Response of *Heliothis virescens* (Lepidoptera: Noctuidae) Strains to *Bacillus thuringiensis* Cry1Ac Incorporated Into Different Insect Artificial Diets

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ABSTRACT Susceptibility to the Cry1Ac toxin from *Bacillus thuringiensis* in tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is usually measured by performing bioassays under laboratory conditions. Accurate comparison of Cry1Ac susceptibility among *H. virescens* samples conducted in different places is challenged by several important methodological aspects, especially if different insect artificial diets are used to perform bioassays. In this study, we compared Cry1Ac susceptibility of four different-origin *H. virescens* colonies when challenged with this toxin incorporated into four different insect artificial diets. Our data show that Cry1Ac susceptibility was lower in all the *H. virescens* colonies for one of the commercial diets (Bio-Serv). Bio-Serv diet was one of the least significantly consumed diets by larvae of the four different colonies, which indicates that insects encountered less Cry1Ac toxin due to lower consumption of diet. Larvae fed Bio-Serv diet also seemed to display slower Cry1Ac toxin activation compared with larvae fed any of the other three diets tested. In contrast, a wheat germ–soybean diet (ARS) was one of the most consumed diets by the four *H. virescens* colonies. The increased consumption of ARS diet probably led to the high level of Cry1Ac susceptibility observed in all the *H. virescens* colonies. Our data highlight the importance of using common diets and use a standard tobacco budworm colony when comparing Cry1Ac susceptibility between diverse *H. virescens* strains or across time.

KEY WORDS tobacco budworm, Cry1Ac-susceptibility, insect artificial diet comparison, larval growth

The tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is a polyphagous insect of important economic consequences in diverse cropping systems (Fitt 1989). Due to the intensive insecticide applications aiming for its control, *H. virescens* has acquired resistance to a wide range of synthetic insecticides (Sparks 1981, Luttrell et al. 1987, Hardee et al. 2001, Terán-Vargas et al. 2005, Zenner de Polanía et al. 2008). Development of transgenic cotton, *Gossypium hirsutum* L., varieties expressing Cry toxins from *Bacillus thuringiensis* (Bt) cotton has greatly enhanced environmentally sound control of *H. virescens.* Transgenic Bt cotton expressing Cry1Ac toxin, the most active Bt toxin against *H. virescens*, efficiently controls populations of tobacco budworm in the field and reduces the need for multiple sprays (e.g., MS, ≥12 applications versus 1.5 applications per crop season currently). However, this insect is still considered one of the most important pests of cotton (Williams 2008) and is one of the insect species that has demonstrated potential to develop high levels of resistance to Cry1Ac toxin in laboratory selection experiments (Ferré and Van Rie 2002).

Due to the importance of this insect and the ecological benefits that the implementation of the Bt transgenic agricultural biotechnology has had, the U.S. Environmental Protection Agency currently requires Bt cotton registrants to monitor for resistance evolution in insect populations targeted by Bt cotton (Matten and Reynolds 2003). Bioassays in which larvae are exposed to *B. thuringiensis* toxins incorporated into artificial diet are used to generate these data. There are numerous published reports on the effect of *B. thuringiensis* on tobacco budworm and bollworm, *Helicoverpa zea* (Boddie), that provide detailed methodologies (Dulmage et al. 1978; Ames and Harper 1985; Luttrell et al. 1999; Blanco et al. 2007, 2009a), but to our knowledge none of them has addressed the influ-

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ence that different insect artificial diets may have on the response of this insect to Cry1Ac.

The goal of this study was to compare the performance of four different tobacco budworm colonies on four different insect artificial diets with and without Cry1Ac. The experiments described herein also provide those involved in insect rearing practices with information regarding maintenance of tobacco budworm colonies. More importantly, these experiments can demonstrate the impact of insect artificial diets on Cry1Ac susceptibility.

Materials and Methods

Insects. Neonate *H. virescens* larvae were obtained from four different laboratory colonies. 1) The Agricultural Research Service (referred herein to as ARS colony) from Stoneville, MS, was established in 1971 from larval collections in wild plant hosts, maintained on soybean–wheat germ-based insect artificial diet (referred to ARS diet hereafter, without the infusion of feral insects since 2002; Blanco et al. 2009b). 2) The YDK colony from the North Carolina State University was established in 1988 from a field collection in Yadkin County, NC (Gould et al. 1995), maintained on corn meal–soybean flour-based diet (modified from Burton 1970, referred to as NCSU diet hereafter) before this study and kept at the ARS facility on ARS diet for one generation before the initiation of this study. 3) The Benzon colony was obtained from a commercial vendor (Benzon Research, Carlisle, PA), of unknown origin, maintained for the past 7 yr without the infusion of new moths. This colony also was maintained at the ARS facility on ARS diet for one generation before the initiation of this experiment. 4) The Monsanto colony provided by the Monsanto laboratory at Union City, TN. This colony originated from a field collection in Northeastern Mississippi in 2001, was infused with feral insects in 2007 and maintained on meridic Bio-Serv diet (F9781B, Bio-Serv, Frenchtown, NJ). This colony also was maintained at the ARS facility on ARS diet for one generation before the initiation of this experiment.

