would fit into the 6.0×7.6-m square, which corresponded to the standard size of the special screened greenhouses used in Japan. Therefore, the number of pollen donors in this study was chosen to present a high risk of outcrossing.

### *Data monitoring*

Meteorological data, including air temperature, luminous intensity, precipitation, and wind speed and direction at the experimental field, were measured using a Vantage Pro weather station (Davis Instruments Corp., Haywood, CA, USA) during the flowering period (Figure 2D). Temperature and humidity data were also collected inside the greenhouse (instruments from Hioki Co., Ueda, Japan) during the flowering period.

We measured the height of all yellow maize plants and approximately 800 silver maize plants once a month. The flowering condition of both male and female flowers was determined once a week on the same specimens used for plant height growth after flowering started at the beginning of July 2004. When male flowers mature, the anthers emerge from the tassel suspended on filaments, and pollen is released from an opening at the tip of the anther. Even a slight breeze or vibration will release the pollen into the air when mature (Aylor 1990). Therefore, we counted a plant as having flowered (male) when the anthers emerged from the tassel. We counted a plant as having flowered (female) when the silks emerged from the ear. On both male and female flowers, flowering rates were calculated by dividing the number of flowered plants by the total number of plants examined and transforming the result into a percentage.

### *Sampling and analysis*

On 1 July 2004, just before the tassels opened, the entire greenhouse was covered by 1-mm, fine-mesh nylon. On 3 August 2004, all yellow maize plants were harvested and silver maize fruits were sampled in 16 different directions (N, NNE, NE, ENE, E, ESE, SE, SSE, S, SSW, SW, WSW, W, WNW, NW, and NNW) from the greenhouse to the edge of the experimental field (Figure 1). After sampling, the number of yellow kernels and the total number of kernels were counted on each silver maize fruit to calculate the outcrossing rate using the following formula:

$$\text{Outcrossing rate (\%)} = \frac{\text{number of yellow kernels}}{\text{row number} \times \text{line number of fruit}} \times 100$$

## Results

### *Weather conditions during the flowering period*

Observations by the Mito meteorological observatory point at Tsukuba, 5 km southwest of the experimental field, indicated that total precipitation in June 2004 was less than the average total precipitation in June from 1990 to 2000 (89 mm versus 133 mm). However, the total precipitation in July 2004 was almost the same as the average for July from 1990 to 2000 (146 mm versus 139 mm). The Tsukuba precipitation data for July were also similar to the data recorded by our observational equipment (131 mm; Figure 3A). In contrast, the rain frequency in July 2004 was biased (Figure 3A). Further,

