

## Pollen-Mediated Gene Flow in California Cotton Depends on Pollinator Activity

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### ABSTRACT

Many cotton (*Gossypium hirsutum* L.) pollination studies have been performed in the southern USA, but no data exist for California. In this study, we measured pollen-mediated gene flow (PGF) in four directions over 2 yr from herbicide-resistant source plots in upland cotton in the California cotton growing region and in a region with high pollinator activity. In addition, samples were taken from fields of conventional varieties at varying distances from fields planted with herbicide-resistant varieties to assess PGF under commercial production conditions. A seedling herbicide bioassay confirmed by DNA tests was used to measure PGF. PGF was independent of direction from the source plot and declined exponentially with increasing distance from 7.65% at 0.3 m to less than 1% beyond 9 m when there was high pollinator activity. In the absence of high pollinator (honeybee, *Apis mellifera* L.) populations, PGF was less than 1% beyond 1 m. Pollen flow in commercial fields was consistent with the experimental plot data, with only 0.04% PGF detected at 1625 m (1 mile). This study confirms that PGF decreases exponentially with distance in cotton grown under California conditions and is low in the absence of pollinators, although sporadic occurrence of PGF can be detected up to 1625 m.

CALIFORNIA produces only 4% of the cotton in the USA but exports 25% of the American crop and 5% of the seed (California Department of Agriculture, 2002; USDA, 2003). In 2003, 73% of cotton in the USA was transgenic, including almost 40% of cotton in California. In the USA, transgenic (biotech) cultivars are not separated from nonbiotech cultivars once the introduced trait has been approved by government agencies. Pollen-mediated gene flow (PGF) or other sources of adventitious presence (e.g., seed contamination or mechanical mixtures) can therefore pose problems for export of cottonseed into countries where the biotech trait may not be approved or deregulated. Cotton is generally considered to be a self-pollinating crop, but it is often cross-pollinated and the majority of cultivars are a mixture of closely related pure lines. The flowers are visited by honeybees, bumblebees (*Bombus* spp.), and melissodes (*Melissodes* spp.) bees. Studies document that provision of honeybees can increase both seed and lint yield of cotton via improved pollination, and outcrossing rates are affected by bee activity (McGregor 1976).

Outcrossing rates reported for cotton vary depending

on the location, the time period, and how measurements are taken. In the 1950s comprehensive studies using visual phenotypic traits reported 10% outcrossing in Texas to 47% in Tennessee (Simpson, 1954; Simpson and Duncan, 1956). These studies reported 28% outcrossing in Mississippi, but a mean of 2% outcrossing was reported in similar locations in Mississippi 20 yr later (Meredith and Bridge, 1973). The authors suggested the differences were due to a reduction in wooded areas and the heavy use of pesticides resulting in a decrease in bee pollinators. Also in Mississippi, Umbeck et al. (1991) measured pollen transfer to non-transgenic rows of cotton planted up to 25 m from a 4 ha field of cotton carrying the *nptII* gene. PGF dropped below 1% at distances beyond 7 m, but continued to be detectable at a distance of 25 m in solid-seeded cotton. Studies summarizing data (>15 000 samples) in Arizona, Arkansas, Mississippi, and North Carolina showed that PGF decreased exponentially with increasing distance from the pollen source and was below 1% beyond 10 m at all locations, although PGF was detected at 20 m (Kareiva et al., 1994). The same authors observed similar trends in South Africa and Argentina, although greater PGF was detected in these locations because of the presence of different pollinators. In a recent study, PGF was measured on a field scale across Arizona, Mississippi, and Texas by means of herbicide-resistance assays and ELISAs for presence of insect-resistance genes from *Bacillus thuringiensis* (Bt) (Berkey et al., 2003). In Arizona, a 55 m nonplanted area was enough to maintain varietal purity. In Mississippi, all samples had less than 0.25% outcrossing, with distances beyond 3.7 m having no significant PGF. In Texas, PGF fell to nonsignificant levels from controls at distances greater than 14 m to the west and 25 m to the east of the pollen source. As cotton pollen is not effectively carried by wind (McGregor, 1976), it is unlikely that the directional differences were due to wind-blown pollen but could be due to the effects of wind on pollinator movement. As the advent of Bt cotton has reduced insecticide applications (Carpenter and Gianessi, 2001), pollinator activity in fields may have increased in recent years (Betz et al., 2000).