Neonates used for this study from the four tobacco budworm colonies were obtained from two different parental moth mating scenarios: 1) from enclosing 20 females and 20 males in a 1.9-liter container (42538L2, Consolidated Plastics, Twinsburg, OH), referred to as "cohort"; and 2) from enclosing one female and one male in 500-ml containers (42505L1, Consolidated Plastics), referred to as "single-pair" mating (Blanco et al. 2008). During each replication, four cohorts and 36 single-pairs were set up from each *H. virescens* colonies. During enclosing, moths were offered 15 ml of 10% sucrose solution placed in the bottom of each container in a plastic cup (T-125, Solo, Urbana, IL) with a paper tissue (Kleenex, Kimberly-Clark, Roswell, GA) stuffed in it. The containers were capped with cloth (batist, Zweigart, Piscataway, NJ) and kept in incubators at 28 ± 0.4°C, 75 ± 10% RH, and a photoperiod of 14:10 (L:D) h. Eggs laid on the cloth from each mating scenario's container from each *H. virescens* colony were removed daily for four consecutive days, placed inside plastic bags (94601, Ziploc, Crawfordsville, IN), most of the air inside the bag was removed, and the bags were closed and stored under the previously described environmental conditions.

Table 1. Ingredients of three of the insect artificial diets used in this study

Ingredient	Amount to prepare 1.0 liters of diet		
	ARS	NCSU	Pinto bean
Acid mix	2.5 ml		
Agar	11.2 g	14.3 g	10.8 g
Ascorbic acid		3.2 g	2.8 g
Aureomycin	1.0 g		
Corn-soy blend		162 g	
Dry milk		8.4 g	
Formaldehyde			0.6 ml
Methyl hydroxybenzoate	1.0 g		1.6 g
Nutrisoy flour	41.2 g		
p-Hydroxybenzoic acid methyl ester		1.8 g	
Pinto bean meal			91.2 g
Sorbic acid	1.0 g	0.9 g	0.8 g
Sugar	41.2 g		
Torula yeast		9.2 g	27.3 g
Vitamin mix	9.5 g	3.4 g	
Water	924 ml	796 ml	821 ml
Wesson salt	9.5 g		
Wheat germ	35.1 g		42.7 g

Insect Artificial Diets. Four different diets were used in this study: 1) the ARS soybean–wheat germ-based diet (modified from Shaver and Raulston 1971, Blanco et al. 2009b); 2) the NCSU corn meal–soybean flour-based diet (modified from Burton 1970); 3) pinto bean, *Phaseolus vulgaris* L.-based diet (modified from Byers et al. 1977); and 4) Bio-Serv tobacco budworm diet (F9781B), a wheat germ-based diet prepared following manufacturer's instructions. A description of the ingredients of the first three diets is presented in Table 1.

Cry1Ac-Susceptibility Bioassays. A series of 15 concentrations of lyophilized MVP II insecticidal powder containing 20% Cry1Ac when in liquid form (Mycogen Corporation, San Diego, CA) were incorporated into each of the four different insect artificial diets using a Magic Bullet (Homeland Housewares, http://www.homelandhousewares.com/index.php) for 12 s. Initially, every colony was tested one to two times, by using previously unpublished information to obtain its approximate severe growth inhibition and death (EC_{50}) (Siegfried et al. 2000) and from that information a narrower range of concentrations was used for the experiment. Cry1Ac concentrations ranged between 0.02 and 2.14 μg of Cry1Ac per ml of diet for each of the four artificial diets that were tested with the ARS and Monsanto colonies, between 0.21 and 7.00 μg of Cry1Ac per ml of diet for each of the four artificial diets tested with the Benzon colony, and between 4.50 and 67.50 μg of Cry1Ac per ml of diet in each of the four artificial diets tested with the YDK colony. Untreated control (0 μg of Cry1Ac) diet also was used for all the diets and insect colonies. We poured 1 ± 0.15 ml of each of the 16 concentrations of

a particular diet individually into one cell of a 16-cell division square of a bioassay tray (BAW-128, C-D International, Pitman, NJ), totaling eight replications of the 16 concentrations of a particular diet per bioassay tray. One ≤24 h-old tobacco budworm neonate from a particular colony's mating scenario was used per cell. Larvae on diet were covered with self-adhesive membranes (BIO-CV-16, C-D International) and then stored for 7 d under the previously described environmental conditions. Each colony using the cohort mating scenario had at least 16 replications per concentration per artificial diet. Each colony using the single-pair mating scenario had at least 20 replications per concentration per artificial diet. This whole process involving the two mating scenarios and the four insect colonies was repeated between two to five times in different dates.