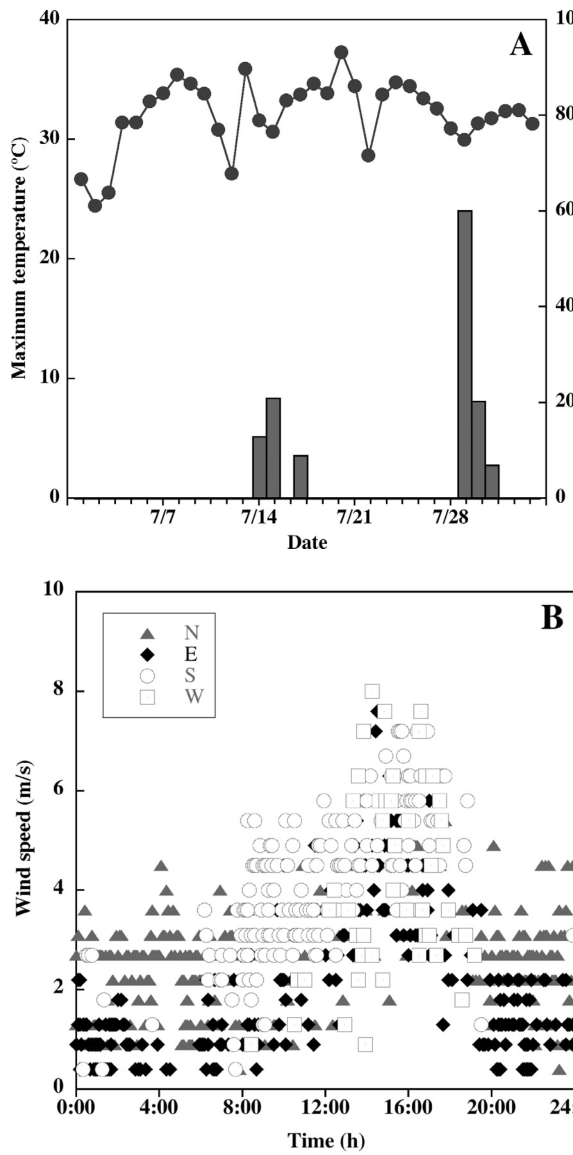


Figure 3. Meteorological conditions in the experimental field during the flowering period. (A) Daily maximum temperature (circles) and precipitation (bars) recorded during the flowering period. (B) Representative data for changes in diurnal wind direction during the flowering period. The plotted data were collected every 3 days during the flowering period in the four cardinal directions.

according to the Tsukuba observations, the average maximum temperature in July 2004 was 31.7°C, which was higher than average maximum temperature of 28.3°C from 1990 to 2000. In our experimental field, the daily maximum temperature was often above 35°C, with an average of 32.1°C (Figure 3A). These severe weather conditions in July 2004 may have suppressed plant growth.

Wind directions changed frequently, with an average wind speed of about 3.0 m/s (data not shown). Figure 3B shows the maximum wind speed in the N, E, S, and W directions for 5-min intervals on 10 days (every 3 days from 3 to 30 July). The figure clearly shows micrometeorological features in the diurnal change of wind direction at the experimental field. From 20:00–6:00, winds were mainly from the E and N; around 6:00, a mainly S wind began to blow and strengthened until around 14:00. After that time, a strong W wind developed, but weakened around 18:00. The conditions observed in the experimental field may have affected the dispersal direction of maize pollen.

### Plant growth conditions

Yellow maize plants grew to an average height of 148±36 cm (Figure 4), which is similar to the average height of *Zea mays* L. cv. Honey Bantam (120–180 cm). However, the silver maize attained an average height of 100±39 cm (Figure 4), which is slightly shorter than the average height of Silver Honey Bantam (120–180 cm). These data suggest that pollen emitted from yellow maize could scatter longer distances than that from the silver maize because of the height differences in pollen release points. Both yellow and silver maize had tassel lengths of approximately 30 cm. Most yellow and silver maize plants had two 20–30 cm long fruits per plant. The ears were located at the middle of plants, between 20 and 80 cm in height. Almost all ears were successfully fertilized, and the mean number of kernels on sampled fruit of silver maize was 576±85 kernels.

### Flowering timing

The male and female flowers on yellow and silver maize began to flower at the beginning of July and finished flowering by the beginning of August (Figure 5). The first male flower flowered earlier than the female flower for both yellow and silver maize (Figure 5). The male flowers on yellow and silver maize plants began to flower at almost the same time, but the male flowering rate of silver maize increased earlier than that of yellow maize (Figure 5). The female flowering rate of silver maize increased earlier than that of yellow maize until mid-July. The female flowering rate of yellow maize, however, caught up to that of silver maize and remained higher until harvest (Figure 5). Consequently, the flowering timing of male yellow maize flowers and female silver maize flowers synchronized best, and may have resulted in increased opportunity for outcrossing.

