RESISTANCE OF BOLLWORM, *HELICOVERPA ZEA*, TO CRYIA(C) TOXIN

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Abstract

In 1998, third-instar bollworm, *Helicoverpa zea*, larvae were collected from the ears of *B.t.* sweet corn grown in Plymouth and Clayton, North Carolina. Larvae were transferred to artificial diet containing 0.1 *ug*/ml of CryIA(c) toxin. A selection experiment was performed to determine the rate of adaptation to varying levels of this toxin. We found that after only 6 generations of selection, the selected colony had developed about 50-fold resistance to CryIA(c) toxin. Nearly 100-fold resistance was seen after 10 generations. The LC_{50} values for the control strain, the selected strain, the selected female by control male cross and the control female by selected male cross were 2.78, 240.98, 141.62, and 277.32 *ug*/ml, respectively for the F_8 generation. This suggests that resistance to *B.t.* can be inherited as a dominant or incompletely dominant trait. The selected strain was only 3 times more resistant to CryIIA than the susceptible lab colony.

Introduction

Transgenic crops expressing the endotoxin proteins of *Bacillus thuringiensis* have recently become an issue of debate. Many researchers have addressed the possibility of insect adaptation to these *B.t.* toxins (Tabashnik 1994; Gould et al. 1992, 1995; Luttrell et al. 1999). Transgenic plants expressing these *B.t.* toxins typically provide season-long protection against various insect pests. This continuous protection may have its drawbacks. Target pests are exposed to these toxins throughout the season, thus causing intense selection pressure and possibly increased rates of resistance development. Tabashnik (1994) and Kennedy and Whalon (1995) reported on the most effective methods of resistance management and ways to decrease the rate of insect adaptation. Recent studies have focused on the development of insect resistance to a single or to several *B.t.* endotoxins. Gould et al. (1995) reported that a field collected strain of *Heliothis virescens* developed >500-fold resistance to CryIA(c) toxin after 19 episodes of selection. This same study reported high levels of cross-resistance for *H. virescens* to other toxins including, CryIA(a), CryIA(b) and CryIF. Moar et al. (1995) reported high levels of resistance in beet armyworm to the CryIC toxin after 21 generations of selection. Most recently, Luttrell et al. (1999) reported LC_{50} values 100 times greater for *Helicoverpa zea* after only 7 generations of selection with CryIA(c). With more *B.t.* crops slated for commercialization, it is imperative to determine the resistance potential so that appropriate management strategies may be employed.

This study reports on a strain of *Helicoverpa zea* that was selected for resistance to CryIA(c). The dose-mortality tests demonstrate increased levels of resistance to CryIA(c) over 13 generations of selection. Also reported are data concerning the inheritance of the resistance trait.

Materials and Methods

In July-August 1998, third-instar bollworm larvae were collected from CryIA(b) producing sweet corn at Tidewater Research Station, Plymouth, NC and Central Crops Research Station, Clayton, NC. Only third-instar larvae and above were collected from *B.t.* sweet corn to increase the chances of recovering insects with resistance genes. In total, 354 larvae were collected for the F_0 generation. These larvae were transferred to artificial diet containing the CryIA(c) toxin. The original concentration of toxin in the diet was 0.1 *ug* of toxin per ml of diet. A control colony was collected from a nearby farm in the Plymouth area which was ~ 1 mile from any *B.t.* crops. These individuals were placed on artificial diet containing no toxin. Both colonies were kept in rearing facilities at North Carolina State University at 27-30ºC, 55-60% relative humidity and a photoperiod of 14:10 (L:D) h. Adults were fed a 5% sugar solution.

Adult F_0 moths were mated using single pairs. Adults (1 male and 1 female) were placed in cardboard buckets along with a cotton ball soaked with a 5% sugar solution. Adults were paired according to their time of emergence. A piece of cheesecloth was placed over top of the bucket to allow a substrate for oviposition. Cheesecloths were checked daily for the presence of eggs; and if eggs were laid, cheesecloths were removed and placed into plastic bags until neonates hatched. At hatch, neonates were placed on their appropriate diet (BT or NBT) with a fine camel hair paint brush. This procedure was carried out through the entire study. Beginning with the F_4, single pair matings were no longer used to sustain the original selected or control lines. Mass mating (i.e. > 10 moths per bucket) was used for the remainder of the study unless otherwise noted. At ten days, all larvae were weighed. Other data recorded included, larval duration, pupal weight, pupal duration and % survivorship for each generation.