The authors are not aware of any outcrossing studies for cotton in California, where both the environment and the varieties differ from those in the southeastern USA. The California crop is approximately one-third Pima (*G. barbadense* L./*G. hirsutum*) and two-thirds Acala (*G. hirsutum*, upland) cottons, with 39% of the upland cotton being transgenic (primarily herbicide resistant) (USDA 2003). Field inspectors for the California Crop Improvement Association have observed Acala/Pima cot-

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**Abbreviations:** Bt, *Bacillus thuringiensis*; ELISA, enzyme-linked immunosorbent assay; GUS,  $\beta$ -glucuronidase; *nptII*, neomycin phosphotransferase II; PCR, polymerase chain reaction; PGF, pollen-mediated gene flow.

ton hybrids in fields intended for certified seed production (hybrids are taller and slightly earlier). The foundation or registered seed from which these fields were planted was produced under isolation standards of 400 m (0.25 mile, 1320 ft) from similar types and 800 m (0.5 mile, 2640 ft) from widely different types (i.e., between Acala and Pima types). Although the frequency of outcrossing generally meets certified seed production standards (1 in 9000 plants for foundation seed in the field, <http://ccia.ucdavis.edu/ccia/cottonsts.htm>; verified 17 March 2005), concern exists that contamination may be more frequent than is visibly evident. Furthermore, since morphological differences are not pronounced among the progeny of these hybrids, there is no indication of the level of contamination in the subsequent year's commercial crop. These very low levels of contamination that meet certification standards have not adversely affected crop production or market quality in the past. However, scrutiny of seed lots using sensitive assays for even rare contamination by transgenic events has raised the standards for seed genetic purity in certain markets. The goals of this research were to evaluate PGF with and without the addition of honeybees in upland cotton grown in California.

## MATERIALS AND METHODS

PGF was tested in summer 2001 and 2002 in two field locations, Shafter Research & Extension Center, Shafter, CA, and Kearney Research & Extension Center, Parlier, CA, and in commercial fields in the San Joaquin Valley in 2000 through 2002. Shafter Center and the surrounding area are essentially a cotton monoculture managed conventionally for insect control. Kearney Center is a mixed cropping area including bee-pollinated crops such as orchards, melons, and alfalfa seed. We chose the Kearney location to minimize competition and migration of introduced bees to neighboring crops that may be preferred for pollen or nectar collection. Honeybees will forage the food source nearest their hive with the maximum reward and avoid collecting pollen or nectar from sources where competition from different colonies is high (Gary et al., 1972; Visscher and Seeley, 1982). These two locations represent the commercial cotton production environment (Shafter) and a worst-case environment such as a cotton field adjacent to a bee-pollinated crop (Kearney) in California. As the majority of commercial transgenic cotton in California is Roundup Ready (Monsanto Corp., St. Louis, MO), seeds of BXN (Calgene Inc., Davis, CA; bromoxynil-resistant) cotton cv. Nova and the near-isogenic, nontransgenic cv. Maxxa provided by California Planting Cotton Seed Distributors (CPCSD) were used to minimize detection of PGF from outside the experimental plots. A herbicide bioassay (described below) on 2000 seeds from the Maxxa seedlot detected no bromoxynil-resistant seedlings. At each site, an 8- × 8-m source plot of Nova was surrounded by 60 × 60 m (0.36 ha) seeded with Maxxa in 0.76-m (30 in) spaced rows. Plots were managed through the growing season by standard methods for commercial cotton production. At Kearney, preplant herbicides Treflan (DowAgroSciences, Zionsville, IN; trifluralin:  $\alpha,\alpha,\alpha$ -trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine) and Dual [Ciba-Geigy Corporation, Greensboro, NC; metolachlor: 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide] were applied for weed control and cotton was planted on 1 May 2001 and 2 April 2002 with no insecticides applied throughout the growing season. At Shafter, cotton was planted on similar dates, but Temik 15G [Union Carbide Corp., Houston, TX; 2-methyl-