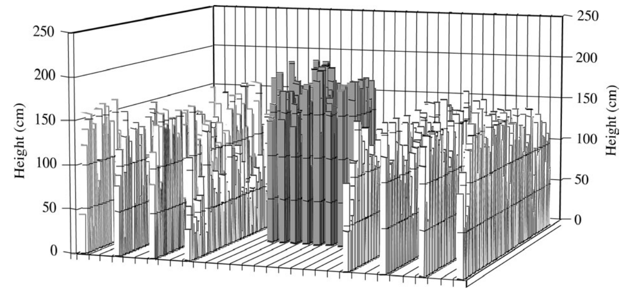


Figure 4. Plant heights of yellow (gray bars) and silver (white bars) maize before the harvest. Plants heights were measured for all yellow and approximately 800 silver maize and used to calculate mean heights and standard deviations.

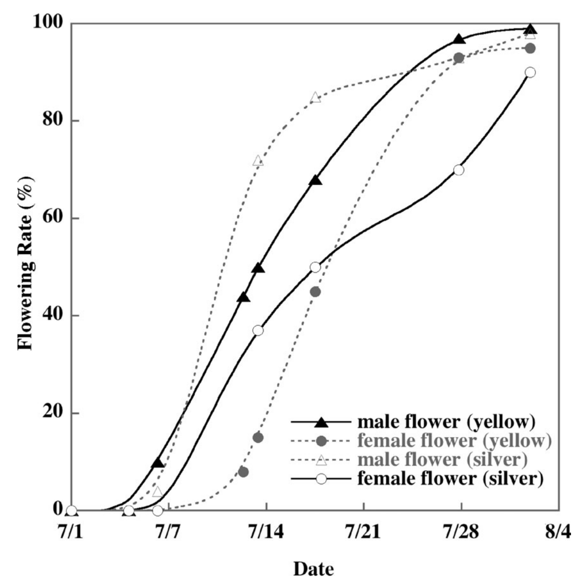


Figure 5. Flowering rate of yellow and silver maize during the flowering period.

### Outcrossing rate

The outcrossing rate decreased as the distance between yellow and silver maize increased (Figures 6A, B), decreasing sharply when silver maize plants were beyond 4 m of the yellow maize; outcrossing gradually decreased toward the edges of the field and finally converged at a minimum rate of 0.17% (Figure 6C). In a female recipient ear with 576 grains, there was at most 1 yellow grain at a 0.17% outcrossing rate. The outcrossing rate was high in silver maize adjacent to yellow maize, where the mean outcrossing rate was 32.8%. A 1.0% outcrossing rate was observed 6.3 m from the yellow maize (Figure 6C). We observed that 58% of all yellow kernels outcrossed with all sampled silver maize $<$1 m from the yellow maize, 96% $<$5 m, and 99% $<$10 m. Figure 6D shows that a higher rate of outcrossing occurred mainly in the north at a greater distance from pollen donor plants.

