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Evaluation of dietary effects of transgenic corn pollen expressing Cry3Bb1 protein on a non-target ladybird beetle, *Coleomegilla maculata*

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Abstract

A transgenic corn event (MON 863) has been recently developed by Monsanto Company for control of corn rootworms, *Diabrotica* spp. (Coleoptera: Chrysomelidae). This transgenic corn event expresses the *cry3Bb1* gene derived from *Bacillus thuringiensis* (Berliner), which encodes the insecticidal Cry3Bb1 protein for corn rootworm control. A continuous feeding study was conducted in the laboratory to evaluate the dietary effect of MON 863 pollen expressing the Cry3Bb1 protein on the survival, larval development, and reproductive capacity of the non-target species, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). First instar *C. maculata* (less than 24 h old) and newly emerging adults (less than 72 h old) were fed individually on a diet mixture containing 50% of MON 863 pollen, non-transgenic (control) corn pollen, bee pollen (a component of normal rearing diet), or potassium arsenate-treated control corn pollen. In the larval tests, 96.7%, 90.0%, and 93.3% of *C. maculata* larvae successfully pupated and then emerged as adults when fed on MON 863 pollen, non-transgenic corn pollen, and bee pollen (normal rearing) diets, respectively. Among the larvae completing their development, there were no significant differences in the developmental time to pupation and adult emergence among the transgenic corn pollen, non-transgenic corn pollen, and bee pollen diet treatments. All larvae fed on arsenate treated corn pollen diet died as larvae. For tests with adults, 83.3%, 80.0%, and 100% of adult *C. maculata* survived for the 30 days of the test period when reared on diets containing 50% of MON 863 pollen, non-transgenic corn pollen, and bee pollen respectively. While the adult survival rate on MON 863 pollen diet was significantly less than that on the bee pollen diet, there was no significant difference between the MON 863 and non-transgenic corn pollen treatments. During the period of adult testing, an average of 77, 80, and 89 eggs per female were laid by females fed on the MON 863 pollen, control corn pollen, and bee pollen, respectively; no significant differences were detected in the number of eggs laid among these treatments. These results demonstrate that when offered at 50% by weight of the dietary component, transgenic corn (MON 863) pollen expressing Cry3Bb1 protein had no measurable negative effect on the survival and development of *C. maculata* larvae to pupation and adulthood nor any adverse effect on adult survival and reproductive capacity. Relevance of these findings to ecological impacts of transgenic Bt crops on non-target beneficial insects is discussed.

Introduction

Transgenic corn expressing Cry1Ab protein, derived from the soil bacterium, *Bacillus thuringiensis* (Bt) Berliner subsp. *kurstaki*, was first introduced in the U.S. in 1996 for the control of European corn borer, *Ostrinia nubilalis* Hübner (Bolin et al., 1996). Because of its high efficacy and improved Bt toxin delivery system, this transgenic corn cultivar has since become increasingly adopted by farmers in the United States (U.S.) as an alternative to chemical sprays

against *O. nubilalis* (Rice & Pilcher, 1998). Although the use of transgenic corn for control of *O. nubilalis* has reduced insecticide use in many corn growing regions (see review in Shelton et al., 2002), the other key corn pest, corn rootworm (mainly western corn root worm, *Diabrotica virgifera virgifera* Le Conte, and northern corn rootworm, *Diabrotica barberi* Smith and Lawrence), continue to require frequent soil-applied insecticide treatments in many U.S. corn growing regions (Pilcher & Rice, 1998; Kuhar et al., 1997; Davis & Coleman, 1997). With the development of physiological resistance to several classes of insecticides and adaptation to crop rotation, corn rootworms as a group have become one of the most challenging pests to control and require effective alternatives to the soil insecticide and crop rotation-based control strategies (Krysan et al., 1984; Foster et al., 1986; Sammons et al., 1997; O'Neal et al., 1999).

Recently, Monsanto Company has developed a genetically modified corn event that expresses the *cry3Bb1* gene derived from *B. thuringiensis*. The *cry3Bb1* gene encodes the insecticidal Cry3Bb1 protein, which has insecticidal activity against Coleoptera species such as corn rootworms, *Diabrotica* spp. and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Donovan et al., 1992). Recent field trials have demonstrated that this transgenic corn event expressing Cry3Bb1 protein is highly efficacious against larvae of *Diabrotica* species (Monsanto Company, unpublished data) and can provide corn growers an effective tool for managing corn rootworms. This transgenic corn event, known as MON 863, is currently under review by the U.S. Environmental Protection Agency pending commercial release (U.S. EPA, 2002).