Severe growth inhibition and death (EC_{50}) values were obtained from Proc Probit Log Normal analysis from SAS program version 9.1 (SAS Institute 2001) by pooling dead and L1 larvae into a "molting inhibition" category 7 d after neonates were placed on diet. Probit analyses were conducted for each single-pair from each colony and insect artificial diet and only those single-pairs that produced significant ($P \leq 0.05$) slope in the four diets were selected for further analysis. Data obtained from pooling the response of all the selected single-pairs or from cohort replications for a particular insect artificial diet and insect colony were used for probit analysis. Also, EC_{50} values from probit analyses obtained with each selected single-pair from each colony and insect artificial diet were analyzed by analysis of variance (ANOVA) and means separated by Proc Mixed test (Blanco et al. 2007).

Insect Artificial Diet Consumption and Larval Growth. To quantify the amount of diet eaten by larvae from the four different insect colonies, 12 ± 2 ml of each of the four artificial diets without Cry1Ac were poured into 30 plastic cups. When the poured diet reached room temperature (23 ± 2°C) and before larval infestation, the weight of each cup was individually recorded. Twenty-five cups of each diet were infested with a ≤16-h old neonate, and five cups had no larvae to serve as a measure of diet's water loss. All cups were closed with paper lids and stored as described previously for 7 d. After this period, larvae and diet remaining in the cups, with the frass removed with a fine camel hair brush, as well as control cups were individually weighed. The whole process to assess diet consumption for each tobacco budworm colony was repeated two times in different dates.

The amount of diet consumed was calculated by subtracting the weight of the remaining diet without frass from the original diet weight (adjusted for water loss obtained from the average of the five control cups) for each cup of each diet and tobacco budworm colony. Larval weight and consumption data were analyzed by ANOVA and means separated by least significant difference (LSD).

Isolation of Larval Midgut Fluids and Analysis of Cry1Ac Processing. To test for differences in the proteolysis of Cry1Ac in larvae fed different diets, we followed previously reported protocols (Karumbaiah et al. 2007), with minor modifications. For these assays, we used neonates of the Monsanto colony fed on each of the four diets without toxin for a 7-d period. This strain was selected to detect alterations in Cry1Ac protoxin activation because it had the highest differences in susceptibility to Cry1Ac when comparing among diets. Whole guts from six fourth instar larvae were dissected, extracted and pooled in 200 μl of molecular grade water in a microfuge tube. After homogenization by vortexing for 30 s, samples were cleared from gut tissue and debris by centrifugation (21,000 × *g* for 5 min). Supernatants were collected as midgut fluid samples and stored at −80°C until used.

MVP II powder (1.0 g) was resuspended in 8 ml of 50 mM Na_2CO_3, pH 9.8, and 0.1% 2-β-mercaptoethanol and sonicated three cycles. Then, the sample was diluted with one-half volumes of 50 mM Na_2CO_3, pH 9.8, and 1 M NaCl, and after sonicating as described above, the sample was incubated at room temperature for 3 h. Samples were cleared by centrifugation (21,000 × *g* for 5 min), and supernatants were kept as solubilized Cry1Ac protoxin.

Before testing for Cry1Ac proteolysis, midgut fluids and Cry1Ac protoxin samples were quantified by fluorometry (Qubit, Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Midgut fluids (1 μg of total protein) were incubated with Cry1Ac protoxin (20 μg) for 2 or 20 min at room temperature and then diluted one-half with sample buffer (Laemmli 1970) and heat denatured (100°C for 10 min). Proteins in each sample (≈10 μg per lane) were separated by SDS-10% polyacrylamide gel electrophoresis and gels stained for total protein after electrophoresis (Protoblue Safe, National Diagnostics, Atlanta, GA).

Results and Discussion

Results from this study show a consistent trend in the susceptibility of *H. virescens* to Cry1Ac and larval growth and consumption among four different artificial diets. Less Cry1Ac susceptibility was obtained with Bio-Serv diet in four *H. virescens* colonies, whereas in most of the cases the diet that produced the highest susceptibility was ARS diet. Consumption and larval weight was consistently higher with ARS diet.

Cry1Ac susceptibility of neonates obtained from mating cohorts of the ARS colony differed significantly among artificial diets (Table 2). The highest Cry1Ac susceptibility was observed when ARS larvae were exposed to NCSU diet, whereas the lowest Cry1Ac susceptibility was obtained with the Bio-Serv diet. The same results were observed for larvae from the Benzon colony. Larvae from the YDK and Monsanto were also significantly less susceptible to the Bio-Serv compared with the other diets (Table 2). Overall, lower Cry1Ac susceptibility was obtained from Bio-Serv diet in all the tobacco budworm colonies when neonates were obtained from cohorts of 20 females and 20 males.

