

# Absence of toxicity of *Bacillus thuringiensis* pollen to black swallowtails under field conditions

C. L. Wraight, A. R. Zangerl, M. J. Carroll, and M. R. Berenbaum\*

Department of Entomology, University of Illinois, 320 Morrill Hall, 505 South Goodwin, Urbana, IL 61801

Contributed by M. R. Berenbaum, May 4, 2000

A single laboratory study on monarch butterflies has prompted widespread concern that corn pollen, engineered to express *Bacillus thuringiensis* (*Bt*) endotoxin, might travel beyond corn fields and cause mortality in nontarget lepidopterans. Among the lepidopterans at high potential risk from this technology is the black swallowtail butterfly, *Papilio polyxenes*, whose host plants in the midwestern U.S. are located principally in narrow strips between roads and crop fields. A field study was performed to assess whether mortality of early instar black swallowtails was associated either with proximity to a field of *Bt* corn or by levels of *Bt* pollen deposition on host plants. Potted host plants were infested with first instar black swallowtails and placed at intervals from the edge of a field of *Bt* corn (Pioneer 34R07 containing Monsanto event 810) at the beginning of anthesis. We confirmed by ELISA that pollen from these plants contained Cry1Ab endotoxin ( $2.125 \pm 0.289$  ng/g). Although many of the larvae died during the 7 days that the experiments were run, there was no relationship between mortality and proximity to the field or pollen deposition on host plants. Moreover, pollen from these same plants failed to cause mortality in the laboratory at the highest pollen dose tested (10,000 grains/cm<sup>2</sup>), a level that far exceeded the highest pollen density observed in the field (200 grains/cm<sup>2</sup>). We conclude that *Bt* pollen of the variety tested is unlikely to affect wild populations of black swallowtails. Thus, our results suggest that at least some potential nontarget effects of the use of transgenic plants may be manageable.

Concerns have been raised that environmental impacts have not yet been fully assessed for such genetically engineered crop plants as *Bacillus thuringiensis* (*Bt*) corn, designed to express *Bt* endotoxin for control of *Ostrinia nubilalis* (European corn borer). Authors of a single laboratory study (1) suggested that endotoxin expressed in pollen and distributed throughout the environment may present risks to lepidopteran nontarget species and in particular to *Danaus plexippus*, the monarch butterfly, which feeds on asclepiadaceous weeds that grow in close proximity to cornfields throughout the midwestern United States. Although embraced by the popular press, this brief report has been criticized in the scientific press (2–4), in part because the study did not address dynamics of endotoxin encounters under field conditions. Moreover, this study examined effects on only one species, despite the fact that other species may be at equal or greater risk from exposure to *Bt* endotoxins in pollen.

The eastern black swallowtail *Papilio polyxenes* occurs throughout eastern North America, ranging from southern Canada to Florida; it also occurs west along the eastern Rockies into northern Mexico (5). The larvae feed almost entirely on species of apiaceous species, several of which (e.g., *Daucus carota*, *Conium maculatum*, *Pastinaca sativa*) are found in pastures, along roadsides, edges of cultivated fields, and in waste places, habitats similar to those of the hosts of *D. plexippus* (6). Larval development requires from 2 to 3 weeks, depending on temperature and host plant (7), with three or more generations each season throughout much of the range. Thus, *Papilio polyxenes* represents a nontarget species with a life history that

makes exposure to *Bt* endotoxin in corn pollen likely throughout the Midwest.

We performed a field experiment in which early instar *P. polyxenes* were placed in an array along the edge of a field of *Bt* corn to determine impact of pollen ingestion under field conditions. In addition, we conducted laboratory bioassays to determine the range of toxicity of pollen from this and one other transgenic corn event.