As part of an ecological impact assessment for transgenic Bt crops, possible impacts of this transgenic corn event expressing Cry3Bb1 protein on relevant non-target beneficial insects, such as predators in the order Coleoptera, should be evaluated. The lady bird beetle, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae), a North American native species, is a very common and abundant predator found in many cropping systems (including corn) throughout the U.S. Both adults and larvae of *C. maculata* are predaceous, feeding on aphids (e.g., corn leaf aphids), thrips, and eggs and larvae of some lepidopterous pests (Hoffmann & Frodsham, 1993). When insect prey are scarce, they also utilize plant (including corn) pollen as a supplemental food source. Although *C. maculata* larvae reared solely on pollen are often not able to complete development (Pilcher & Obrycki, 1994), pollen may make up 50% of their total diet (Hoffmann & Frodsham, 1993; Riddick & Barbosa, 1998; Don Vacek, USDA-APHIS Mission Plant Protection Center, pers. comm.). Thus, pollen produced by transgenic Bt corn plants expressing the Cry3Bb1 protein may be a risk factor affecting populations of *C. maculata* in or near cornfields.

In this study, a laboratory bioassay was developed for evaluation of the potential dietary effect of transgenic Bt corn pollen expressing Cry3Bb1 protein on both larvae and adults of *C. maculata*. Both larvae and adults were offered diets consisting of 50% of transgenic (Bt), non-transgenic (control) corn pollen, and potassium arsenate-treated control pollen (a reference treatment). Survival, development, and reproductive potential were compared among the different treatments to determine the effect that the transgenic corn pollen may have on any of these observed response variables.

Materials and methods

Insect sources. Both larvae and adults of *C. maculata* used in the study originated from egg masses obtained from the USDA-APHIS Mission Plant Protection Center (Mission, TX). Egg masses of *C. maculata* were shipped by FedEx One Day Freight Service from the Mission Plant Protection Center to Monsanto (St. Louis, MO) in Styrofoam coolers on a weekly basis during the period of the study. Upon receipt of the shipment, egg masses were placed immediately in plastic Petri-dishes (8 cm in diameter) lined with filter paper and incubated in a Percival growth chamber (Percival Scientific, Inc, Boone, IA) at about 27 °C.

First instars (within 24 h after hatching) were used in different diet treatments. For tests with adults, *C. maculata* larvae were reared until pupation in a ventilated plastic box (approximately 1000 ml) placed in the Percival growth chamber using a semi-artificial diet comprised, by weight, of 50% lyophilized eggs of Mexican fruit fly, *Anastrepha ludens* (Loew), and 50% bee pollen. This diet was developed by the USDA-APHIS Mission Plant Protection Center and used successfully for rearing both larvae and adults of *C. maculata* in the laboratory (Riddick & Barbosa, 1998; Don Vacek, USDA-APHIS Mission Plant Protection Center, pers. comm.). Adults were used 72 h after emerging from pupae.

Pollen source. The transgenic corn pollen for this study originated from the corn event MON 863, which had been genetically transformed with a linearized DNA fragment derived from the plasmid ZMIR13L. The transformation vector (linearized DNA fragment of ZMIR13L) contains coding sequences for the *cry3Bb1* gene and a selectable marker (neomycin phosphotransferase II). The promoter used for transformation of the corn event MON 863 consisted of four tandem copies of active sequence 1 (4-AS1) and a single portion of the sequence 35 (35S) from the cauliflower mosaic virus (Odell et al., 1985; Lam & Chua, 1990; U.S. EPA 2002). The non-transgenic corn pollen used in this study originated from the corn line MON 846 (also known as RX670), which had not been genetically modified, but had background genetics representative of the test event MON 863. For tests with larvae, both transgenic and non-transgenic pollen were collected in 1999 from corn plants grown at the Monsanto Field Station (Monmouth, IL). For tests with adults, both transgenic and non-transgenic corn pollen were collected from corn plants grown in 1999 on the Monsanto Argentina Research Farm (Fontezuela, Salto and Rojas, Argentina).

For pollen collection, flowering tassels were contained in paper bags and then each tassel stalk was hand-shaken 3–5 times. All pollen shed into the bag was sifted using a sieve of approximately 200 microns, and then poured into 50 ml centrifuge tubes. Pollen collected from different corn plants of the transgenic event or non-transgenic control line were pooled and thoroughly mixed together prior to use in the study. All pollen samples collected from the transgenic corn event and non-transgenic control line were properly labeled and placed in dry ice within about 1 h of field collection. Pollen samples were then shipped on dry ice to Monsanto (St. Louis, MO) via FedEx express freight service (in the U.S.) or World Courier express service (in Argentina). Upon receipt at Monsanto, all pollen samples were stored at −80 °C for about nine months prior to use in the study.