2-(methylthio)propionaldehyde-*O*-(methylcarbomoyl)oxime] and Digon 400 EC [Wilber-Ellis, Fresno, CA; dimethoate: *O,O*-dimethyl *S*-(*N*-methylcarbomoyl) methyl phosphorodithioate] were applied in the beginning and middle of June to control insects. The nearest BXN cotton was >1625 m (1 mile) away for all experiments except in 2002, a BXN field was located 220 m to the north of the Shafter trial. Four beehives were placed at a single corner of the experiment in Kearney at the beginning of the flowering and pollination period to ensure a high level of pollinators. No pollinators were introduced at Shafter.

Commercial fields in the San Joaquin Valley neighboring either Roundup Ready or BXN cotton were sampled at distances between 25 m and 1625 m (1 mile) from the herbicide-resistant pollen source. The fields were separated by open space. The first samples were taken at the edge of the herbicide-susceptible field and at 200, 400, 800, and 1625 m away from the pollen source field. Two fields were sampled in 2000, one in 2001 and two in 2002.

At maturity, cotton bolls were harvested from lower, middle, and upper flowers of Maxxa plants at 0.3 (adjacent plants), 1 (adjacent rows), 3, 9, and 30 m from the edge of the Nova plot in the center of the field. Samples were taken on four diagonals (SE, SW, NE, and NW) from the central plot. A sample was also taken from the central transgenic Nova plots. Each cotton boll sample (1.8 kg) was ginned and delinted by small-scale facilities at CPCSD, providing a minimum of 6000 seeds per sample. Each seed sample was bioassayed individually for bromoxynil tolerance as described in Jeanes et al. (2003). Approximately 2000 seeds from each sample were planted in flats containing potting soil (200 or 400 seeds/flat) and grown in a greenhouse in Davis, CA, with a constant temperature of 30°C and a 16-h photoperiod. A row of 25 control Nova seeds was also planted in each flat. Seedlings were allowed to grow to the cotyledon–first leaf stage. The total number of seeds germinated was recorded. Buctril 4 EC (Bayer CropSciences, Research Triangle Park, NC; bromoxynil: 3,5-dibromo-4-hydroxybenzotrile) was applied at the equivalent rate of 1.1 kg a.i./ha (1.0 lb a.i./acre). A second application was sprayed 3 d later to treat any late-germinating seedlings. The number of surviving plants was scored 7 d after the initial Buctril application. PGF resulting in herbicide tolerance was calculated as:

$$\text{PGF (\%)} = \left[ \frac{\text{(number of plants surviving)}}{\text{(number of plants germinated)}} \right] \times 100$$

Samples from commercial fields adjacent to BXN cotton were bioassayed in the same way. For samples taken from fields adjacent to Roundup Ready cotton, the rolled towel bioassay was used to measure resistance of seedlings to Roundup Ultra (Savoy et al., 2001).

Leaf tissues from up to five plants per 2000 seed sample that survived the bromoxynil bioassay were collected and frozen at –80°C. Samples were ground and DNA was extracted with the Qiagen DNeasy kit (Qiagen, Valencia, CA). Seventy random seedling samples were tested by polymerase chain reaction (PCR) for the presence of the BXN transgene for 2001 field trials and 169 seedling samples were tested from the 2002 trials at Emergent Genetics (Scott, MS) using proprietary primers. Primers for an endogenous cotton gene were utilized for each sample as a positive control to ensure that DNA was of sufficient quality for PCR amplification.

Before data analysis, negative samples based on DNA tests (i.e., false positives for resistance in the bioassay) were subtracted from the number of plants surviving for those samples actually tested by PCR; values for samples not confirmed by PCR tests were not adjusted. Data were subjected to analysis

of variance by Agrobase v.II (Agronomix Inc., Winnipeg, MB, Canada). To address heterogeneity of variances among locations, data were transformed by the square root of  $(X + 0.5)$  function in Agrobase v. II where  $X$  equals PGF. Regression analysis was performed on the means for each location by Microsoft Excel XP and confidence intervals were determined. Confidence intervals for PGF were calculated by equations outlined by Redmund et al. (2001):

$$pUL = [(d + 1)F1 - \alpha] / [(n - d) + (d + 1)F1 - \alpha]$$

where pUL is the upper confidence limit,  $d$  is the number of plants surviving the bioassay,  $n$  is the total number of plants.  $F1 - \alpha$  is the  $F$  statistic at  $P = 0.05$  with  $2d + 2$  and  $2n - 2d$  degrees of freedom.