## Materials and Methods

**Field Experiment.** This experiment was performed at the University of Illinois Phillips Tract research area located 1.5 km northeast of Urbana, along the narrow edge of a field of *Bt* corn measuring 30 m wide with rows running 400 m long. The corn tested was Pioneer variety 34R07, which contains Monsanto event 810 and expresses the *Cry1Ab* gene in its pollen. The first of two plots, consisting of an array of five rows of five potted wild parsnip plants (*Pastinaca sativa*) grown from seed collected in Phillips Tract, was placed midway along the eastern edge of the field on July 12, 1999, 24 h after the initiation of pollen release from the field. Three days later, a second array was constructed adjacent to the south edge of the first array. The five rows of each array were spaced 0.5, 1, 2, 4, and 7 m from the field's edge, and the plants within a row were spaced 1 m apart. Both arrays were placed along the eastern edge of the field to maximize the potential for prevailing westerly winds to carry pollen into the test plots. Ten first instar black swallowtails, hatched from eggs obtained from wild-caught females and from females in a laboratory colony, were placed on each plant. Pollen falling on each plant was monitored by a wire-staked microscope slide covered with a thin coat of petroleum jelly (Vaseline). The slides provided an accurate measure of total pollen deposition but probably overestimated the amount of pollen remaining on parsnip foliage. The number of live larvae on each plant was recorded daily for 7 days. The condition of surviving larvae was determined by weighing each larva at the end of the 7 days. Rain fell on July 17, 19, and 20. To prevent the loss of pollen, we retrieved all of the slide traps on July 17 and replaced them with fresh traps on July 18. This second set of traps was removed before the next rainfall and was not replaced. Pollen counts for each array were summed for the two trap samples.

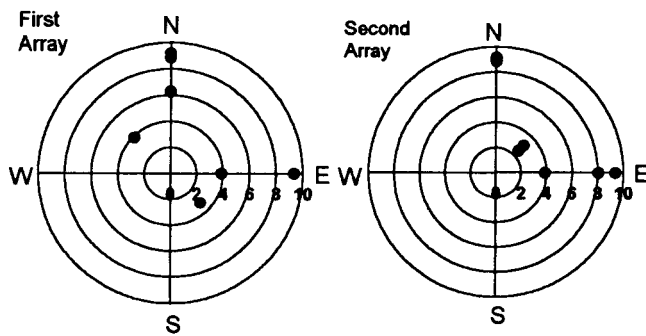
**Laboratory Bioassay.** The Pioneer 34R07 pollen used in laboratory bioassays was collected directly from plants in the field by shaking tassels inside a large plastic bag. The pollen then was brought back to the laboratory, sifted to remove contaminants, and frozen at  $-20^{\circ}\text{C}$ . Non-*Bt* pollen was collected from five rows of non-*Bt* corn (Pioneer 3489) that were planted at the same time as the *Bt* variety. Pollen from Novartis variety Max 454, known

Abbreviation: *Bt*, *Bacillus thuringiensis*.

\*To whom reprint requests should be addressed. E-mail: maybe@uiuc.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.130202097. Article and publication date are at [www.pnas.org/cgi/doi/10.1073/pnas.130202097](http://www.pnas.org/cgi/doi/10.1073/pnas.130202097)



**Fig. 1.** Average wind speeds (km/h) and wind directions during the times that each array was monitored in the field. Direction refers to the direction that the wind traveled. Data were obtained from the Illinois State Water Survey station located 6 km southwest of the field site.