Analysis of the transgenic corn pollen by a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) three days prior to the study established that levels of the Cry3Bb1 protein in the transgenic corn (MON863) pollen used in tests with both larvae and adults ranged from 37.4–101.0 μg/g fresh weight of pollen, with a mean concentration of 77.1 μg/g fresh weight of pollen. No Cry3Bb1 protein was detected in any of the non-transgenic control corn (MON 846) pollen used in both tests.

Diet treatment. For tests with both *C. maculata* larvae and adults, there were four diet treatments based on four types of pollen used: transgenic (MON 863) pollen, non-transgenic control (MON 846) pollen, potassium arsenate-treated control (MON 846) pollen, and bee pollen. The arsenate-treated pollen was used as a positive control, while the bee pollen was included in the study as an assay control because bee pollen is a component of the normal rearing diet for *C. maculata* (Riddick & Barbosa, 1998). The use of potassium arsenate for a positive control treatment was based on the observation that arsenical compounds have been used for many years for the control of various insect pests and that control is achieved through ingestion by the pest species and not simply contact with these toxins (Metcalf et al., 1962). The objective of including the arsenate-treated pollen as a positive control was to qualitatively demonstrate that the test system could detect the effects of toxicants administered through the diet. However, we point out that this positive control should not serve as a reference for comparison of the amount of pollen consumed by *C. maculata* in different pollen treatments.

All the diets consisted of equal amounts by weight of lyophilized Mexican fruit fly eggs and pollen. The lyophilized Mexican fruit fly eggs used in all the diet treatments were obtained from the USDA-APHIS (Mission, TX) and the bee pollen used in the assay control were purchased from Vitamin World (St. Ann, MO). Both the fruit fly eggs and the bee pollen were ground with a mortar and pestle and put through a 650-micron sieve (American Scientific Product, McGaw Park, IL) prior to inclusion in the diet mixtures. Fruit fly eggs and bee pollen were not put through the finer sieves (200 micron) used previously for the corn pollen because of the difficulty in grinding these materials to the size of a corn pollen grain. However, this should not have an impact on the study because it is not necessary to grind these two components for rearing of *C. maculata* (Riddick & Barbosa, 1998; Don Vacek, USDA-APHIS Mission Plant Protection Center, pers. comm.). To ensure the homogeneity of the diet mixture, fly eggs and transgenic corn pollen, non-transgenic corn pollen, arsenate-treated corn pollen, or bee pollen were thoroughly mixed in a centrifuge tube by manually rolling the tube until all the components (fly eggs and pollen grains) were visually uniform. All the diets were prepared one day before initiation of the study and stored in a −20 °C freezer between uses during the study. Sub-samples of both Bt (MON 863) and non-Bt (MON 846) corn pollen were also stored

in the same −20 °C freezer and analyzed by ELISA at the end of the study to determine the stability of the Cry3Bb1 protein under those storage conditions.

To generate the positive control pollen, the potassium arsenate salt (purchased from Sigma Chemical Company, St. Louis, MO) was first dissolved in deionized water (at a concentration of 198 μg/ml for larval tests and 2000 μg/ml for adult tests) and then mixed with the control (MON 846) pollen in a crucible at a ratio of 0.2 g pollen per ml of the potassium arsenate solution. Prior to inclusion in the diet, the mixture of potassium arsenate solution and the non-transgenic corn pollen was dried in a ventilated hood under room temperature for 48 h. The dried potassium arsenate-treated control pollen (hereafter referring to as positive control pollen) was used in the diet mix. A pre-trial assay established that corn pollen treated with these doses of potassium arsenate was lethal to *C. maculata* larvae and adults.

Testing procedure. For tests with both larvae and adults, newly hatching larvae or emerging adults were selected randomly and placed in individual test arenas containing the diet mix for appropriate treatments. The test arenas consisted of polystyrene petri dishes (10 cm in diameter, 1.5 cm depth) with covers which allowed for ventilation; a disc of filter paper (8.5 cm in diameter) was placed on the bottom of each petri dish to absorb moisture and provide a walking surface for the test insect. Each test arena contained one larva or adult. A total of ten individual larvae or adults per diet treatment were tested in each assay, and each assay was replicated three times with different batches of insects for each replicate.

Water and diet mixes were presented to test larvae or adults on a glass microscope slide. Diet was placed at one end of the microscope slide and a small piece ($\approx 1 \times 1 \times 0.2$ cm) of sponge moistened with water was placed at the other end. During the experiment, test arenas were placed in an incubator at a target temperature of $\approx$ 27 °C and photoperiod of L14:D10 h. For each test insect (larva or adult), diets and water were changed on a daily basis. The amount of the diet presented to each insect on each day was not quantified; however, no test larva or adult depleted the diet supply in one day.

