Toxicity of Transgenic Corn Tissues Expressing Insecticidal Protein of *Bacillus thuringiensis* Berliner to Southwestern Corn Borer (Lepidoptera: Crambidae) in the Laboratory[1]

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Abstract Laboratory studies were conducted to compare the response of *Diatraea grandiosella* (Dyar) larvae to lyophilized transgenic corn tissue expressing the Cry1Ab endotoxin protein of *Bacillus thuringiensis* (Berliner) (Bt) with conventional corn tissue incorporated into a BioServ™ artificial diet. Whorl leaf, stalk, shank, husk, silk or kernel tissues were tested independently in the diet. Larvae fed diet containing conventional corn weighed more and were longer in length than larvae fed diet containing Bt corn for all tissue types included in the study. The number of larvae that survived depended on the tissue type and age of the tissue. Larvae fed diet with kernel tissue expressing Bt toxin had greater weight and body length than larvae fed the other Bt tissue types. The negative effects of Bt corn tissues expressing the Cry1Ab endotoxin protein on growth of *D. grandiosella* was observed, even at the diluted concentrations of toxic tissues incorporated into the diet in this study.

Key Words *Bacillus thuringiensis*, southwestern corn borer, Cry1Ab toxicity

Recent advances in genetic engineering have allowed for the insertion of *Bacillus thuringiensis* Berliner (Bt) genes and resultant expression of Bt toxins in crop plants and have helped to overcome the inadequacies of Bt insecticides (Gasser and Fraley 1989). Corn, *Zea mays* L., has been genetically engineered with the insertion of the Bt crystaline toxin (Cry) genes (Andow and Hutchinson 1998).

Transgenic crops expressing insecticidal toxins offer growers the chance to take advantage of the toxin's safety and effectiveness against targeted pests without major changes in their management practices. One of the concerns in the use of transgenic crops is the potential development of resistance in targeted pests. Unlike foliar sprays, plants expressing Bt toxins may expose insects to selection for an entire growing season, causing continuous selection pressure. Several major crop pest species, including the tobacco budworm, *Heliothis virescens* (F.), have already demonstrated the ability to develop resistance to *B. thuringiensis* toxins in the laboratory (McGaughey and Whalon 1992). Successful selection for a resistant trait in a laboratory environment may not reflect the selection process in the field or the resistance mechanisms that evolve (ILSI HESI 1998).

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The widespread use of crops with this Bt technology for insect pest control will contribute to the development of pest populations that will be resistant to the Bt toxin, if resistant management programs are not implemented (Gould 1988, McGaughey and Whalon 1992, Tabashnik 1994). A high-dose refuge management strategy has been developed that can effectively delay corn borer resistance to genetically modified pesticidal corn (Hurley et al. 1999). This strategy has three essential components: (1) plant tissue must be very toxic so that resistance is functionally recessive, (2) resistant alleles must be sufficiently rare, so that nearly all resistant alleles will be found in heterozygous genotypes, and (3) a spatial arrangement of transgenic plots interspersed with nontoxic refuges must be used to maximize the probability that resistant homozygotes will mate with susceptible homozygotes produced within the non-Bt refuge delaying resistance within the species (Andow and Hutchinson 1998).

It is important that transgenic plants maintain their high toxicity throughout the entire growing season to maintain a high dose/refuge strategy. Plant genetics, tissue type, plant age, and environmental conditions influence concentration of the toxin within the Bt crops (ILSI HESI 1998). Toxin type and concentration can vary among corn hybrids, plants within a hybrid, plant parts of a single plant, and time of the growing season (Andow and Hutchinson 1998). Laboratory and field studies on transgenic crops performed over only part of a plant generation or part of a season may not provide sufficient data to evaluate strategies for resistance management (Onstad and Gould 1998). The effects of changes in toxin concentration on the evolution of resistance will depend on the timing and magnitude of the changes, insect phenology, and dose-response performances (Andow and Hutchinson 1998).

This study was designed to compare the relative toxicities of Bt corn plant structures with similar structures from their near-isogenic parent line at different stages of plant growth for effects on *Diatraea grandiosella* (Dyar) larvae. Differences in toxicities of plant structures within hybrids were also compared.