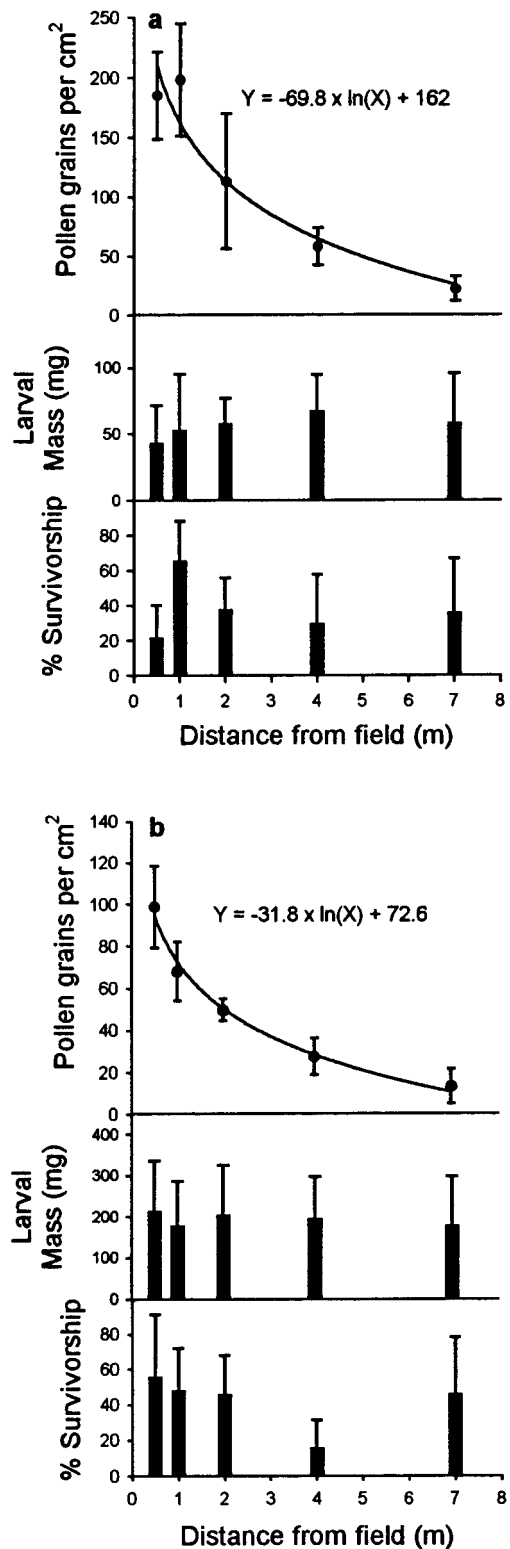
to be insecticidal, was collected and provided to us by John Obrycki (Iowa State University, Ames). Disks, measuring 33.6 mm<sup>2</sup>, were cut from greenhouse-grown wild parsnip foliage by using a paper punch. We pipetted 12.5 μl of a suspension of pollen in acetone onto each treatment disk. Stock suspensions of pollen ranged from 40 mg/ml to 0.0040 mg/ml, yielding doses of 10,000, 1,000, 100, 10, and 1 grain/cm<sup>2</sup>, respectively. Doses were extrapolated from an average (101.5 grains/cm<sup>2</sup>) of counts of pollen grains in six 12.5-μl aliquots of the 0.40 mg/ml solution. We also assayed pollen from Novartis variety Max 454 and a Pioneer non-*Bt* pollen (variety 3489) at 10,000 grains/cm<sup>2</sup>. The non-*Bt* Pioneer variety was selected for its otherwise similar genetic makeup to the *Bt* variety. An acetone control consisted of disks treated only with 12.5 μl acetone. A single first instar was placed together with a leaf disk into a well of a plastic 96-well serum plate. All together, 48 larvae were assayed for each treatment and control. After the wells were filled, the plate was covered with glass and placed inside a plastic box with standing distilled water to maintain humidity. If a disk was completely consumed, it was replaced with an untreated disk. The number of larvae surviving was recorded daily for 3 days.

**Endotoxin Quantification.** Levels of Cry1Ab endotoxin in pollen were quantified by ELISA. Pollen was removed from the freezer and ground with a mortar and pestle and subsequently placed in a Wiggle Bug Amalgamator (Crescent Dental Manufacturing, Chicago) for 1 min. A 100-mg quantity of each pollen type then was analyzed by using a kit from Agdia (Elkhart, IN) designed to quantify Cry1Ab endotoxin in transgenic crops. Two sets of aliquots from each pollen type were analyzed.

**Results**

For 3 of the 7 days that the first array was in place, winds were from the west, whereas westerly winds were recorded for 5 of the 7 days that the second array was in place (Fig. 1). In the first array, pollen load declined steeply from a mean of 210 pollen grains/cm<sup>2</sup> at a distance of 0.5 m from the cornfield to a mean of 26 grains/cm<sup>2</sup> at a distance of 7 m (Fig. 2a). In the second array, pollen load declined from an average of 100 grains/cm<sup>2</sup> adjacent to the field to 11 grains/cm<sup>2</sup> at the furthest distance (Fig. 2b). No significant relationships between larval survivorship or mass were detected either as a function of distance from the edge of the field or as a function of pollen deposition (Table 1).