Observations. For tests with larvae, all the test insects were monitored daily for changes in developmental stage until they died or completed their development to pupae and adults. In addition to observations of the developmental stage and mortality, newly emerging adults (less than 24 h old) were also weighed. For tests with adults, test insects were also monitored daily for mortality until they died, or for a maximum of 30 days after the initiation of testing. In addition to observations of the adult mortality, the numbers of eggs laid (if any) by each test adult were counted daily from the day when eggs were first observed, and then removed from the test arena.

Data analysis. A randomized complete block design with three blocks (i.e., replicates) for each diet treatment was used in the experiment. For the larval experiment, the response variables analyzed included the proportion of the larvae that successfully developed into pupae and adults, developmental time (days) from larvae to pupae and adults, and also weight of the newly emerging adults. For the experiment with adults, the response variables included the percentage of surviving adults at the end of 30 days of exposure to different treatments as well as the number of eggs laid per female adult in each treatment.

For the survival rate of test larvae to pupae and adults as well as test adults, the following linear model was applied to fit the data: $y = \mu + \text{diet} + \text{rep} + e$, where y is the survival rate of test larvae or adults, μ the mean survival rate, diet the type or treatment of the diet, rep the number of replicates of the treatment; and e the error term of the model. To fit the model, data were transformed using arcsin function and then analyzed by the PROC ANOVA procedure (SAS Institute, 1995). The Least Square Difference (LSD) procedures were used for pairwise comparison of mean survival rate among different diet treatments. Because all the pupae developed from the larvae subsequently emerged to adults, the survival rate of the test larvae to pupae was identical to that of the test larvae to adults. The number of eggs produced per replicate was adjusted to the mean number of eggs laid per female and then analyzed using the same ANOVA procedure.

For the developmental time of test larvae to pupation and adult emergence and weight of the newly emerging adults, the following linear model was applied to fit the data: $y = \mu + \text{diet} + \text{rep} + \text{diet}^{*}\text{rep} + \text{resid}$, where y is the developmental time (days) from larvae to pupa and adults or the weight of emerging adults, μ the mean developmental time or mean weight, rep the number of replicates of the treatment, diet the type or treatment of the diet, diet*rep the interaction of rep with diet, and resid the residual effect of

the model. Data on the developmental time and the weight were fitted using PROC MIXED procedure (SAS Institute, 1995). The Least Square Difference (LSD) procedures were used again for pairwise mean comparisons among different diet treatments. Because all the test larvae fed on the arsenate-treated pollen diet died before pupation, there were no observations on the developmental time from larvae to pupae and adults, nor observations on the weight of adults for this treatment. Thus the arsenate-treated pollen treatment was not included for these analyses.

Results

Effect on larvae. The tests were terminated when the larvae of *C. maculata* in different treatment groups had either died or completed development to pupae and adults. The survival rate of *C. maculata* from larvae to pupation and/or to adult emergence was as follows: 96.7% for transgenic corn (MON 863) pollen, 90.0% for non-transgenic corn (MON846) pollen, 93.3% for bee pollen (assay control), and 0% for arsenate-treated (positive control) corn pollen (Table 1).

ANOVA showed that there were no significant differences in the proportion of larval survival or development to pupae and adults between the assay control and the test or control pollen groups; nor were there any significant differences between the test and control pollen groups. There were significant differences in the larval survival and development to pupae and adults between the larvae in the reference pollen group and transgenic corn pollen, non-transgenic corn pollen, or assay control groups. The 100% mortality of the larvae in the arsenate-treated pollen group demonstrated that *C. maculata* larvae had actively fed on the pollen; thus the assay was effective in determining the dietary effect of the corn pollen on the larvae.

Among the larvae completing development, there were no significant differences in the development time to pupation and to adult stages among the transgenic corn pollen, non-transgenic corn pollen and assay control groups (Table 1). Also there were no significant differences in the mean weight of the adults emerged from the larvae reared between any of the transgenic corn pollen, non-transgenic corn pollen and assay control groups (Table 1).