## RESULTS

Good plant growth and flower production were achieved at both experimental sites in the 2 yr of this study. As herbicide-susceptible Maxxa is the recurrent parent for development of herbicide-resistant Nova, their flowering was synchronous, thus ensuring ample opportunity for PGF via cross fertilization. As a result of standard agronomic practices using pesticides to control insect pests in cotton, no bees and very few insects were observed at the Shafter site in both years. In contrast, many honeybees were observed working cotton flowers at the Kearney site.

A total of 160 000 seeds (2000 seeds/sample  $\times$  4 directions  $\times$  5 distances  $\times$  2 locations  $\times$  2 yr) were sampled for herbicide resistance due to PGF. Although not all positive seedlings were assayed by DNA analyses, a minimum of five positive seedlings (out of 2000 planted/sample) were assayed for all samples when available. If there were less than five positive seedlings in a sample, all positive seedlings were subjected to DNA analysis. In 2001, of 70 DNA samples of individual seedlings (from seeds harvested from experimental plots) that survived the herbicide bioassay, 22 were negative for the presence of the transgene. In 2002, 33 of 149 seedlings from experimental plots and 16 of 20 seedlings from field samples that were tested by PCR showed negative results. This indicates that there were moderate to high rates of false positives in the bioassay tests. Values for the DNA-tested plots were corrected, but as not all samples were confirmed by PCR, our data likely overestimate the actual extent of PGF. In addition, two samples suspected of being outliers (Shafter SW 2001, 1 m and field 2002-1, 1625 m) were bioassayed a second time followed by DNA testing of surviving seedlings. Those values were adjusted to the average of the two assays after correcting for false positives based on the DNA tests.

Analysis of variance was conducted with data averaged over distances from the pollen source. The analysis indicates that locations were significantly different ( $P = 0.02$ ) and that samples collected at different directions from the pollen source did not differ significantly ( $P = 0.97$ ). This was confirmed by analyses of individual experiments (data not shown). Analysis of variance was thus conducted on data with distance from pollen source and year as independent variables. Bartlett's test for

homogeneity of variances was conducted for transformed PGF data. Although Bartlett's test indicated that variances were not homogeneous across experiments for original data, Bartlett's tests on transformed data were nonsignificant when combined over years for Kearney ( $P > 0.05$ ) and Shafter ( $P > 0.05$ , data not shown). The data were thus analyzed for each location with year and distance as independent variables. Each mean for distance was based on data points collected from eight samples  $\times$  2000 seeds/sample = 16 000 seeds. Analysis of variance on transformed data indicated that PGF differed significantly with distance for Kearney ( $P < 0.001$ ) and for Shafter ( $P < 0.001$ ). Regression analysis was conducted for each location representing environments with and without pollinators. PGF decreased from 7.65% at 0.3 m to 0.67% at 9 m and 0.32% at 30 m at the Kearney site where honeybees were provided; a negative exponential curve explained over 99% of the variance at this site (Fig. 1). Confidence intervals indicate that at worst, 0.41% PGF is expected at 30 m with honeybees present. At Shafter, where no honeybees were provided, PGF decreased from 4.86% at 0.3 m to 0.30% at 1 m and remained at 0.03% at longer distances; the upper confidence limit was below 0.08% beyond 1 m (Fig. 1).