When probit analysis also was performed pooling the response data of neonates whose parents were

Table 2. Severe growth inhibition and death (EC_{50}) obtained with the same concentrations of *B. thuringiensis*' Cry1Ac per insect colony, incorporated into four different insect artificial diets tested with four different *H. virescens* colonies

Diet	*n*	Slope ± SE	Significance of slope		EC_{50} (μg/ml)		Goodness of fit	
			χ^2	Prob.	Dose	95% FL	χ^2	Prob.
ARS colony								
ARS	48	1.3403 ± 0.142	88.1	<0.0001	0.410	0.342–0.485	5.8	0.55
NCSU	64	0.7072 ± 0.065	115.3	<0.0001	0.142	0.103–0.184	7.5	0.87
Pinto bean	64	1.4530 ± 0.163	79.2	<0.0001	0.225	0.189–0.265	12.1	0.51
Bio-Serv	64	0.7489 ± 0.062	142.9	<0.0001	0.836	0.664–1.024	15.4	0.28
YDK colony								
ARS	64	1.3458 ± 0.183	53.6	<0.0001	10.267	7.449–12.654	16.6	0.11
NCSU	64	1.0568 ± 0.182	33.5	<0.0001	9.691	4.919–13.775	20.8	0.07
Pinto bean	64	1.4719 ± 0.229	41.0	<0.0001	10.708	7.686–13.150	8.7	0.55
Bio-Serv	64	1.4532 ± 0.241	36.2	<0.0001	39.047	33.039–45.155	4.0	0.91
Benzon colony								
ARS	32	1.3389 ± 0.112	140.8	<0.0001	0.486	0.406–0.567	13.8	0.38
NCSU	32	0.8783 ± 0.075	135.4	<0.0001	0.269	0.209–0.331	6.3	0.93
Pinto bean	32	1.4551 ± 0.135	116.2	<0.0001	0.571	0.482–0.662	8.1	0.77
Bio-Serv	32	1.2151 ± 0.170	50.8	<0.0001	2.630	2.022–3.474	20.3	0.05
Monsanto colony								
ARS	40	1.1473 ± 0.102	124.5	<0.0001	0.699	0.584–0.812	7.0	0.72
NCSU	40	0.9728 ± 0.080	147.3	<0.0001	1.566	1.316–1.831	7.7	0.73
Pinto bean	40	1.3808 ± 0.114	144.6	<0.0001	0.777	0.673–0.880	15.4	0.21
Bio-Serv	37	0.756 ± 0.102	54.0	<0.0001	3.993	2.938–5.939	3.8	0.79

enclosed as single-pair, a similar trend was noticed. The ARS colony produced significantly different EC_{50} values with all the diets but on a different pattern from the one obtained with the cohort data. ARS diet produced significantly higher susceptibility to Cry1Ac followed by pinto bean, NCSU and Bio-Serv diets (Table 3). Larvae from the Benzon colony also demonstrated some differences between the cohort and single-pair response. For this colony, ARS and pinto bean diets produced significantly higher susceptibility to Cry1Ac, obtaining an intermediate level of susceptibility with NCSU diet and significantly less susceptibility with Bio-Serv diet. For YDK, the highest Cry1Ac susceptibility also was observed for the ARS diet, whereas intermediate susceptibility was observed for NCSU and pinto bean diets. In agreement with the cohort bioassays, the lowest Cry1Ac susceptibility was observed for the larvae fed Bio-Serv diet (Table 3).

Comparing Cry1Ac susceptibility between cohort and single-pair mating scenarios within a colony, results showed that significantly higher Cry1Ac susceptibility was obtained with the pooled response of single-pairs of the ARS colony and ARS diet, whereas significantly lower susceptibility was obtained with NCSU and pinto bean diets and the pooled response of single-pairs. The Benzon colony followed a consistent pattern with the four diets, obtaining significantly higher Cry1Ac susceptibility in the cohort data. The YDK colony was also significantly more Cry1Ac-susceptible when tested as cohort, but only with two diets (NCSU and pinto bean). Bio-Serv diet gave the least

Table 3. Severe growth inhibition and death (EC_{50}) obtained with the same concentrations of *B. thuringiensis*' Cry1Ac per insect colony, incorporated into four different insect artificial diets tested with pairs of four different *H. virescens*