Effect on adults. While no mortality occurred in transgenic corn pollen, non-transgenic corn pollen and assay control diet treatments in the first two weeks of tests, all adults fed on arsenated-treated corn pollen diet died within seven days of diet exposure (Figure 1). The total mortality of the adults in the reference pollen treatment demonstrated that *C. maculata* adults had actively fed on the pollen; thus the assay was effective in determining the dietary effect of toxicant in the corn pollen on adults of *C. maculata*. At test termination 30 days after exposure to diet treatments, the survival rate of adult *C. maculata* was 83.3% for transgenic corn pollen, 80.0% for non transgenic corn pollen, 100% for assay control (bee pollen), and 0% for the reference (arsenate-treated) corn pollen (Table 2). Statistical analysis of the data showed that there were no significant differences in the adult survival rate between the transgenic and non-transgenic corn pollen treatments. There were significant differences in the survival rate of adults between the reference pollen and transgenic corn pollen, non-transgenic corn pollen, or assay control diet. The percentage of adults surviving on transgenic and non-transgenic corn pollen diets was significantly less than those adults on the assay control diet. This indicated that corn pollen as a diet component for adult *C. maculata* might be poorer in nutrition than bee pollen, which has been used as an optimal diet component for rearing *C. maculata* in other studies (Riddick & Barbosa, 1998). During the entire period of adult testing, an average of 77, 80, and 89 eggs per female were laid by test females fed on the MON 863 pollen, control corn pollen, and bee pollen, respectively. No significant differences were detected in the number of eggs laid among these treatments (see Table 2).

Stability of Cry3Bb1 protein in test corn pollen. Analysis of sub-samples of transgenic corn pollen by ELISA at the end of study indicated that the level of the Cry3Bb1 protein ranged from 49.3 to 135.0 μg/g pollen for the test transgenic corn pollen stored in a -20 °C freezer for approximately 49 days (during the course of the study). Compared with previously estimated levels of the variant Cry3Bb1 protein (37.4–101.0 μg/g of pollen), this result confirmed that there was no reduction in the level of the Cry3Bb1 protein in the test Bt corn pollen stored at -20°C during the course of the study.

Discussion

Results from this study demonstrated that, when offered as a dietary component (at a concentration of

Table 1. Survival and development of *Coleomegilla maculata* from larvae to adult emergence on different diet mixes of lyophilized fruit fly eggs and pollens

Diet mix of fruit fly eggs (50%) mixed with 50% of:	Replicate	Number of larvae tested	Number of larvae developing to adults[a]	Percent survival (±SE) to adults (%)[b]	Days to pupation (Mean ± SE)[b]	Days to adult emergence	Adult mass (mg, mean ± SE)[b] (mean ± SE)[b]
Transgenic corn pollen (MON863)	A	10	10	100	12.3 ± 0.84	16.3 ± 0.84	11.2 ± 0.58
	B	10	10	100	12.8 ± 0.83	17.1 ± 0.89	9.7 ± 0.86
	C	10	9	90	12.9 ± 0.96	16.9 ± 0.96	11.7 ± 0.42
	Grand	N = 30	n = 29	Mean = 96.7 ± 3.3a	12.7 ± 0.54a	16.8 ± 0.55a	10.9 ± 0.39a
Control corn pollen (MON846)	A	10	10	100	12.4 ± 0.60	16.4 ± 0.60	10.9 ± 0.56
	B	10	9	90	13.2 ± 1.88	17.2 ± 1.88	10.7 ± 0.88
	C	10	8	80	13.0 ± 0.78	17.0 ± 0.78	10.6 ± 0.54
		N = 30	n = 27	Mean = 90.0 ± 5.5a	12.9 ± 0.56a	16.9 ± 0.57a	10.7 ± 0.40a
Bee pollen	A	10	9	90	14.8 ± 0.70	18.8 ± 0.70	10.8 ± 0.67
	B	10	10	100	13.4 ± 1.00	17.4 ± 1.00	10.2 ± 0.67
	C	10	9	90	13.2 ± 0.68	17.2 ± 0.68	10.1 ± 0.42
	Grand	N = 30	n = 28	Mean = 93.3 ± 4.6a	13.8 ± 0.55a	17.8 ± 0.56a	10.4 ± 0.39a
Arsenate-treated pollen	A	10	0	0			
	B	10	0	0			
	C	10	0	0			
	Grand	N = 30	n = 0	Mean = 0.0 ± 0.0b			
ANOVA:							
F =	–	–	–	26.7	1.21	1.05	0.47
df =	–	–	–	3 and 6	2 and 4	2 and 4	2 and 4
P =	–	–	–	0.0007	0.3892	0.4309	0.6548

[a]All pupae successfully emerged to adults; thus the number of larvae that developed to pupation was equal to the number that emerged to adults.
[b]Grand means followed by the same letter in the same column are not significantly different at the 0.05 error level according to ANOVA and LSD tests. Standard errors (SE) for grand means of percent survival were calculated using the formula of square root of [p * (1-p)/N], where p = percent of survival and N = the number of the larvae tested.