Materials and Methods

Corn was planted on 3 May 2000 on the Rodney Foil Plant Science Research Farm in Oktibbeha Co. in northeast Mississippi. Two hybrids expressing the Cry 1Ab endotoxin protein of Bt originating by event MON810 and their near-isogenic parent lines were included in the study. The hybrids were 'Pioneer 31B13' (Bt), 'Pioneer 3223' (near isogenic parent line of Pioneer 31B13), 'Pioneer 33V08' (Bt) and 'Pioneer 3394' (near isogenic parent line of Pioneer 33V08). Seeds were provided by Pioneer Hi- Bred International (Des Moines, IA). The corn hybrids were planted in four replicated plots (each plot 4 rows by 100 feet) for each hybrid in a randomized complete block design to be used primarily in a separate study to observe effects of the transgenic materials on naturally occurring insect pests under field conditions. Cultural practices recommended for corn production in this area were used and no insecticides were applied.

Diatraea grandiosella larvae feed on most plant parts, including leaves, stalk, husks, silks and kernels. Small lots of plant tissue were collected from each represented treatment in the above experimental design at three growth stages: midwhorl (26 June—whorl), blister (14 July—stalk, shank, husk, and silk), and dough (1 August—stalk, shank, husk, silk, and kernel) stages. Individual sampled material for selected growth stage and for replications of each treatment was combined for analysis. Tissue was collected from a total of 25 plants of each hybrid, divided according

to plant structures, placed into Zip-loc™ (Johnson & Son, Inc., Racine, WI) freezer bags, and placed immediately into a refrigerated box for transportation to the laboratory. Tissues were placed in a freezer at −18°C and later lyophilized using procedures of Stewart et al. (2001). Lyophilized tissues were stored at the same temperature until they were used.

Tissues from collections on individual sample dates were ground into a fine powder using a mortar and pestle. A diet (Bio-Serv™, Frenchtown, NJ) for *D. grandiosella* was prepared and allowed to cool to approximately 51°C before the plant tissue was incorporated into the diet using a high-speed Osterizer Galaxie™ blender (Oster Corp., Milwaukee, WI). Approximately 2 ml of diet was poured into 5-ml cells within trays containing 24 wells. One neonate *D. grandiosella* larvae, obtained from the USDA/ARS Laboratory in Starkville, MS, was placed into each cell using a camel hairbrush. Larvae were taken from egg masses collected from cages containing approximately 500 *D. grandiosella* moths. Trays were shrink-wrapped and heat-sealed, and four small holes were punched in the cover of each cell using pins embedded in a rubber cork.

A preliminary test was conducted on 28 March 2001 to measure the response of *D. grandiosella* larval feeding on different amounts of the lyophilized plant tissue in the artificial diet. One concentration from this test was chosen to be used for evaluations of all plant tissues collected. In the preliminary test, husk tissue was weighed to the nearest µg using an analytical balance (Denver Instrument Company, Denver, CO.) and incorporated into the artificial diet. The response of larvae to husk tissue (collected during blister stage) of Pioneer 31B13 (Bt) and its near-isogenic parent line, Pioneer 3223, was tested at concentrations of 15,000, 1,500, 150 and 15 µg/ml of diet. Ninety-six larvae (4 trays of 24 cells) were allowed to feed on each specific treatment diet for 6 days. A total of 768 larvae were included in this test using the two hybrids. Larval survival and body length and weight were analyzed using PROC GLM, and least significant means was used for separation of treatment effects (SAS Institute 1989).

Table 1. Response of *D. grandiosella* larvae fed for six days on artificial diet with lyophilized husk tissue at four concentrations

Hybrid	Concentration µg/ml	% survival	Body length (mm)	Weight (mg)
3223	15	84.4 ± 2.0b	8.1 ± 0.3a	6.9 ± 0.6a
3223	150	86.5 ± 7.1b	7.5 ± 0.3a	5.9 ± 0.8ab
3223	1500	92.7 ± 3.1ab	7.9 ± 0.3a	6.4 ± 0.2ab
3223	15000	88.5 ± 8.9b	7.3 ± 0.3a	5.5 ± 0.4b
31B13	15	90.6 ± 6.0b	7.5 ± 0.3a	5.8 ± 0.2ab
31B13	150	97.9 ± 2.1a	6.3 ± 0.3a	3.8 ± 0.4c
31B13	1500	60.4 ± 2.7c	2.7 ± 0.3b	0.3 ± 0.1d
31B13	15000	50.0 ± 5.1c	2.2 ± 0.4b	0.1 ± 0.5d

Means ± SE within a column not followed by the same letter are significantly different ($P \leq 0.05$, PROC GLM, protected LSM).