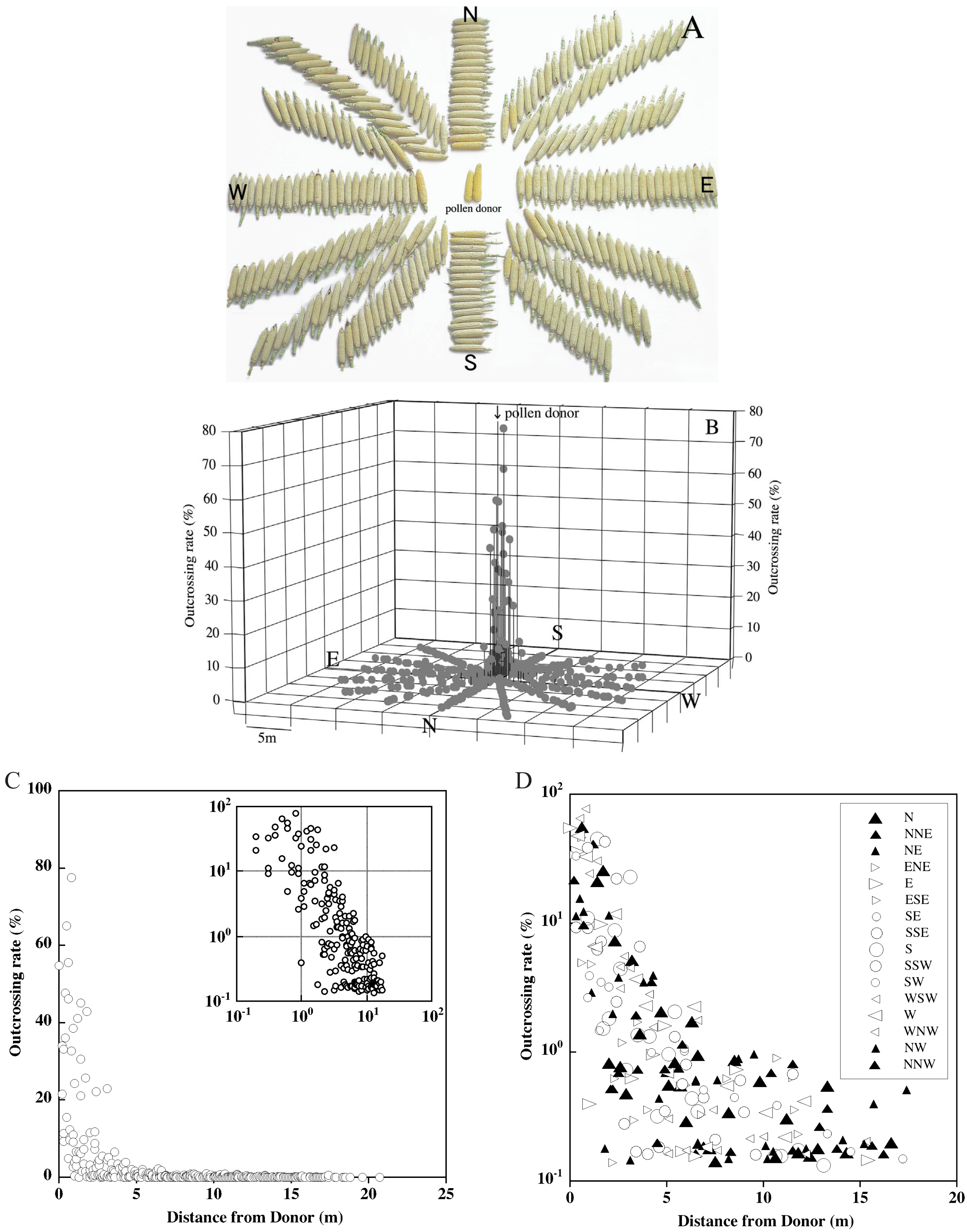


Figure 6. Outcrossing between yellow and silver maize. (A) The silver maize harvest, arranged according to location in the experimental field. The yellow maize samples in the center were from the pollen donor. (B) Percent outcrossing in maize versus distance from the source of yellow maize measured as a percent of yellow maize kernels on silver maize fruits. The three-dimensional plot presents a comprehensive image of outcrossing dispersal in the experimental field. The pollen release point was located at the cross-point between the N–S line and E–W line. (C) Relationship between the outcrossing rate and distance from the pollen donor source. Data plotted on the linear scale suggest a definable dispersal-length scale (large plot), whereas the log–log plot of the same data indicates the existence of a long-tail characteristic of dispersal by atmospheric turbulence (small plot). (D) Relationships among outcrossing rate, distance from pollen donor source, and wind direction on a linear–log plot.

## Discussion

During the flowering period, the severe weather conditions, including biased rainfall and high temperatures, may have suppressed plant growth in silver maize. During the flowering period, the difference in air temperature between the outside and inside of the greenhouse located in the maize field was between 5°C and 8°C at 100 klux in luminous intensity (data not shown). Even though the maximum temperature inside the greenhouse often reached >40°C, the yellow maize grew better than the silver maize. The difference of approximately 48 cm in average plant height between yellow and silver maize, and the corresponding difference in vertical tassel position, may have given some pollen release advantage to yellow maize, allowing pollen to scatter longer distances than that from silver maize. In contrast, despite the severe weather conditions that continued throughout the flowering period, both yellow and silver maize yielded satisfactory products at harvest (Figure 6A).