Table 2. Thirty-day survival and egg production of adult *Coleomegilla maculata* exposed to diets containing pollen from various transgenic and non-transgenic sources

Treatment group	Replicate	Number of adults started	Number of females	Percent Survival (±SE) at the end at the trial (day 30) (%)[a]	Number of eggs laid per female (mean ± SE)[a,b]
Transgenic corn pollen	A	10	8	90	62.9 ± 15.6
(MON 863)	B	10	8	80	87.86 ± 16.1
	C	10	7	80	80.60 ± 27.9
	Grand	N = 30	n = 23	83.3 ± 6.8b	77.1 ± 7.4a
Control pollen	A	10	5	70	59.60 ± 25.2
(Mon 846)	B	10	5	80	49.60 ± 18.2
	C	10	4	90	133.50 ± 49.6
	Grand	N = 30	n = 14	80.0 ± 7.3b	80.9 ± 26.5a
Bee Pollen	A	10	2	100	91.0 ± 31.0
	B	10	5	100	96.4 ± 28.2
	C	10	5	100	80.6 ± 25.6
	Grand	N = 30	n = 12	100 ± 0.0a	89.3 ± 4.6a
Arsenate-pollen	A	10	Not determined	0	–
	B	10	Not determined	0	–
	C	10	Not determined	0	–
	Grand	N = 30	–	0 ± 0.0c	–
ANOVA					
F =				96.13	0.1354
df =				3 and 6	2 and 4
P =				0.0001	0.8772

[a]Grand means followed by the same letter in the same column are not significantly different from each other according to ANOVA and LSD tests. Standard errors (SE) for grand means of percent survival were calculated using the formula of square root of [p * (1-p)/N], where p = percent of survival and N = the number of the adults tested.
[b]Includes only those females that laid eggs

50% by weight of the diet composition), transgenic Bt corn (MON 863) pollen containing the Cry3Bb1 protein had no adverse effect on the survival and development of *C. maculata* larvae to adults, nor on the survival and reproductive potential (egg production) of adult *C. maculata*. In another laboratory study, Lundgren & Wiedenmann (2002) exposed both *C. maculata* larvae and adults to combinations of a constant number of living insect prey (one adult aphid every 2–4 days) with mixtures of transgenic corn (MON 863) pollen and non-transgenic corn pollen (Hybrid 34G81, Pioneer Hi-Bred International, Johnston, IA). Although the dietary exposure regime used in Lundgren & Wiedenmann (2002) was different from the one used in the study reported here, no differences were observed in fitness parameters such as larval and adult survivorship, larval developmental time, and reproductive potential for *C. maculata* larvae fed on a pollen mixture containing only non-transgenic corn pollen or 25, 50, 75, and 100% of the transgenic corn pollen. Together these findings suggest that the use of the transgenic corn event MON 863 expressing Cry3Bb1 protein for corn rootworm control poses minimal risk to both larvae and adults of *C. maculata*.

Marvier (2001) criticized certain regulatory non-target organism studies designed with small sample sizes (n = 4 or less), pointing out that the inadequate sample sizes could hinder statistical detection of small treatment differences when the amount of variability is large among replicates. In the study reported here, a total of 120 insects were tested individually in three replicates of four treatments for both tests with larvae and adults of *C. maculata*. In both tests, the magnitude of differences was very small between transgenic and

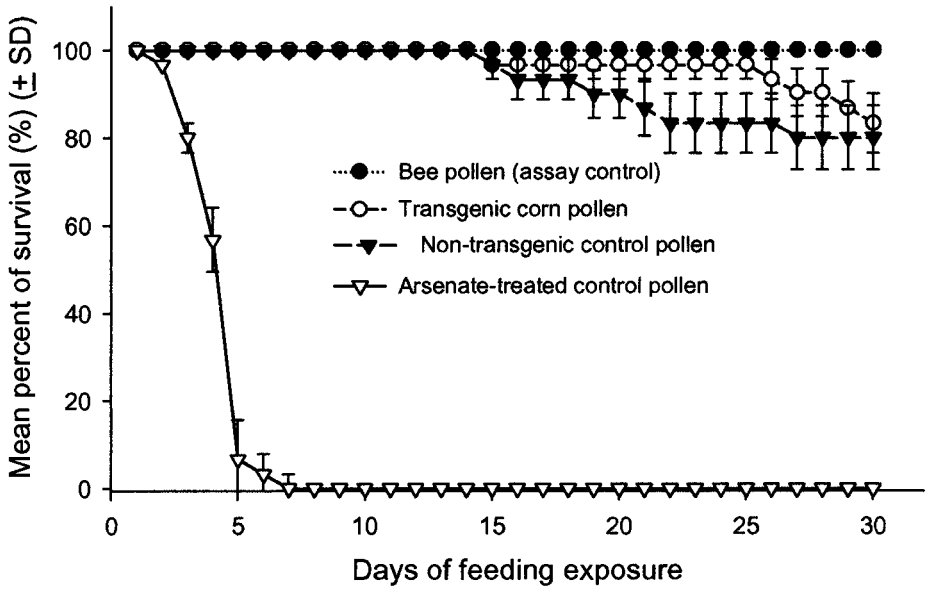

Figure 1. Daily survivorship of *C. maculata* adults fed four diet treatments.