Diet	*n*	Slope ± SE	Significance of slope		EC_{50} (μg/ml)		Goodness of fit	
			χ^2	Prob.	Dose	95% FL	χ^2	Prob.
ARS colony								
ARS	120	1.0249 ± 0.052	383.2	<0.0001	0.210	0.183–0.239	21.9	0.06
NCSU	146	1.0757 ± 0.045	549.1	<0.0001	0.424	0.380–0.469	20.9	0.09
Pinto bean	114	1.3230 ± 0.083	253.2	<0.0001	0.326	0.282–0.372	15.6	0.07
Bio-Serv	89	1.0786 ± 0.059	331.3	<0.0001	0.938	0.852–1.029	13.6	0.39
YDK colony								
ARS	133	0.9666 ± 0.050	366.7	<0.0001	12.112	10.677–13.470	4.7	0.98
NCSU	132	1.1733 ± 0.058	397.7	<0.0001	23.279	21.563–24.897	19.3	0.11
Pinto bean	131	1.1951 ± 0.073	264.5	<0.0001	22.334	20.028–24.458	20.2	0.89
Bio-Serv	91	1.3026 ± 0.088	216.1	<0.0001	37.546	35.054–40.057	15.5	0.27
Benzon colony								
ARS	54	1.0444 ± 0.096	117.9	<0.0001	0.790	0.621–0.961	19.5	0.06
NCSU	54	0.7908 ± 0.060	169.1	<0.0001	1.226	1.027–1.441	11.5	0.31
Pinto bean	54	1.2659 ± 0.095	177.2	<0.0001	0.905	0.794–1.015	11.5	0.39
Bio-Serv	53	0.7388 ± 0.130	31.9	<0.0001	34.047	16.375–142.010	15.2	0.29
Monsanto colony								
ARS	79	1.7669 ± 0.141	155.1	<0.0001	0.905	0.795–1.009	19.1	0.06
NCSU	77	1.356 ± 0.083	264.2	<0.0001	1.922	1.739–2.109	11.3	0.33
Pinto bean	76	1.307 ± 0.152	73.4	<0.0001	3.334	2.725–4.072	17.5	0.09
Bio-Serv	74	1.3373 ± 0.113	138.3	<0.0001	5.056	4.494–5.758	11.4	0.24

Table 4. Severe growth inhibition and death concentration (EC_{50}) obtained from the offspring of moth pairs of four different *H. virescens* colonies exposed to the same concentrations of Cry1Ac per insect colony

Diet	*n*	Mean ± SE EC_{50} (μg/ml)	Avg LSD[a]
ARS colony			
ARS	120	0.247 ± 0.057c	0.169
NCSU	146	0.417 ± 0.055b	0.169
Pinto bean	114	0.325 ± 0.057bc	0.169
Bio-Serv	89	0.786 ± 0.071a	0.169
YDK colony			
ARS	44	15.418 ± 3.793c	10.986
NCSU	43	26.562 ± 3.978b	10.986
Pinto bean	40	24.438 ± 3.837bc	10.986
Bio-Serv	38	59.327 ± 4.081a	10.986
Benzon colony			
ARS	27	0.769 ± 0.186b	0.614
NCSU	27	0.975 ± 0.228b	0.614
Pinto bean	18	1.103 ± 0.186b	0.614
Bio-Serv	13	6.334 ± 0.269a	0.614
Monsanto colony			
ARS	25	0.938 ± 0.233d	0.655
NCSU	25	1.758 ± 0.233c	0.655
Pinto bean	25	3.538 ± 0.233b	0.655
Bio-Serv	24	5.092 ± 0.238a	0.655

Means within insect colony followed by a common letter do not differ significantly based on LSD comparison at $P < 0.05$.

[a] Since data is not balanced within the no. of replicated per diet of pairs, LSDs vary between comparisons therefore an average LSD is presented per insect colony.

amount of Cry1Ac susceptibility variation between cohort and single-pair data, whereas pinto bean produced the greatest difference between the two mating scenarios (Tables 2 and 3).

The EC_{50} mean response of individual single-pairs (Table 4) followed a similar pattern to that obtained with the data of all the single-pairs pooled (Table 3). This is not a surprising result because data obtained to generate the pooled response shown in Table 3 was obtained from the individual response. Interestingly, the mean EC_{50} response of individual single-pairs of the Benzon colony to Bio-Serv diet (Table 4) significantly differs from the value obtained from pooling all the individual single-pairs into one analysis (Table 3). The EC_{50} values of the individual response of single-pairs agree with the previous analyses showing that Bio-Serv diet gives the lowest Cry1Ac susceptibility for all the tobacco budworm colonies.

One factor that might have contributed to the differences in response to the four diets is the intrinsic rate of diet consumption, assuming that greater consumption indicates greater Cry1Ac ingestion. Figure 1 shows that larvae consumed significantly greater amounts of ARS diet, indicating that lower concentrations of Cry1Ac in this diet might have produced lower EC_{50} values in the four insect colonies due to higher ingestion. However, this pattern relating diet consumption and Cry1Ac susceptibility was only observed for data obtained from individual single-pairs (Table 2). Even though Bio-Serv diet, depending on the *H. virescens* colony, was significantly less consumed than other diets, there was no correlation between Cry1Ac susceptibility and diet consumption (Fig. 1).