Table 2. Survival, weight, and length of *D. grandiosella* larvae fed for 7 days on artificial diet with lyophilized corn tissue collected during whorl and blister stages

Tissue	Parameter	Hybrid			
		3223	31B13	3394	33V08
Whorl*	% Survival	100.0 ± 5.9a	89.6 ± 4.0a	100.0 ± 3.4a	83.4 ± 5.9a
	Weight (mg)	18.0 ± 1.2a	0.8 ± 0.1c	15.7 ± 0.5b	1.0 ± 0.1c
	Length (mm)	10.5 ± 0.3a	3.6 ± 0.1b	10.4 ± 0.2a	4.0 ± 0.2b
Stalk**	% Survival	98.0 ± 9.2a	77.1 ± 5.2a	91.7 ± 14.8a	79.2 ± 9.9a
	Weight (mg)	25.6 ± 1.4a	0.6 ± 0.1b	22.8 ± 2.0a	0.9 ± 0.1b
	Length (mm)	11.1 ± 0.3a	3.1 ± 0.1b	10.6 ± 0.3a	3.3 ± 0.1b
Shank**	% Survival	73.0 ± 5.2b	70.8 ± 7.2b	95.8 ± 5.4a	39.6 ± 8.6c
	Weight (mg)	22.0 ± 1.6a	0.4 ± 0.1b	23.9 ± 0.5a	0.5 ± 0.1b
	Length (mm)	10.8 ± 0.3a	2.1 ± 0.1c	10.0 ± 0.2b	2.4 ± 0.1c
Husk**	% Survival	87.5 ± 5.4a	81.2 ± 2.1ab	75.0 ± 3.4b	70.8 ± 2.4b
	Weight (mg)	24.1 ± 1.0a	0.7 ± 0.1c	20.1 ± 1.7b	0.7 ± 0.1c
	Length (mm)	11.5 ± 0.2a	3.4 ± 0.1c	10.6 ± 0.5b	3.3 ± 0.1c
Silk**	% Survival	89.6 ± 11.0a	83.3 ± 4.8a	87.6 ± 8.0a	87.5 ± 8.0a
	Weight (mg)	19.7 ± 0.9a	0.8 ± 0.2b	20.5 ± 1.8a	0.8 ± 0.1b
	Length (mm)	9.4 ± 0.2a	3.4 ± 0.1b	9.6 ± 0.3a	3.4 ± 0.2b

Means ± SE within a row not followed by the same letter are significantly different ($P < 0.05$, LSD).
* Mid-whorl stage.
** Blister stage.

The response of *D. grandiosella* larvae to all lyophilized plant structures collected in 2000 was tested on 4 April and 25 April 2001. Forty-eight *D. grandiosella* neonates were tested (2 trays of 24 larvae) for each hybrid and tissue combination for each sample date. Based on preliminary studies, 48 larvae was considered a sufficient number to separate possible differences between various tissue types. Neonates were handled as in the above preliminary concentration tests, and larval survival, length and weight were recorded after 7 days. Larval survival was recorded for insects in one-half of each test tray (4 groups of 12 cells). Each one-half of a test tray was considered a replication (12 cells). Total weight of larvae for each replication was divided by the number of larvae surviving in the replication to obtain average weight of each group. This weighted average (weighted by number of larvae surviving on a specific diet) was used in analysis of the data. Body length of each surviving larva was recorded in mm. Larval survival, length, and weight were analyzed by PROC GLM, and means were separated by least significant difference (LSD) (SAS Institute 1989).