non-transgenic corn pollen treatments (Tables 1 and 2) in the mean percent survival of larvae (96.7% vs. 90.0%) and adults (83.3% vs. 80.0%), larval development time to pupation (12.7 vs. 12.9 days) and adults (16.8 vs. 16.9 days), and weight of emerging adults of *C. maculata* (10.9 vs. 10.7 mg). In addition, the variation among the replicates in those response variables was very small (as evidenced by the values of the standard errors associated with the grand means – Tables 1 and 2). Together, these results suggest that the likelihood of incorrectly accepting the null hypothesis would have been small in these tests, and no biologically significant differences would have been missed by the statistical analysis.

Although the amount of corn pollen consumed by *C. maculata* larvae and adults was not measured in the study reported here, nor in Lundgren & Wiedenmann (2002), there was no reason to assume that test larvae and/or adults preferred insect prey (such as lyophilized Mexican fruit fly eggs or living aphids) to the transgenic corn pollen. In fact, Mexican fruit fly eggs are not natural prey items for *C. maculata*; the lyophilized eggs alone do not support complete development of both larval and adult *C. maculata* for laboratory rearing (Don Vacek, USDA-APHIS Mission Plant Protection Center, pers. comm.). In addition, the total mortality of both larvae and adults exposed to arsenate-treated corn pollen diet in this study indicated that *C. maculata* did not avoid corn pollen mixed with immobile insect prey (i.e., lyophilized Mexican fruit fly eggs). Nevertheless, additional studies are needed to quantify the proportion of different types of corn pollen and fruit fly eggs actually consumed by test larvae and adults of *C. maculata*.

Plant pollen serves as a supplemental food source and can make up a maximum of 50% of the total natural diets of *C. maculata* larvae and adults (Smith, 1965; Hoffmann & Frodsham, 1993; Pilcher & Obrycki, 1994; Pilcher et al., 1997; Riddick & Barbosa, 1998). Smith (1965) and Pilcher & Obrycki (1994) showed that *C. maculata* reared solely on pollen (without alternative prey such as aphids and fruit fly eggs) were not able to complete development. In nature, corn pollen is not likely to be the sole food source because other alternative plant pollens and insect prey (such as aphids and lepidopteran insect eggs) are likely to be abundant in and/or near corn fields (e.g., Cottrell & Yeargan, 1998). Recent studies indicate that levels of the Cry protein in phytophagous insects feeding on Bt crops (if any) are usually many times lower than in the Bt corn plants, and aphids (phloem feeders) do not pick up plant-produced Cry protein when feeding on Bt plants (Head et al., 2001; Dutton, 2002). Thus, field exposure of *C. maculata* to plant-produced Cry3Bb1 via tri-trophic interactions with phytophagous prey, if any, should be very low, and much less important than exposure via direct feeding on corn pollen. Although there are exposure

situations in the field where *C. maculata* might feed exclusively on corn pollen alone for a short time period (e.g., a few days), the concentration (50%) of the transgenic corn (MON 863) pollen in the diet evaluated in the study reported here should represent a worst case for long-term exposure in the field, especially considering that both larvae and adults are continuously exposed to the test diets for the entire period of larval development and 30 days of adult feeding. Nevertheless, further tests are warranted with *C. maculata* and/or other coleopteran predators under realistic field conditions for exposure to corn pollen and insect prey, especially if a new transgenic corn event expresses a significantly higher level of Cry3Bb1 protein (or a different class of Coleoptera-active insecticidal proteins) than the event MON 863 evaluated in the study reported here.

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References

Bolin, P. C, W. D. Hutchison & D. W. Davis, 1996. Resistant hybrids and *Bacillus thuringiensis* for management of European corn borer (Lepidoptera: Pyralidae) in sweet corn. Journal of Economic Entomolology 89: 82–91.

Cottrell, T. E. & K. V. Yeargan, 1998. Influence of a native weed, *Acalypha ostryaefolia* (Suphorbiaceae), on *Coleomegilla maculata* (Coleoptera: Coccinellidae) population density, predation, and cannibalism in sweet corn. Environmental Entomology 27: 1375–1385.

Davis, P. M. & S. Coleman, 1997. Managing corn rootworms (Coleoptera: Chrysomelidae) on dairy farms: the need for a soil insecticide. Journal of Economic Entomology 79: 303–310.