In the laboratory bioassays involving leaf disks treated with pollen, the pollen containing endotoxin associated with event 810 (Pioneer 34R07) had no effect on survivorship of neonate black swallowtails; there were no differences in survivorship



**Fig. 2.** Pollen loads, larval mass, and survivorship of early instar black swallowtails as a function of distance from the edge of field of *Bt* corn. (a) First array. Plotted data are the mean and SD of pollen counts associated with the five plants in each row. The regression was based on average total pollen counts at each distance from the edge of the field ( $F = 32.3$ ,  $df = 1,3$ ,  $P = 0.0108$ ,  $r^2 = 91.5$ ). (b) Second array. The regression for pollen count as a function of distance from the field was based on averaged pollen counts at each distance ( $F = 285$ ,  $df = 1,3$ ,  $P = 0.0005$ ,  $r^2 = 98.9$ ). Total pollen counts were based on the summed counts for the two sequentially placed traps in each pot.

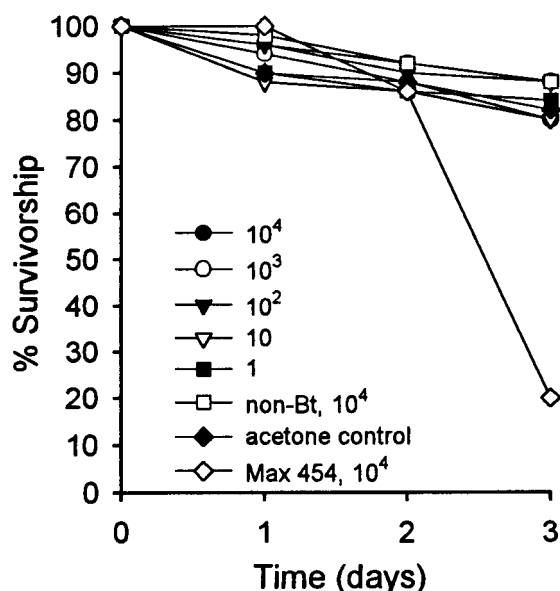
**Table 1. Regressions of survivorship and larval mass on distance from edge of corn field and pollen deposition**

Array	Degrees of freedom	Mean squares	F statistic	Probability	Regression coefficient
<b>First</b>					
Survivorship vs. pollen					
Linear model	1	193.5	0.635	0.484	0.116
Residual	3	304.6			
Survivorship vs. distance					
Linear model	1	35.9	0.100	0.772	-1.13
Residual	3	357.1			
Mass vs. pollen					
Linear model	1	154.4	2.98	0.183	-0.103
Residual	3	51.8			
Mass vs. distance					
Exp. model	1	0.074	7.016	0.077	0.128
Residual	3	0.011			
<b>Second</b>					
Survivorship vs. pollen					
Exp. model	1	0.002	0.014	0.913	-0.088
Residual	3	0.143			
Survivorship vs. distance					
Log model	1	212.7	1.799	0.273	-6.21
Residual	3	118.4			
Mass vs. pollen					
Exp model	1	0.0073	1.183	0.356	0.168
Residual	3	0.0062			
Mass vs. distance					
Log model	1	142.1	0.528	0.526	-5.65
Residual	3	277.0			

All analyses are based on means. Only the best fitting of the linear, logarithmic, and power models are presented.

between larvae fed disks containing this pollen and larvae fed disks containing either a non-*Bt* pollen or acetone (Fig. 3). However, there was a significant effect of the Max 454 pollen

associated with event 176. Antibody assays of Cry1Ab endotoxin revealed that the pollen expressing the 176 event contained more than 40 times as much endotoxin on average ( $90.5 \pm 2.6$  ng/g) as the pollen expressing the 810 event ( $2.1 \pm 0.3$  ng/g).



**Fig. 3.** Survivorship of black swallowtails on *Bt*-, control-, and non-*Bt* pollen-treated leaf disks of wild parsnip. At the end of the third day, only the Max 454 pollen caused significant mortality compared with the acetone control ( $X^2 = 31.4, P < 0.0001$ ) and non-*Bt* Pioneer variety ( $X^2 = 38.9, P < 0.0001$ ).