Results

The preliminary test with *D. grandiosella* larvae feeding on four concentrations of husk tissue collected during the blister stage for Pioneer 3223 and Pioneer 31B13 indicated a hybrid by concentration interaction for survival ($F = 55.19$; df = 3, 24; $P < 0.0001$), body length ($F = 35.33$; df = 3,24; $P < 0.0001$), and body weight ($F = 35.33$; df = 3,24; $F < 0.0001$) (Table 1). No significant differences were detected between hybrids at the lowest concentration of tissue (15 µg/ml of diet) for survival, body length or weight of larvae. The two highest concentrations of Pioneer 31B13 tissue caused significant stunting and mortality of larvae. About 60% survival was observed in larvae fed 1500 µg of lyophilized Bt tissue per ml of diet; this concentration was chosen as the discriminatory dose used in subsequent evaluations.

Larvae that fed 7 days on diet with whorl, stalk, shank, husk, silk or kernel tissue from the conventional corn hybrids, Pioneer 3223 and Pioneer 3394, collected during midwhorl, blister or dough plant growth stages had significantly greater body length and were heavier than larvae that fed on diet with their respective Bt hybrids, Pioneer 31B13 and Pioneer 33V08 (Tables 2, 3). Larval survival varied based on

Table 3. Survival, weight, and length of *D. grandiosella* larvae fed for 7 days on artificial diet with lyophilized corn tissue collected during dough stage

		Hybrid			
Tissue	Parameter	3223	31B13	3394	33V08
Stalk*	% Survival	100.0 ± 4.8a	79.2 ± 7.2c	98.9 ± 2.1ab	83.3 ± 7.6bc
	Weight (mg)	19.9 ± 0.7a	0.7 ± 0.1b	18.6 ± 1.7a	0.8 ± 0.1b
	Length (mm)	8.5 ± 0.3a	3.2 ± 0.1b	8.6 ± 0.2a	3.4 ± 0.2b
Shank**	% Survival	95.8 ± 5.4a	85.4 ± 8.6ab	100.0 ± 6.3a	64.6 ± 7.1b
	Weight (mg)	12.3 ± 0.8b	0.4 ± 0.1c	13.7 ± 0.4a	0.3 ± 0.1c
	Length (mm)	9.5 ± 0.3a	2.9 ± 0.1b	9.7 ± 0.2a	2.8 ± 0.1b
Husk**	% Survival	100.0 ± 6.0a	89.6 ± 4.0a	100.0 ± 3.4a	83.3 ± 6.0a
	Weight (mg)	18.0 ± 1.2a	0.8 ± 0.1c	15.7 ± 0.5b	1.0 ± 0.1c
	Length (mm)	10.5 ± 0.3a	3.6 ± 0.1b	10.4 ± 0.2a	3.9 ± 0.2b
Silk**	% Survival	100.0 ± 3.4a	89.6 ± 12.0a	100.0 ± 2.1a	91.7 ± 3.4a
	Weight (mg)	15.9 ± 0.6a	0.4 ± 0.1b	16.7 ± 0.9a	0.6 ± 0.1b
	Length (mm)	9.8 ± 0.3a	2.9 ± 0.1b	9.3 ± 0.2a	3.1 ± 0.2b
Kernel**	% Survival	98.0 ± 2.1a	91.7 ± 3.4a	98.0 ± 4.0a	85.4 ± 4.0a
	Weight (mg)	17.7 ± 1.0a	4.2 ± 0.6b	20.0 ± 1.0a	3.4 ± 0.2b
	Length (mm)	8.7 ± 0.2a	6.2 ± 0.2b	9.2 ± 0.2a	5.4 ± 0.3c

Means ± SE within a row not followed by the same letter are significantly different ($P < 0.05$, LSD).

* Mid-whorl stage.