Donovan, W. P., M. J. Rupar, A. C. Slaney, T. Malvar, M. C. Gawron-Burke & T. B. Johnson, 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to Coleoptera species. Applied Environmental Microbiology 58: 3921–3927.

Dutton, A. H. Klein, J. Romeis & F. Bigler, 2002. Uptake of Bt-toxin by herbivores on transgenic maize and consequences for the predator *Chrysoperla carnea*. Ecological Entomology 27 (in press).

Foster, R. E., J. J. Tollefson, J. P. Nyrop & G. L. Hein, 1986. Value of adult corn rootworm (Coleoptera: Chrysomelidae) population estimates in pest management decision making. Journal of Economic Entomology 79: 303–310.

Head, G. P., C. R. Brown, M. E. Groth & J. J. Duan, 2001. Cry1Ac protein levels in phytophagous insects feeding on transgenic corn: implications for secondary exposure risk assessment. Entomologia Experimentalis et Applicata 99: 37–45.

Hoffmann, M. P. & A. C. Frodsham, 1993. Natural Enemies of Vegetable Pests. Cooperative Extension, Cornell University, Ithaca, NY.

Kuhar, T. P., R. R. Youngman & C. A. Laub, 1997. Risk of western corn rootworm (Coleoptera: Chrysomelidae) damage to continuous corn in Virginia. Journal of Entomological Science 32: 281–289.

Krysan, J. L., J. J. Jackson & A. C. Lew, 1984. Field termination of egg diapause in *Diabrotica* with new evidence of extended diapause in *D. barberi* (Coleoptera: Chrysomelidae). Environmental Entomology 13: 1237–1240.

Lam, E. & N. Chua, 1990. GT-1 binding site confers light responsive expression in trangenic tobacco. Science 248: 471–474.

Lundgren, J. G. & R. N. Wiedenmann, 2002. Effects of coleopteran-specific Cry3B toxin from transgenic corn pollen on the fitness of a non-target species, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae). Environmental Entomology (in press).

Marvier, M. 2001. Ecology of transgenic crops. American Scientist 89: 160–167.

Metcalf, C., W. Flint & R. Metcalf, 1962. Destructive and Useful Insects: Their Habits and Control. McGraw-Hill Book Company, New York, NY.

Odell, M., F. Mag & N. Chua, 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

O'Neal, M. E., M. E. Gray & C. A. Smith, 1999. Population characteristics of a western corn rootworm (Coleoptera: Chrysomelidae) strain in east-central Illinois corn and soybean fields. Journal of Economic Entomology 92: 1301–1310.

Pilcher, C. D. & J. J. Obrycki, 1994. Feeding, development and survival of the lady beetle *Coleomegilla maculata* (Coleoptera: Coccinellidae) on transgenic *Bacillus thruringiensis* corn pollen, pp. 261–263. In Proceedings, The Integrated Crop Management Conference, 30 November – 1 December 1994. Iowa State Extension, Iowa State University, Ames.

Pilcher, C. D. & M. E. Rice, 1998. Management of European corn borer (Lepidoptera: Crambidae) and corn rootworms (Coleoptera: Chrysomelidae) with transgenic corn: a survey of farmer perceptions. American Entomologist 44: 36–44.

Pilcher, C. D, J. J. Obrycki, M. E. Rice & L. C. Lewis, 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology 26: 446–454.

Rice, M. E. & C. D. Pilcher, 1998. Potential benefits and limitations of transgenic Bt corn for management of the European corn borer (Lepidoptera: Crambidae). American Entomologist 44: 75–78.

Riddick, E. W. & F. Barbosa, 1998. Impact of Cry3Aa-intoxicated *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and pollen on consumption, development, and fecundity of *Coleomegilla maculata* (Coleoptera: Coccinellidae). Annals of Entomological Society of America 91: 303–307.

Sammons, A. E., C. R. Edwards, L. W. Bledsoe, P. J. Boeve & J. J. Stuart, 1997. Behavioral and feeding assays reveal a western corn rootworm (Coleoptera: Chrysomelidae) variant that is attracted to soybean. Environmental Entomology 26: 1336–1342.

SAS Institute Inc., 1995. SAS Statistical Software, version 3. SAS Institute, Campus Drive, Cary, NC.

Shelton, A. M., J.-Z. Zhao & R. T. Roush, 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annual Review of Entomology 47: 845–881.

Smith, B. C., 1965. Growth and development of coccinellid larvae on dry foods (Coleoptera: Coccinellidae). Canadian Entomologist 97: 760–768.

U. S. EPA (United States Environmental Protection Agency), 2002. Notice of a public comment period for the application to resister a new plant-incorporated protectant: *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production in corn event MON 863 (EPA file symbol 524-LEI: Docket Control Number OPP-30509B). Federal Register 67: 11333–11334.