In the experimental field, the wind direction was changeable, but some micrometeorological patterns were found in daily wind direction changes. Around 6:00, mainly S wind began and then strengthened until around 14:00. The low wind speed threshold for maize pollen release affects its dispersal (Aylor 1990). However, maize pollen was released mainly during dry and drying conditions, and typically, the major portion of the daily release usually occurred during mid-morning to mid-day (Ogden et al. 1969, Jarosz et al. 2003). The experiments suggest that the S wind from mid-morning to mid-day had important implications and was influential in controlling the direction of pollen dispersal, and a higher rate of outcrossing occurred mainly in the north at a greater distance from pollen donor plants. Therefore, wind direction was thought to be an important factor in raising the outcrossing rate, specifically in the leeward direction.

Pollen viability is one factor in outcrossing. Maize pollen is sensitive to dehydration (Barnabas 1985, Buitink et al. 1996). Luna et al. (2001) measured maize pollen longevity by exposing it outdoors in winter in Mexico; they found a relative loss in pollen viability of 80% in 1 h and 100% loss in 2 h. However, maize pollen viability ranges from 3 h to 9 days, depending on the environmental conditions, with cool temperatures and high relative humidity allowing for longer survival times (Glover 2002). Because daytime relative humidity values in the experimental field during the flowering period were approximately 50–90% (data not shown), field conditions may not have affected pollen longevity.

Numerous studies have measured actual gene flow using the percentage of outcrossing (e.g., Jones and Brooks 1950, Jemison and Vayda 2001, Loos et al. 2003). One of the most widely cited studies on actual outcrossing in maize was conducted by Jones and Brooks (1950). They planted a strip of a yellow dent field maize variety, cv. Yellow Surecropper, as the pollen donor, and a strip of a white-kernel sweet corn variety, cv. Honey June, as the female recipient, separated by various distances downwind from the strip of Yellow Surecropper. In that study, 0.12–0.32% (0.2% on average) outcrossing was observed over 500 m from the pollen donor plants, and 0.4–2.5% (1.19% on average) at 200 m. Under suitable atmospheric conditions, another study noted that maize pollen can cross with other cultivars of maize up to 800 m away (Eastham and Sweet 2002).

Experiments similar to those of the present study using the same maize species in a larger, 4.5-ha field with a maximum distance of 400 m were conducted in Tsumagoi, Nagano Prefecture, by researchers from the National Institute for Agro-Environmental Sciences of Japan (NIAES 2004). The mean outcrossing rate of silver maize over 2 years of research was 1.2% at 50 m, 0.23% at 100 m, and 0.04% at 400 m (Matsuo et al. 2004). The NIAES conducted previous research at an approximately 0.14-ha field in Tsukuba, with a maximum distance of 55.5 m between recipient and donor plants (Matsuo et al. 2002). According to the Tsukuba-based NIAES research, the outcrossing rate of 1.0% occurred at 50.1-m distance between pollen and donor plants. The present study was conducted in a field of approximately 0.15 ha with a maximum distance of 23.7 m between recipient and donor plants; a 1.0% outcrossing rate occurred at 6.3 m and 0.17% rate occurred at 20 m (Figure 6C). The special screened greenhouse covered by 1-mm fine mesh was thus considered somewhat less effective in reducing the longest distance of pollen dispersal. Data plotted on a linear scale suggest a definable dispersal length scale; a log–log plot of the same data indicates the presence of a long-tail characteristic of dispersal by atmospheric turbulence, implying a linear decrease in the outcrossing percentage with distance (Aylor et al. 2003). The same data on a log–log plot (small plot in Figure 6C) suggest that 1.0% outcrossing occurred at approximately 10 m from the pollen donor, indicating the potential of outcrossing in maize.