### Discussion

Although the rate of mortality through the first two instars of development was high in our field experiment (Fig. 2), we found no evidence that pollen from corn contributed to that mortality. The high mortality was more likely caused by predation; for example, on two occasions while larvae were being counted, we observed predaceous arthropods in the act of attacking them. Not only were we unable to attribute any of the mortality to pollen exposure, it also appears likely that exposure to much higher concentrations of this pollen would not affect survivorship. At 10,000 grains/cm<sup>2</sup>, pollen did not affect survivorship of first instar black swallowtails in the laboratory. This concentration of pollen was 40 times higher than the highest measure of pollen exposure in the field and is probably higher yet than the actual pollen concentrations in the field on wild parsnip leaves, which are smooth and therefore likely to shed pollen. Although it is possible that longer-term exposure to *Bt* pollen may affect survivorship or reproduction in both the field and laboratory, it did not appear that such an effect was likely; there were no obvious differences in size of the larvae as a function of event 810 pollen dose at the conclusion of the assay and no significant weight differences among larvae as a function of distance from the corn field or pollen level.

The use of plants genetically engineered for resistance to pests has been touted as an environmentally compatible alternative to synthetic organic insecticides (8), offering advantages such as reduced impacts on nontarget species and decreased contami-

nation of groundwater, soil, and air. Although there is potential for nontarget impacts, particularly on close relatives of target species, our study suggests that under field conditions such impacts can be managed by use of constructs with tissue-limited expression or, at the very least, by event selection. Larvae of the black swallowtail, by virtue of their multivoltine life history and broader host range in the Midwest, are as, if not more, likely to encounter corn pollen between late June and mid-August during its 8- to 10-day period of anthesis than are larvae of the monarch butterfly, yet under actual field conditions no mortality directly or indirectly attributable to ingestion of endotoxin-containing corn pollen could be detected in our study. This is not to say that monarch butterflies are unaffected by *Bt* corn pollen; however, field studies as well as appropriately controlled laboratory studies are necessary before such a conclusion can be drawn.

Losey *et al.* (1) expressed an urgent need for data “to evaluate the risks associated with this new agrotechnology and to compare these risks with those posed by pesticides and other pest-control tactics.” According to the Environmental Protec-

tion Agency, more than 80 million pounds of conventional insecticides and miticides (active ingredients) were applied by the agricultural sector as recently as 1997 (<http://www.epa.gov/oppead1/pestsales/97pestsales/table3.htm>). The broad nontarget effects of these conventional pesticides are well documented and constitute a significant environmental problem (9). The rational and careful use of genetically engineered crops, although not demonstrably entirely without nontarget impacts, has considerable potential to reduce nontarget impacts broadly and substantially by reducing inputs of less selective chemical pesticides. Such technology thus may potentially contribute to preserving agroecosystem biodiversity relative to other pest management options.

We thank E. Green, R. Petersen, D. Skirvin, K. Lustofin, C. Wraight, and S. Wraight for assistance in the fieldwork and F. Gould and P. Raven for comments on the manuscript. This work was supported by a grant to C.L.W. from the University of Illinois Environmental Council.

1. Losey, J. E., Rayor, L. S. & Carter M. E. (1999) *Nature (London)* **399**, 214.
2. Hodgson, J. (1999) *Nat. Biotechnol.* **17**, 627.
3. Beringer, J. E. (1999) *Nature (London)* **399**, 405.
4. Shelton, A. M. & Roush, R. T. (1999) *Nat. Biotechnol.* **17**, 832.
5. Opler, P. A. & Krizek, G. O. (1984) *Butterflies East of the Great Plains* (Johns Hopkins Univ. Press, Baltimore).
6. University of Illinois at Urbana Champaign (1979) *Weeds of the North Central States*, North Central Regional Pub. No. 36, Circular 718 (Univ. of Illinois Agricultural Experiment Station, Urbana).
7. Blau, W. S. (1981) *Oecologia* **48**, 116–122.
8. Liu, K. (1999) *Food Technol.* **53**, 42–48.
9. Benbrook, C. M. (1996) *Pest Management at the Crossroads* (Consumers Union, Yonkers, NY).