** Blister stage.

Table 4. Percent survival, body weight (mg) and body length (mm) of *D. grandiosella* larvae 7 days after being fed a diet with lyophilized corn tissue

Parameter stage	Hybrid	Tissue type				
		Stalk	Shank	Husk	Silk	Kernel
Survival						
Blister	3223	98.0 ± 9.2a	72.9 ± 5.2a	87.5 ± 5.4a	89.6 ± 11.0a	—
	31B13	77.1 ± 5.2a	70.8 ± 7.2a	81.2 ± 2.1a	83.3 ± 4.8a	—
	3394	91.7 ± 14.8a	95.8 ± 5.4a	75.0 ± 3.4a	87.5 ± 8.0a	—
	33V08	79.2 ± 10.0a	39.6 ± 8.6b	70.8 ± 2.4a	87.5 ± 8.0a	—
Dough	3223	100.0 ± 4.8a	95.8 ± 5.8a	100.0 ± 5.9a	100.0 ± 3.4a	97.9 ± 2.1a
	31B13	79.2 ± 7.2a	85.4 ± 8.6a	89.6 ± 4.0a	89.6 ± 12.0a	91.7 ± 3.4a
	3394	98.0 ± 2.1a	100.0 ± 6.3a	100.0 ± 3.4a	100.0 ± 2.9a	97.9 ± 4.0a
	33V08	83.3 ± 7.6a	64.6 ± 7.1a	83.3 ± 5.9a	91.7 ± 3.4a	85.4 ± 4.0a
Weight						
Blister	3223	25.6 ± 1.4a	22.0 ± 1.6ab	24.1 ± 1.0a	19.7 ± 0.9b	—
	31B13	0.6 ± 0.1ab	0.4 ± 0.1b	0.7 ± 0.1ab	0.8 ± 0.2a	—
	3394	22.8 ± 2.0a	23.9 ± 0.5a	20.1 ± 1.7a	20.5 ± 1.8a	—
	33V08	0.9 ± 0.1a	0.5 ± 0.1a	0.7 ± 0.1a	0.8 ± 0.1a	—
Dough	3223	19.9 ± 0.7a	12.3 ± 0.8c	18.0 ± 1.2ab	15.9 ± 0.6b	17.7 ± 1.0ab
	31B13	0.7 ± 0.1b	0.4 ± 0.1b	0.8 ± 0.1b	0.4 ± 0.1b	4.2 ± 0.6a
	3394	18.6 ± 1.7ab	13.7 ± 0.4c	15.7 ± 0.5bc	16.7 ± 0.9b	20.0 ± 1.0a
	33V08	0.8 ± 0.1bc	0.3 ± 0.1d	1.0 ± 0.1b	0.6 ± 0.1cd	3.4 ± 0.2a

Table 4. Continued.

Parameter stage	Hybrid	Tissue type				
		Stalk	Shank	Husk	Silk	Kernel
Length						
Blister	3223	11.1 ± 0.3a	10.8 ± 0.3a	11.5 ± 0.2a	9.4 ± 0.2b	—
	31B13	3.1 ± 0.1a	2.1 ± 0.1b	3.4 ± 0.1a	3.4 ± 0.1a	—
	3394	10.6 ± 0.3a	10.0 ± 0.2a	10.6 ± 0.5a	9.6 ± 0.3a	—
	33V08	3.3 ± 0.1a	2.4 ± 0.1b	3.3 ± 0.1a	3.4 ± 0.2a	—
Dough	3223	8.5 ± 0.3c	9.5 ± 0.3b	10.5 ± 0.3a	9.8 ± 0.3ab	8.7 ± 0.2c
	31B13	3.2 ± 0.1bc	2.9 ± 0.1c	3.6 ± 0.1b	2.9 ± 0.1c	6.2 ± 0.2a
	3394	8.6 ± 0.2c	9.7 ± 0.2b	10.4 ± 0.2a	9.3 ± 0.2b	9.2 ± 0.2bc
	33V08	3.5 ± 0.2c	2.8 ± 0.1d	4.0 ± 0.2b	3.1 ± 0.2cd	5.4 ± 0.3a

Means ± SE within a row not followed by the same letter are significantly different ($P \leq 0.05$, LSD).

tissue type and plant stage. Although larvae were alive after 7 days, very little feeding was observed on the diet in many of the cells with Bt tissue.