The results suggest that the extent of gene flow from outcrossing was mainly dependent on the scale of pollen release and dispersal, based on distances between source and recipient populations. It is not easy to compare the results of our study with those of the NIAES because the experimental conditions, including the ratio of pollen donor plants to recipient plants, the location, and the flowering synchrony, were different. However, our results indicate that the special screened greenhouse covered by 1-mm mesh was effective in reducing the amount of pollen dispersal from the greenhouse. Our field experiment was designed for a high possibility of outcrossing between the donor and recipient maize to investigate a threshold for the greatest risk of genetic contamination in the case of a physical contaminant. First, the openings of the special screened greenhouse were at least four times bigger than those of the greenhouses commonly used by domestic institutes in risk-assessment studies. Second, the inside of the greenhouse was filled with 200 pollen donors, a number considered sufficient for usual risk assessment practices. Moreover, later seeding and the indirect effects of three typhoons (numbers 7, 8, and 10) caused comparatively stronger winds in the experimental field during the flowering period. For these reasons, in a practical risk assessment, the possibility of outcrossing around a special screened greenhouse with standard regulations is predicted to be much lower.

For biosafety risk assessment, it is difficult to scientifically determine an outcrossing threshold rate of interfering transgene escape from GM plants. In fact, the GM labeling controversy in global agricultural trade involves more than science. Politicians and environmental groups in Europe and elsewhere have contended that GM labeling relates to consumer choice and consumer rights, rather than health alone. The Europeans are taking a precautionary approach. Alternatively, the United States, Canada, and Japan are using science-based risk-assessment procedures (Carter and Gruere 2003). In the European Union (EU), regulations on foodstuff labeling provide European consumers with comprehensive information on the contents and composition of food products, including information on genetic modification. The allowable adventitious presence level for EU-approved varieties of genetically modified organisms (GMOs) for use in food and feed is 0.9% (European Commission 2003). The Japanese government requires mandatory labeling when GM material is present in the top three raw ingredients and accounts for 5% or more of the total weight (Carter and Gruere 2003). Japan's regulations also require that labels indicate the presence of non-GM labels at the same tolerance levels, if produced with identity preservation.

If we pursue the risk assessment from the strictest threshold, such as the 0.9% required by the EU, the results of this study indicate that related plant species must be thoroughly removed within approximately 10 m of pollen donor plants located in special screened greenhouses. Although maize requires isolation distances from contaminating sources of 200 m to maintain crop purity at 99% for commercial cultivation, some other crops require greater distances (Agricultural Biotechnology Support Project [ABSP] 2003). For example, sunflower, watermelon, and onion required isolation distances of 800, 800, and 1600 m, respectively (ABSP 2003). However, because these crops are insect pollinated, a thorough insect-control regime in the special screened greenhouse during the period of risk assessment can be effective for typical insect pollinators, such as bees. Flowering timing is also critical for gene flow via pollen dispersal. Thus, in addition to spatial separation, time separation can also be effective for de-synchronizing outcrossing in some GM plants, such as maize. Time separation is easily accomplished, especially in greenhouse cultivation, where flower timing can be staggered during risk assessment.

This study examined how the special screened greenhouse affected outcrossing in a wind-pollinated plant with a high potential for long-distance pollen release; the chosen threshold is not sufficient for all GM plants. For example, a study of commercial canola showed that pollen moved over 3 km from the pollen source (Rieger et al. 2002). It is important to include previously determined information, such as pollen size and pollination form, when studying plants that have a greater potential to release pollen for longer distances than maize (Watanabe et al. 2006). Various studies have examined crop pollen dispersal in both wind- and insect-pollinated species, including cotton (Kareiva et al. 1994), potato (Skogsmyr 1994, Conner and Dale 1996), oilseed rape (Morris et al. 1994, Lavigne et al. 1996), alfalfa (St. Amand et al. 2000), and ryegrass (Giddings et al. 1997). Finally, all biosafety risk assessments must be conducted carefully.

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