The results for samples taken from commercial fields neighboring either glyphosate- (Roundup) resistant or bromoxynil-resistant cotton are consistent with those from experimental plots. The values from field samples of bromoxynil-resistant cotton (2002-1) do not differ significantly from those from field samples of glyphosate-resistant cotton (Fig. 2). PGF percentages for field samples taken at approximately 30 m (average 0.34%) were slightly higher than in experimental plots at the same distance from the source (average 0.17% over both locations). The 30 m sample was taken from the edge of the field closest to the herbicide-resistant field and the fields were separated by open space instead of solid-seeded cotton as it was the case for the plots. PGF was sporadic beyond 30 m, ranging from 0.01 to 0.10% at distances between 200 and 1625 m. PGF was on average below 0.1% at 400 m, the minimum distance required for isolation of foundation class seed fields in the USA (California Crop Improvement, 2005, [http://ccia.ucdavis.edu/CCIA/standards\\_frame.htm](http://ccia.ucdavis.edu/CCIA/standards_frame.htm)). An average PGF of 0.04% was detected at 1625 m on the basis of samples taken from three different sites in 3 yr. Because of the low and sporadic nature of PGF beyond 30 m, only 24% of the variation could be explained with the best fit curve compared to 99% at distances below 30 m with honeybee pollinators (Fig. 1 and 2).

## DISCUSSION

Herbicide resistance provides a simple and effective means to monitor PGF, as any resistant seedlings growing from seeds harvested from herbicide-susceptible trap plants are presumed to result from pollen transfer (Marshall et al., 2001; Messeguer et al., 2001; Berkey et al., 2003). Herbicide resistance allows large numbers of seedlings to be screened, permitting detection of rela-

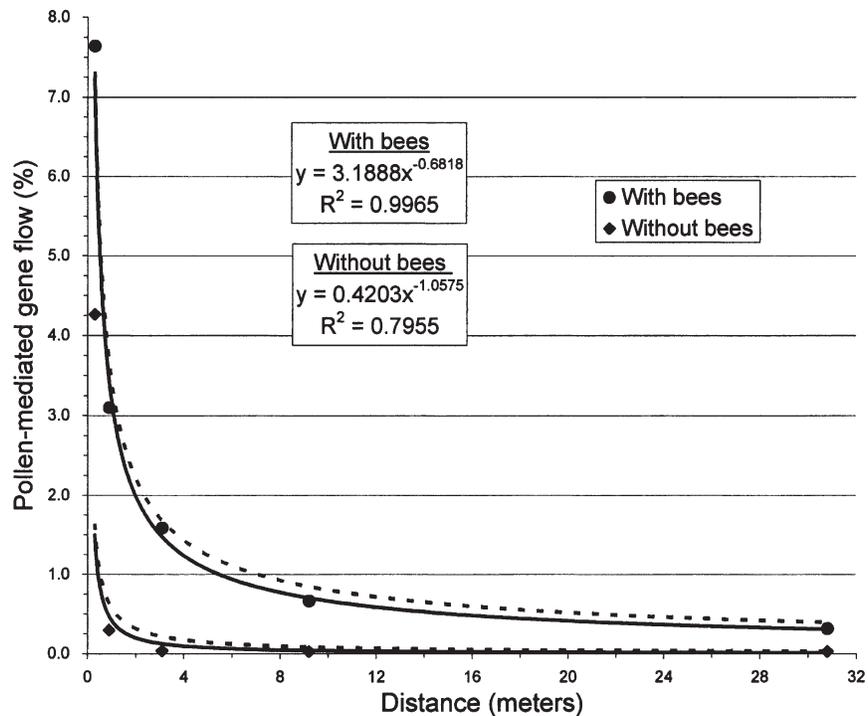


Fig. 1. Pollen-mediated gene flow (PGF) in California with (Kearney, circles) and without (Shafer, diamonds) the addition of honeybees. Each data point represents PGF detected from 16 000 seeds sampled in four directions from a source plot in two growing seasons. Solid lines represent the best fit curve for the two datasets. Broken lines represent 95% upper confidence limit.

tively rare pollen flow events. However, our data highlight the importance of confirming results of the bioassay for bromoxynil resistance with a secondary protein or DNA test. Bromoxynil is a contact herbicide that is

most effective in sunlight when plants are vigorously growing (Jeanes et al., 2003). DNA tests were done on a maximum of five individual plants per 2000 seed sample. A disproportionate number of negative seedling sam-