Beginning with the F_6 generation, multiple concentration diet incorporation bioassays were performed to determine LC_{50}

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estimates for the control and selected strains (Gould et al. 1995). A 5-fold increment serial dilution approach was used to obtain concentrations ranging from 0.064 *ug*/ml to 1000 *ug*/ml of CryIA(c) toxin. Inheritance tests began with the F_8 generation using neonates from reciprocal hybrid crosses and the control and selected strains. The F_{10} generation was tested for resistance to CryIA(c) and cross-resistance to CryIIA using the multiple concentration bioassay. For all multiple concentration bioassays, mortality was recorded after 10 days on artificial diet. All LC_{50} values are based on 10^{th} day mortality readings.

All dose mortality data were analyzed using PROC PROBIT (SAS Institute 1990).

Results

Table 1 shows the total number of corn ears checked for infestation during the summer of 1998. A total of 2600 NBT ears checked contained 2646 larvae. In most of the 7 samples of NBT corn examined, almost 100% of the ears were infested. In some cases more than 1 larvae was present in the NBT ears; but for the most part, the cannibalistic nature of bollworms prevented this occurrence. The estimate of approximately one larva per ear is therefore a significant underestimate of the actual infestation rate of NBT ears. From the 4580 BT corn ears checked, only 354 larvae were found. This represented an infestation rate of 7.7%.

Survivorship data along with concentration of CryIA(c) toxin used in each generation of the selection experiment are presented in Table 2. The survivorship of the F_0 on 0.1 *ug*/ml CryIA(c) diet was 56.2%. This was fairly consistent for the first three generations. F_3 survivorship on *B.t.* diet was 66.1%. The toxin concentration was therefore raised to 1.0 *ug*/ml in the F_4 generation. Survivorship was still high at 58.2%. Therefore, the concentration was again raised in the F_5 generation to 10.0 *ug*/ml. Survivorship for this generation decreased to 43.6%. This was consistent through the F_7 generation. At the F_8 generation, concentration was raised to 40.0 *ug*/ml and survivorship decreased to 25.3%. The same concentration was used through the F_{13} with increasing levels of survivorship as seen in Table 2.

Results from the multiple concentration bioassays using CryIA(c) are presented in Table 3. F_6 selected individuals had an LC_{50} of 137.76 *ug*/ml while the LC_{50} for the control strain was 2.96 *ug*/ml which resulted in a resistance ratio of 46.54. The F_6 colony was being selected on 10.0 *ug*/ml CryIA(c) at the time of this bioassay. The LC_{50} for the F_8 selected individuals was 240.98 *ug*/ml while the LC_{50} for the control strain was 2.78 *ug*/ml of CryIA(c). The resistance ratio had increased to 86.68 for the F_8 individuals. Data for the F_8 reciprocal crosses are presented in Table 4. Progeny from a single pair cross of a selected female with a control male had an LC_{50} of 141.62 *ug*/ml, while progeny from the single pair cross of a control female with a selected male had an LC_{50} of 277.32 *ug*/ml of CryIA(c). The confidence intervals for LC_{50} values of the two crosses did not overlap (Table 4). In the F_{10} generation, the selected strain had an LC_{50} of 280.56 *ug*/ml while the control LC_{50} was 3.00 *ug*/ml. The resistance ratio was 93.52. Table 5 reports the results for the bioassay using CryIIA against the control and selected strains for the F_{10}. The selected strain had an LC_{50} of 45.06 *ug*/ml and the control strain had an LC_{50} of 13.79 *ug*/ml of CryIIA toxin. Although the 3.27 resistance ratio for this bioassay was statistically significant, the level of cross-resistance was very low.