Different plant tissues collected during the blister stage were analyzed to compare feeding responses by *D. grandiosella* larvae on each corn hybrid. Larvae fed diet with shank tissue of Pioneer 33V08 had significantly lower survival ($F = 7.25$; df = 3,12; $P = 0.0049$) and body length ($F = 7.06$; df = 3; 12, $P = < 0.001$) than larvae fed stalk, husk or silk tissues of this hybrid (Table 4). Larvae fed shank tissue from Pioneer 31B13 at blister stage had a lower mean (± SEM) body length (2.1 ± 0.1) than larvae fed husk (3.4 ± 0.1), silk (3.4 ± 0.1) or stalk (3.1 ± 0.1) tissues of this hybrid ($F = 20.25$; df = 3; 164, $P < 0.001$) (Table 4). Larvae fed diet incorporated with silk tissue from Pioneer 3223 at blister stage had lower mean body length (9.4 ± 0.2) than husk (11.5 ± 0.2), stalk (11.1 ± 0.3), or shank (10.8 ± 0.3) tissues of this hybrid ($F = 12.52$; df = 3; 163, $P < 0.001$) (Table 4).

Larvae fed diet with kernel tissue from Pioneer 31B13 had greater body weight ($F = 35.51$; df = 4, 15; $P < 0.001$) (Table 4) and body length ($F = 89.18$; df = 4, 209; $P < 0.001$) (Table 4) than larvae fed diet with husk, shank, silk, or stalk tissues of this hybrid collected during the dough stage of development. Also, larvae fed diet with kernel tissue from Pioneer 33V08 had greater body weight ($F = 114.68$; df = 4, 15; $P < 0.001$) and body length ($F = 32.99$; df = 4, 194; $P < 0.001$) than larvae fed other tissues of this hybrid. Larvae fed diet with shank tissue of Pioneer 3223 had the lowest average body weight (Table 4) when compared with larvae fed other tissues of this hybrid ($F = 11.10$; df = 4, 15; $P < 0.001$). Larvae fed on husk tissue of Pioneer 3394 had the greatest body length compared with larvae fed other tissues of this hybrid ($F = 9.16$; df = 4, 238; $P < 0.001$) (Table 4).

Discussion

In a preliminary study, as the concentrations of Bt husk tissue in the artificial diet increased, *D. grandiosella* larval survival and body length and weight decreased. There may have been factors that affected the larvae other than the increase in concentration of Bt toxin in the diets. Larvae fed diet with higher concentrations of conventional husk tissue also showed some effects of slower growth. These results could be caused by the response of insects to other plant compounds in specific plant structures that can inhibit larval growth. Plants contain a great variety of chemicals that are not used for primary functions of the plant, but may serve as plant defenses to insects. Wiseman et al. (1992) have shown a significant relationship between flavone levels from corn silks and weight of *H. zea* larvae.

Comparisons within a Bt hybrid at the same plant growth stage indicated considerable variability in response of *D. grandiosella* larvae to different plant tissues. The results of this study further indicate that all tissues that may be fed on by targeted insects should be tested for their conformity to the high dose/refuge strategy that is recommended for management of insecticide resistant insect pests. Tissues expressing the Cry1Ab endotoxin did not seem to be uniform in their expression of the Bt toxin within the test hybrids. The actual level of toxin expression was not measured for the various plant tissues. The Bt tissue used in this study was diluted in artificial diet, so it would not give the response of *D. grandiosella* larvae to living tissue in a field situation or its ability to kill heterozygous individuals.

The high dose/refuge strategy recommended for Bt corn requires that plants have toxins in plant tissues at a dose high enough to kill heterozygous individuals that may carry a resistant allele. The U. S. Environmental Protection Agency (1998) accepted

a definition of high dose as 25 times the toxin concentration needed to kill susceptible larvae. Andow and Hutchinson (1998) reported that Bt toxin concentration can vary among corn hybrids, plants within a hybrid, parts of a single plant, and at different plant growth stages. These differences were observed with the Bt hybrids included in the present study. The results indicate that kernel tissue appeared to have the least affect on *D. grandiasella* of the Bt tissues tested. Information from this study relates only to Bt corn hybrids expressing the Cry1Ab endotoxin by means of Event MON810. It would be helpful to test the high dose/refuge strategy assumptions against tissues expressing the lowest amount of toxin at a plant growth stage when targeted insects are present.

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