Another explanation for the lower Cry1Ac susceptibility observed for Bio-Serv diet is the potential alteration of expression of digestive enzymes when larvae are fed this diet in comparison to the ARS, NCSU or pinto bean diets. Heliothines are notorious for their ability to alter their digestive proteases to overcome inhibitors (Brito et al. 2001; Volpicella et al. 2003, 2006). Resistance to Cry1Ac in strains of *H. virescens* has been reported previously to be related to alteration of digestive proteases (Forcada et al. 1996; Ka-

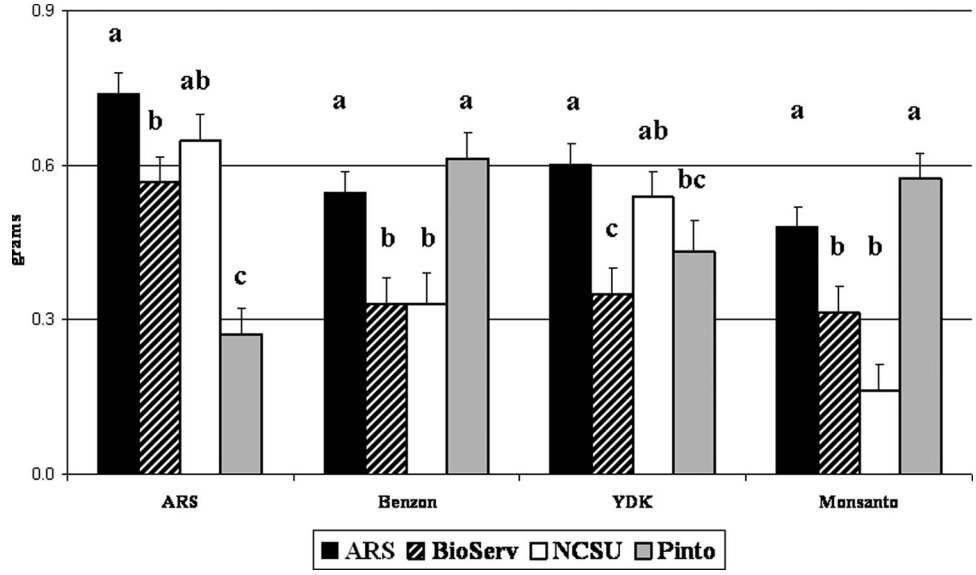

Fig. 1. Consumption of four different artificial diets by four different *H. virescens* colonies. Bars for each insect colony that have common letters are not significantly different at $P < 0.05$.

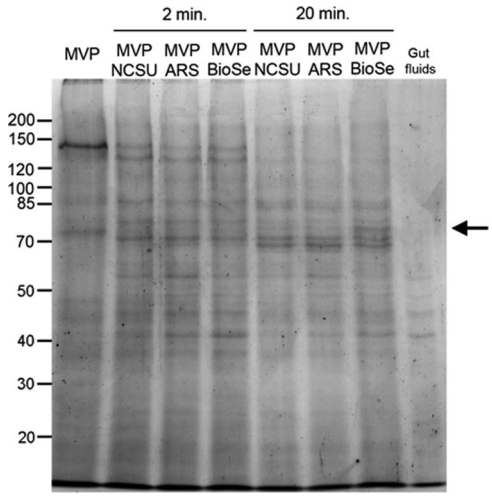

Fig. 2. Proteolysis of Cry1Ac protoxin solubilized from MVP when incubated in midgut fluids from larvae of the Monsanto strain fed NCSU, ARS, or Bio-Serv diet for 2 or 20 min as indicated. Last lane (gut fluids) is a control to detect proteins present in the midgut fluids (no MVP included in this sample). Arrow indicates position of intermediate that is predominant in samples from larvae fed Bio-Serv diet compared with NCSU- or ARS-fed larvae.

rumbaiah et al. 2007). To test whether altered Cry1Ac protoxin processing was involved in lower susceptibility to this toxin when larvae were fed Bio-Serv diet, we compared protoxin processing between larvae from the Monsanto colony fed NCSU, ARS, or Bio-Serv diet. As shown in Fig. 2, a single Cry1Ac protoxin band of ≈135-kDa in size was observed when MVP II was solubilized (lane 1). After a 2-min incubation with midgut fluid extracts from larvae of the Monsanto colony fed NCSU or ARS diet, the protoxin was processed to protein bands ranging from 135 to 70 kDa. After a 20-min incubation, the Cry1Ac toxin band was clearly detected at 68 kDa, with higher mass intermediates also observed. This size for activated Cry1Ac toxin core agrees with previous reports on Cry1Ac from *B. thuringiensis* HD-73 (Adang et al. 1985), which is the toxin core contained in MVP II (Gilroy and Wilcox 1992). In the protoxin sample treated with midgut fluids from larvae fed Bio-Serv diet we observed a prominent 77-kDa intermediate that was not as conspicuous in samples treated with midgut fluids from larvae fed NCSU or ARS diets (see arrow in Fig. 2). This observation may suggest that larvae fed Bio-Serv diet activate Cry1Ac protoxin at a slower rate than larvae fed NCSU or ARS diet, which may support a putative role of digestive protease alteration in lower susceptibility to Cry1Ac. We are currently pursuing further quantitative characterization of the rate of protoxin activation by midgut fluids from larvae fed distinct diets to test this hypothesis.