Discussion

In 1998, untreated NBT sweet corn plots had 100% ear infestation rates in most areas we sampled. Therefore, we would assume that 100% of the *B.t.* plots should have been infested before *B.t.* induced mortality occurred. We found 7.7% of the *B.t.* sweet corn ears infested with *Helicoverpa zea*. Of the original 354 larvae collected, only 199 survived to pupation on 0.1 *ug*/ml of CryIA(c) diet. Although some susceptible larvae can survive on 0.1 *ug*/ml CryIA(c) during a 10-day bioassay, they typically can not reach the pupal stage. Therefore, we assume that most of the 199 survivors had at least a low genetically based tolerance of *B.t.* If we assume that the *B.t.* corn initially had one neonate per ear then the percentage with some genetic tolerance would be 4.3% (199/4580). It has long been recognized that the level of infestation of corn ears estimated by counts of later instar larvae underestimates the true initial neonate infestation rate because of cannibalism (Quaintance and Brues 1905), so the 4.3% estimate of larvae with some genetically based resistance is an overestimate. However, even if 4.3% overestimates the initial resistance allele frequency by an order of magnitude, these results still indicate that the initial genetic variance for resistance is high.

The selection experiment indicates that bollworms have an ability to develop at least 94-fold resistance to the *B.t.* toxin, CryIA(c). As concentration increased throughout the experiment the level of survivorship only decreased marginally. With the exception of F_8 (when selection on 40.0 *ug*/ml diet began), survivorship remained above 30% throughout the study. This shows that the resistant colony was responding to the selection pressure being applied. The concentration of CryIA(c) diet went from 0.1 *ug*/ml to 40.0 *ug*/ml, which is a 400-fold increase. The survivorship for the F_{13} (46%) was not much lower than it was for the F_0 (56.2%) showing that the selected colony developed increased levels of resistance fairly rapidly. This was also supported by the LC_{50} data from the F_6, F_8 and F_{10} that showed resistance ratios of 46.54, 86.68 and 93.52, respectively. These results are

consistent with other data that show selected *H. zea* having ~100 times higher $LC_{50's}$ than controls (Luttrell 1999).

Most studies of *B.t.* resistance indicate that resistance is inherited as a partially recessive trait. If a *B.t.* resistance trait is recessively inherited, then crosses between selected and control individuals should produce offspring that are similar in their susceptibility to the control strain. When reciprocal crosses were performed in this study, we found $LC_{50's}$ which were closer to the selected strain than to the control (comparing LC_{50} values for F_8 in Table 3 with LC_{50} values of crosses in Table 4). In fact, the LC_{50} for a control female crossed with a selected male was slightly higher than the LC_{50} for the selected strain. This suggests that resistance may be dominant or incompletely dominant. Recently, Huang et al. (1999) reported that inheritance of resistance to the *B.t.* toxin found in Dipel ES in the European corn borer was inherited as an incompletely dominant autosomal gene. Gould et al. (1995) also found dominant resistance to CryIIA. However, the level of resistance in those studies was lower than that found in the current study. Preliminary bioassays with *B.t.* cotton (Jackson et al. in prep.) indicate that the resistance in the selected bollworm strain confers higher survival on *B.t.* plants, so dominance of the trait is of significant concern for resistance management of cotton and corn that don't have high dose expression of *B.t.* toxins.

Cross-resistance is also a major concern in agriculture today since many new *B.t.* crops that express different protein endotoxins are soon expected to be commercialized. Gould et al. (1992) have already shown that a lab strain of *Heliothis virescens* has developed resistance to a number of *B.t.* toxins. Since bollworm is a pest on a number of *B.t.* crops that express different endotoxins, it is important that cross-resistance be minimal. We tested our CryIA(c) selected line and the control line on various doses of CryIIA to determine if any cross-resistance was present. We found $LC_{50's}$ of 45.06 *ug*/ml and 13.79 *ug*/ml for the selected and control strains, respectively. This shows a resistance ratio of about 3.27. Although, this cross-resistance does not seem to be biologically important, further studies will be carried out to determine if the selected strain has high levels of cross-resistance to any other *B.t.* toxins.

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References Cited

Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva and W. J. Moar. 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. 89:7986-7990.

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.

Huang, F., L. L. Buschman, R. A. Higgins and W. H. McGaughey. 1999. Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European corn borer. Science 284:965-967.

Kennedy, G. G. and M. E. Whalon. 1995. Managing pest resistance to *Bacillus thuringiensis* endotoxins: constraints and incentives to implementation. J. Econ. Entomol. 88:454-460.

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.