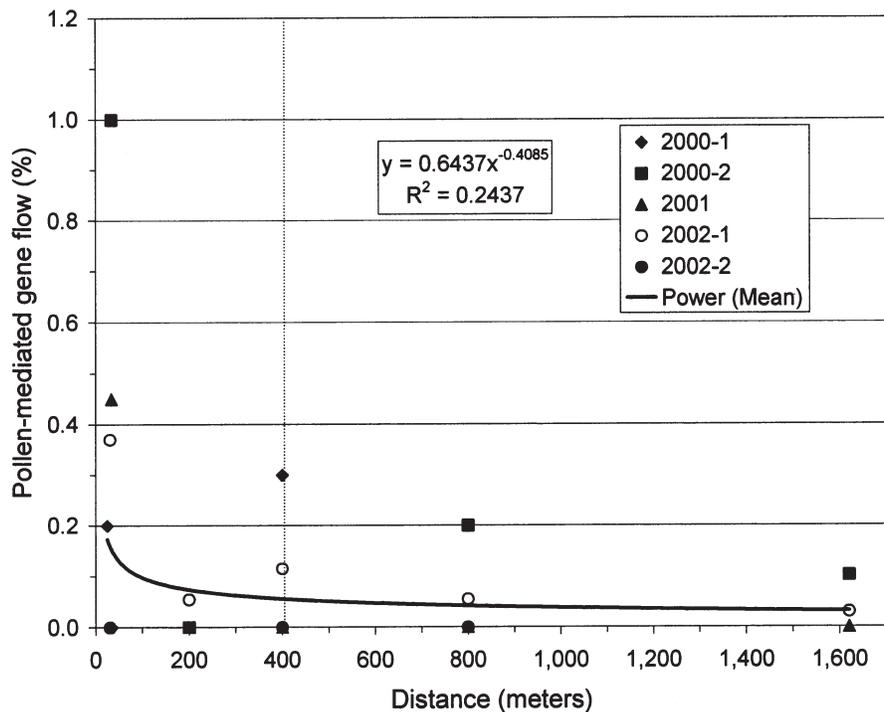


Fig. 2. Pollen-mediated gene flow (PGF) in California collected from neighboring fields separated by open space in five different locations in three years. PGF was calculated on the basis of samples (2000 seeds each) collected at the closest edge of solid-seeded commercial fields (25–34 m), 200, 400, 800, and 1625 m from herbicide-resistant (BXN or Roundup Ready) cotton. Solid line is the best-fit regression curve. Broken vertical line represents the current isolation distance for foundation seed of 400 m.

ples (13/35) in DNA tests in 2002 were from the Kearney NE samples and the 16 negative field seedling samples were also from a single site. All these samples were assayed simultaneously in December 2002–January 2003 when there was a period of cloudiness causing uneven growth and poor expression of symptoms, resulting in false positives (survival of susceptible seedlings) in the bioassay. These results were obtained even under supplemental lighting conditions (see Materials and Methods). On the other hand, no mortality was observed in the control bromoxynil-resistant plants in each flat, suggesting that false negatives (i.e., death of resistant seedlings due to other causes) were very low. Overall, we are confident in the trends in our data, but actual percentages of PGF are likely overestimated because of the occurrence of false positives in the bioassays.

Using bromoxynil in the current study, 25% (55/219) of seedlings were escapes on the basis of DNA analysis. Messeguer et al. (2004) tested gene flow between cultivated and wild rice (*Oryza rufipogon* Griff.) using glufosinate ammonium herbicide resistance as a marker for PGF. Of 71 seedlings surviving the herbicide bioassay, 25 (30%) proved to be escapes after testing using a GUS ( $\beta$ -glucuronidase) assay and progeny analysis. In contrast, in a pollen flow study in alfalfa, we tested 2200 seedlings that survived a bioassay with glyphosate (Roundup). There were no escapes in this bioassay (Teuber et al., 2004). On the basis of DNA analysis, no escapes were observed by Umbeck et al. (1991) for 68 seedlings surviving a kanamycin bioassay. These results emphasize the need to evaluate and confirm bioassay results for gene flow studies.