There were also significant larval weight differences among diets for each of the four *H. virescens* colonies (Fig. 3), corroborating the trend found with the consumption of diet and the expression of digestive enzymes. Larvae grew significantly heavier on the diet that they have been maintained before this experiment, demonstrating the selection process they were subjected to during multiple generations. Although this only applies to three of the four colonies because no information was obtained about the type of diet that Benzon colony is maintained with, it is interesting to note that each colony obtained heavier or similar weights with ARS diet that with the diet they were selected before this study (Fig. 3).

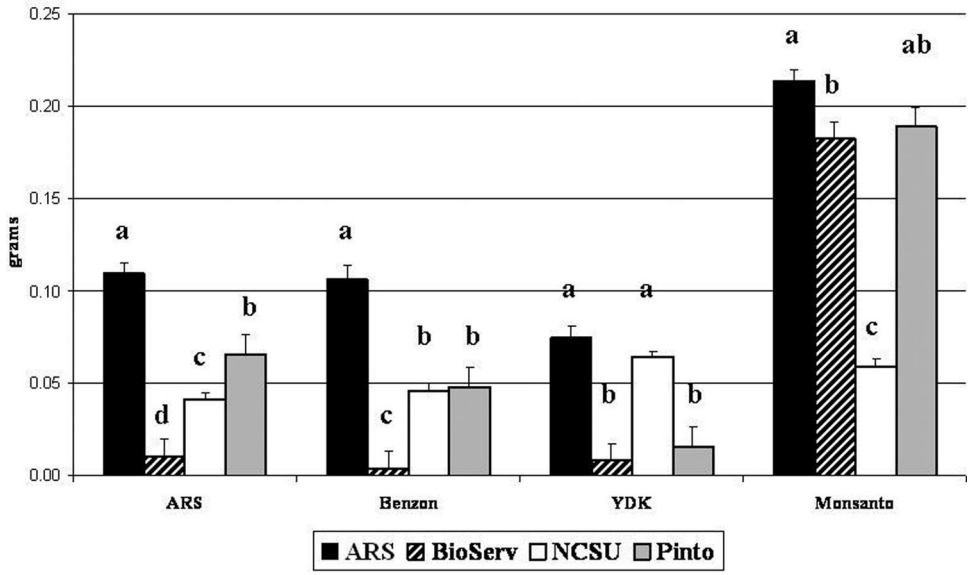

Fig. 3. *H. virescens* larval weights (grams) after 7 d of exposure to four different insect artificial diets. Bars for each insect colony that have common letters are not significantly different at $P < 0.05$.

Results from our study show a consistent trend of lower Cry1Ac susceptibility when larvae were fed Bio-Serv diet. This reduced Cry1Ac susceptibility did not correlate with reduced diet ingestion, but with slower Cry1Ac protoxin activation. Together, these results demonstrate that susceptibility to Cry1Ac can be affected by the type of diet that this protein is incorporated into. Diets such as ARS, NCSU and pinto bean, from which we provide a detailed description, can be produced with minimal effort and offer the possibility of replicating the same manufacturing process. In contrast, the Bio-Serv is a meridic commercial diet and its ingredients are not specifically known. The advantage with the use of Bio-Serv diet was the consistency of EC_{50} values between cohort and single-pair mating scenarios. Our data highlight the importance of considering the diet used when comparing mortality data obtained from bioassays.

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References Cited

Adang, M. J., M. J. Staver, T. A. Rocheleau, J. Leighton, R. F. Barker, and D. V. Thompson. 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. kurstaki HD-73 and their toxicity to *Manduca sexta.* Gene 36: 289–300.

Ames, H. D., and J. D. Harper. 1985. Bioassay of β-exotoxin of *Bacillus thuringiensis* against *Heliothis zea* larvae. J. Invertebr. Pathol. 46: 247–250.

Blanco, C. A., O. P. Perera, D. Boykin, C. Abel, J. Gore, S. R. Matten, J. C. Ramírez-Sagahon, and A. P. Terán-Vargas. 2007. Monitoring *Bacillus thuringiensis*-susceptibility in insect pests that occur in large geographies: how to get the best information when two countries are involved. J. Invertebr. Pathol. 95: 201–207.

Blanco, C. A., O. P. Perera, F. Gould, D. V. Sumerford, G. Hernández, C. A. Abel, and D. A. Andow. 2008. An empirical test of the F_2 screen for detection of *Bacillus thuringiensis*-resistance alleles in tobacco budworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 101: 1406–1414.

Blanco, C. A., D. A. Andow, C. A. Abel, D. V. Sumerford, G. Hernandez, J. D. Lopez, Jr., L. Adams, A. Groot, R. Leonard, R. Parker, G. Payne, O. P. Perera, A. P. Terán-Vargas, and A. Azuara-Domínguez. 2009a. *Bacillus thuringiensis* Cry1Ac resistance frequency in tobacco budworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 102: 381–387.

Blanco, C. A., M. Portilla, C. A. Abel, H. Winters, R. Ford and D. Street. 2009b. Soybean flour and wheat germ proportions in insect artificial diet and their effect on the growth rates of Heliothis virescens (F.) (Lepidoptera: Noctuidae). J. Ins. Sci. (in press).