Moar, W. J., M. Pusztai-Carey, H. Van Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo and M. J. Adang. 1995. Laboratory development of resistance to *Bacillus thuringiensis* by beet armyworm, *Spodoptera exigua*: implications for the field. Proceedings Beltwide Cotton Conferences. 881-886.

Quaintance, A. L. and C. T. Brues. 1905. The cotton bollworm. USDA Bureau of Entomology, bulletin #50. Washington D.C. 155 pp.

Tabashnik, B. E. 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39:47-79.

SAS Institute. 1990. SAS/STAT User's Guide, Vol. 2. SAS Institute, Cary, NC, 795 pp.

Table 1. Total # of sampled sweet corn ears along with total # of bollworm larvae found for each sampling episode at various sites in North Carolina in 1998.

Sample Date	Location	Total # NBT ears sampled / # larvae found	Total # BT ears sampled / # larvae found
July 9	CRS[1]	100 / 163	100 / 8
July 14	TRS[2]	200 / 194	200 / 18
July 15-16	TRS	1000 / 1004	1614 / 135
July 23	TRS	200 / 192	400 / 8
August 3	TRS	400 / 400	1400 / 120
August 6	CRS	500 / 500	500 / 40
August 10	TRS	200 / 193	366 / 25

[1]CRS = Central Crops Research Station, Clayton, NC.
[2]TRS = Tidewater Research Station, Plymouth, NC.

Table 2. Total # of *H. zea* females used to start each generation along with # of bollworm larvae selected, survivorship and concentration of CryIA(c) toxin (*ug*/ml) for each of the first 14 generations of the selection experiment.

Generation	Total # females to start this generation[1]	Total # of neonates selected	% surviving	Toxin concentration (*ug*/ml)
0	-	354	56.2	0.1
1	18	647	58.4	0.1
2	20	418	52.4	0.1
3	18	620	66.1	0.1
4	170	1017	58.2	1.0
5	180	1579	43.6	10.0
6	160	1268	44.3	10.0
7	150	1386	45.2	10.0
8	100	1007	25.3	40.0
9	100	567	36.5	40.0
10	80	625	34.7	40.0
11	80	650	35.7	40.0
12	90	500	46.0	40.0
13	100	635	46.0	40.0

[1] Larvae from generations 1-3 were obtained via successful single pair matings, so only a low number of females contributed eggs to subsequent generations. However, mass mating (10 females per bucket) was used from F_4 on, so female numbers are based on total # of successful buckets (i.e. ones which produced fertile eggs) times 10.

Table 3. Resistance of control and selected *Helicoverpa zea* to CryIA(c) toxin for given generations.

Generation	Slope	LC_{50}[1]	Low[2]	High	RR[3]
F_6 control	2.42	2.96	1.76	4.44	
F_6 selected	2.99	137.76	75.8	196.68	46.54
F_8 control	1.94	2.78	1.77	4.05	
F_8 selected	2.01	240.98	41.62	720.76	86.68
F_{10} control	1.90	3.00	1.59	4.68	
F_{10} selected	1.97	280.56	NC[4]	NC	93.52

[1] LC_{50} values are *ug*/ml.
[2] Low and high 95% fiducial limits from SAS probit analysis.
[3] RR = LC_{50} selected / LC_{50} control.
[4] NC, not calculated by SAS Probit due to poor fit to log/probit model.

Table 4. Resistance of reciprocal hybrid crosses of *Helicoverpa zea* to CryIA(c) toxin for the F_8 generation.

Cross	Slope	LC_{50}[1]	Low[2]	High
SF[3] x CM	2.43	141.62	98.83	195.22
CF x SM	2.59	277.32	196.59	377.01

[1] LC_{50} values are *ug*/ml.
[2] Low and high 95% fiducial limits from SAS probit analysis.
[3] SF = selected female, CF = control female, SM = selected male,
CM = control male

Table 5. Resistance of control and selected *Helicoverpa zea* to CryIIA toxin for the F_{10} generation.

Generation	Slope	LC_{50}[1]	Low[2]	High	RR[3]
F_{10} control	1.83	13.79	7.17	21.71	
F_{10} selected	1.11	45.06	21.78	81.82	3.27

[1] LC_{50} values are *ug*/ml.
[2] Low and high 95% fiducial limits from SAS probit analysis.
[3] RR = LC_{50} selected / LC_{50} control.