PGF in cotton is affected by the presence of honeybee pollinators (Fig. 1). With the low pollinator activity at Shafter (characteristic of cotton-growing regions of California), PGF dropped from 4% at 0.3 m (neighboring plants) to 0.3% at 1 m and remained sporadic and below 0.1% up to 30 m in experimental plots. Samples from commercial fields showed that up to 1% PGF can be detected at the edge of neighboring fields (approximately 30 m from source) when open space separates the fields, but this quickly decreases within 200 m to less than 0.1% on average, with 0.04% PGF detected at 1625 m (1 mile) within a field. Providing bees (four hives adjacent to a 0.36 ha plot) increased the percentage and distance of outcrossing, but PGF was still < 1% beyond 9 m from the source plot (Fig. 1). PGF declined exponentially with distance from the source plot, as was found by Kareiva et al. (1994) across the USA, as well as in Argentina and in South Africa. Kareiva et al. (1994) suggested that the differences in PGF among locations in the USA, Argentina, and South Africa were due to the presence of different natural pollinators. This is consistent with data gathered over time in Mississippi, where PGF has shown a gradual decline from 28 to 1% between 1954 and 1991 (Simpson, 1954; Umbeck et al., 1991), likely as a result of a decrease in natural pollinators because of deforestation and use of pesticides. Our data are also consistent with recent studies showing 0% PGF in neighboring fields when separated by open space of 17 m in Arizona, less than 0.22% at distances beyond 1 m in Mis-

issippi, and less than 1% beyond 10 m in Texas (Berkey et al., 2003).

Many factors such as flower structure, pollen attributes, self-incompatibility, wind, and kind and number of pollinators may affect PGF in plants. Cotton blooms from bottom to top with a cream-colored flower opening in the morning shortly after dawn, turning pink in the afternoon and closing at night, never to reopen. The flowers are self-fertile and pollen grains are large and coated with a viscous material that causes them to adhere to each other and prevents them from being carried by wind (McGregor, 1976). Chaney (1985) studied honeybee activity and their relative preference to visit cotton, alfalfa (*Medicago sativa* L.), corn (*Zea mays* L.), safflower (*Carthamus tinctorius* L.), bindweed (*Convolvulus arvensis* L.), and spikeweed [*Hemizonia pungens* (Hook. & Arn.) Torr. & A. Gray] in California. He showed that even though cotton made up the majority of the crop surrounding the beehive, honeybees worked the source of food that gave the largest reward. For example, honeybees traveled 5 km for maize and 8 km for safflower to collect pollen, bypassing cotton. In fact, cotton was the least collected pollen of the six plants studied, although honeybees readily visit cotton for nectar. Vaissière and Vinson (1994) conducted a similar study in the laboratory by presenting honeybees with pollen from cotton, okra [*Abelmoschus esculentus* (L.) Moench], pumpkin (*Cucurbita pepo* L.), corn, carelessnessweed (*Amaranthus palmeri* S. Watson) and sunflower (*Helianthus annuus* L.), representing pollen grains that vary in shape, size and structure. As a result, there is a large difference in how they are moved from flower to flower. For example, corn has large pollen grains with a smooth surface that is carried short distances by wind. Cotton has large echinate pollen grains with long spikes that are covered in a “viscid” material. Except for okra (also in the Malvaceae), significantly less cotton pollen was collected than from other taxa, even though bees visited cotton pollen with equal frequency and duration. Honeybees took considerably longer and carried smaller cubicular loads with cotton pollen. The cubicular loads were up to 39 times less than for corn and sunflower. The authors ruled out size, repellents, and presence of pollenkitt and concluded that the length of the spines on cotton pollen grains prevent them from packing tightly, thus preventing large cubicular loads. This in turn results in little or no transfer of cotton pollen in the hives, as was demonstrated by Loper and Degrandi-Hoffman (1994). The low reward for honeybees in collecting cotton pollen thus acts as a deterrent for collection and accounts for relatively low PGF observed for an insect-visited crop.

Our studies quantify the extent of PGF in cotton under California conditions in both the presence and absence of added pollinators. In the absence of pollinators, significant PGF was observed only at distances of less than 10 m and the majority of PGF occurred between adjacent plants. This study complements the field-scale study by Berkey et al. (2003) across the southern USA and helps define isolation standards for U.S. cottonseed production for different markets.

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