Brito, L. O., A. R. Lopes, J. R. Parra, W. R. Terra, and M. C. Silva-Filho. 2001. Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitors may be mediated by the synthesis of new proteinases. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 128: 365–375.

Burton, R. L. 1970. A low-cost artificial diet for corn earworm. J. Econ. Entomol. 63: 1969–1970.

Byers, R. A., D. L. Gustine, and B. G. Moyer. 1977. Toxicity of β-nitropopionic acid to *Trichoplusia ni.* Environ. Entomol. 6: 229–232.

Dulmage, H. T., H. M. Graham, and E. Martinez. 1978. Interactions between the tobacco budworm, *Heliothis virescens* and the δ-endotoxin produced by the HD-1 isolate of *Bacillus thuringiensis* var. *kurstaki:* relationship between length of exposure to the toxin and survival. J. Invertebr. Pathol. 32: 40–50.

Ferré, J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 47: 501–533.

Fitt, G. P. 1989. The agroecology of *Heliothis* species in relation to agroecosystems. Annu. Rev. Entomol. 34: 17–52.

Forcada, C., E. Alcácer, M. D. Garcerá, and R. Martínez. 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. Arch. Insect. Biochem. Physiol. 31: 257–272.

Gilroy, T. E., and E. R. Wilcox, inventors; Mycogen Corporation, assignee. 1992 July 7. Hybrid *Bacillus thuringiensis* gene, plasmid and transformed *Pseudomonas fluorescens.* U.S. patent 5,128,130.

Gould, F., A. Anderson, A. Reynolds, L. Bumgarner, and W. Moar. 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88: 1545–1559.

Hardee, D. D., L. C. Adams, and G. W. Elzen. 2001. Monitoring for changes in tolerance and resistance to insecticides in bollworm / tobacco budworm in Mississippi, 1996–1999. Southwestern. Entomol. 26: 365–372.

Karumbaiah, L., B. Oppert, J. L. Jurat-Fuentes, and M. J. Adang. 2007. Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 146: 139–146.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227: 680–685.

Luttrell, R. G., R. T. Roush, A. Ali, J. S. Mink, M. R. Reid, and G. L. Snodgrass. 1987. Pyrethroid resistance in field populations of *H. virescens* (Lepidoptera: Noctuidae) in Mississippi in 1986. J. Econ. Entomol. 80: 985–989.

Luttrell, R. G., L. Wan, and K. Knighten. 1999. Variation in susceptibility of noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis.* J. Econ. Entomol. 92: 21–32.

Matten, S. R., and A. Reynolds. 2003. Current resistance management requirements for Bt cotton in the United States. J. New Seeds 5: 137–178.

SAS Institute. 2001. Version 9.1. SAS Institute, Cary, NC.

Siegfried, B. D., T. Spencer, and J. Nearman. 2000. Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis.* J. Econ. Entomol. 93: 1265–1268.

Sparks, T. C. 1981. Development of insecticide resistance in *Heliothis zea* and *Heliothis virescens* in North America. Bull. Entomol. Soc. Am. 27: 186–192.

Shaver, T. N., Raulston, J. R. 1971. A soybean-wheat germ diet for rearing the tobacco budworm. Ann. Entomol. Soc. Am. 64: 1077–1079.

Terán-Vargas, A. P., J. C. Rodriguez, C. A. Blanco, J. L. Martinez Carrillo, J. Cibrian Tovar, H. Sanchez Arroyo, L. A. Rodríguez del Bosque and D. Stanley. 2005. Bollgard cotton and resistance of the tobacco budworm (Lepidoptera: Noctuidae) to conventional insecticides in

southern Tamaulipas, Mexico. J. Econ. Entomol. 98: 2203–2209.

Volpicella, M., L. R. Ceci, J. Cordewener, T. America, R. Gallerani, W. Bode, M. A. Jongsma, and J. Beekwilder. 2003. Properties of purified gut trypsin from *Helicoverpa zea*, adapted to proteinase inhibitors. Eur. J. Biochem. 270: 10–19.

Volpicella, M., J. Cordewener, M. A. Jongsma, R. Gallerani, L. R. Ceci, and J. Beekwilder. 2006. Identification and characterization of digestive serine proteases from inhibitor-resistant *Helicoverpa zea* larval midgut. J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 833: 26–32.

Williams, M. R. 2008. Cotton insect losses–2007, pp. 927–979. *In* Proceedings of the 2008 Beltwide Cotton Conference, 8–11 January 2008, Nashville, TN. National Cotton Council, Nashville, TN.

Zenner de Polanía, I., J. A. Álvarez Rodríguez, H. A. Arévalo Maldonado, R. Mejía Cruz, and M. A. Bayona, R. 2008. Susceptibilidad de cuatro nóctuidos plaga (Lepidoptera) al gene Cry1Ac de *Bacillus thuringiensis* incorporado al algodonero. Rev. Colomb. Entomol. 34: 41–50.

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