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DIFFERENTIAL RATE OF RESISTANCE DEVELOPMENT TO BT CRY1AC IN COTTON BOLLWORM, *HELICOVERPA ZEA* (BODDIE) WHEN SELECTED USING MVP II AND ACTIVATED TOXIN

Konasale J. Anilkumar and William J Moar
Auburn University
Auburn, AL

Abstract

A susceptible laboratory strain of cotton bollworm, *Helicoverpa zea*, was established from a Monsanto laboratory colony. The baseline susceptibility of this strain to MVP II and Cry1Ac toxin was 24µg/g and 9µg/g diet, respectively. Subsequently, two Cry1Ac-resistant strains of cotton bollworm were selected using MVP II (MR) or activated Cry1Ac toxin (AR). Larvae that molted into second instar within seven days of selection were reared until pupation on regular diet (containing no Cry1Ac). Current resistant ratios for MR and AR strains are 12.6 and 35.9 fold after seven generations of selection, respectively. Selection studies indicated approximately three times quicker resistance development in the AR compared to the MR strain. Additionally, there were higher fitness costs in terms of fertility and fecundity in the MR compared to the AR strain.

Introduction

Cotton bollworm (CBW), *Helicoverpa zea* is one of the major insect pests of cotton in the U. S. It is one of the target pests of Bollgard® that expresses the Cry1Ac insecticidal protein from *Bacillus thuringiensis* (Perlak et al., 1990). Though Bollgard® provides unprecedented control against tobacco budworm (TBW) and pink bollworm (PBW), it is not economically effective against CBW post bloom and/or when the crop is under stress (Jackson et al., 2004). Additionally, Bollgard® does not express a 'high-dose' in all plant parts necessary to kill all CBW larvae, thereby increasing the likelihood of resistance development (Greenplate 1999, and Adamczyk et al., 2001).

Although there is no documented field resistance to Bollgard® by any target pest, laboratory selection studies with TBW, PBW and *Helicoverpa armigera* have indicated the inherent capacity of insects to adapt to Cry1Ac. These results have contributed for insect resistance management (IRM) policy making, but this has not happened with CBW because the study of Cry1Ac resistance in CBW have not been as successful as for other target pests. Because each species has different resistance characteristics (Akhurst et al., 2003, Morin et al., 2003, Bird and Akhurst, 2004, Tabashnik et al., 2003, 2004, and Xie et al., 2005), potentially impacting resistant management strategies (Bates et al., 2005), characterizing Cry1Ac resistance in CBW is critical. Additionally, selection experiments cited above were conducted with MVP II, a commercial formulation containing Cry1Ac protoxin inclusion bodies (Gould et al., 1995). The MVP II formulations used for the previously mentioned selection studies typically contained less than 20% Cry1Ac, meaning at least 80% of the formulation consisted on non Cry1Ac material. Additionally, because CBW has at least a 10-fold increase in tolerance to Cry1Ac and MVP II (Stone and Sims, 1993 and Luttrell et al. 1999), high levels of resistance to MVP II by CBW may not have occurred partly due to the extremely high amounts of non Cry1Ac material present in the concentrations tested. Furthermore, because Bollgard® expresses solubilized Cry1Ac protoxin that is at least partially activated to toxin upon ingestion; we hypothesize that selection using MVP II may not adequately reflect resistance selection occurring *in planta*. Therefore, we initiated selection experiments using MVP II and Cry1Ac toxin to compare resistance characteristics using these two different selection pressures. In this paper we report the rate of resistant development when selected using two sources of Cry1Ac and fitness costs associated with each one of them.

Material and methods

Insect culture

A laboratory susceptible CBW colony was established from a Monsanto (Union City, Tennessee) susceptible colony. Insects were reared on artificial diet (Moar et al., 1995) at 27 ± 1^0C with a photoperiod of 14:10h (light: dark).

Bt proteins

Cry1Ac activated toxin: Cry1Ac protoxin inclusion bodies were produced as a single gene product in *E. coli* using Terrific Broth containing 50µg/ml ampicilin. Inclusion bodies were extracted, solubilized using 50mM CAPS pH 10.5 buffer, and digested using 1mg/ml TPCK-treated trypsin. Activated toxin was purified using high performance liquid chromatography (HPLC) (Moar et al., 1995).

MVP II is a commercial formulation, freeze dried powder containing 19.1% Cry1Ac protoxin inclusion bodies encapsulated in *Pseudomonas fluorescens* cells.

Bioassays

Bioassays were conducted initially to establish the baseline susceptibility values for activated Cry1Ac and MVP II before selection experiments could be initiated.
Five to seven concentrations of Bt proteins were assayed against neonate CBW using diet incorporation assays. Bt proteins were diluted in twenty percent (v/w) distilled water and added to 24 g of artificial diet when the diet temperature was $<60^0$ C. The Bt protein-diet mixture was poured into 32 wells of a 128 well CD International bioassay tray. One "active" neonate was loaded per well and the bioassay trays were incubated at 27 ± 1^0C and 60% RH with a photoperiod of 14:10h (light: dark). Mortality (failure to molt) was recorded after 7 days. Assays were replicated at least three times and the data was analyzed by probit analysis (Finney, 1971) using POLO-plus program (LeOra Software, Berkeley, CA, U.S.A.).

Selection

Bt protein concentrations were prepared in 20% (v/v) of distilled water and incorporated with 80% artificial diet. Artificial diet containing Bt was poured into 96 well microtitre plates and one neonate was placed into each well. Larvae were exposed to MVP II (MR strain) or Cry 1Ac toxin (AR strain) for seven days; only those larvae that molted were selected and reared to pupation on diet containing no Bt protein. Selection concentrations were increased between 2.0 and 2.5 fold depending on percent survivorship.

Resistance ratios (RR) were determined every three generations of selection by comparing LC_{50}'s of the susceptible strain (SS) vs. the resistant strains using diet incorporation bioassays as described above.

Fitness costs

The fitness of insect strains was measured using per cent egg hatch and mating status. Fertility was determined by deducting the number of unhatched eggs from the total number of eggs, and calculating the hatching percentage. The total number of eggs laid and unhatched were counted on the day of egg collection and hatching (4^{th} day), respectively.

Sex and mating status was determined by dissecting dead moths. Female moths were classified as mated or unmated, based on the presence or absence of spermatophores. Female moths were also separated by the number of spermatophores present.

Results

Bioassay results

The baseline susceptibility (LC_{50}) value of SS to MVP II and Cry1Ac toxin were averaged over three generations conducted with a minimum of three replications per generation. The LC_{50} for MVP II was 24.13 µg/g of diet, (95% FL 16.34-35.62 µg/g; slope ± SE: 1.73 ± 0.24) and LC_{50} for activated toxin was 8.89 µg/g diet, (95% FL 5.83-12.53 µg/g; slope ± SE: 1.61 ± 0.41). These values indicted a 2.7 fold difference in susceptibility between the two sources of Cry1Ac.

Response to selection

Selections were conducted on every generation of the AR (currently at generation 9) and MR strain. The MR strain selection was discontinued after 7 generations of selection owing to insufficient larval numbers (discussed below). Significant variation in tolerance was observed in both resistant strains compared to the unselected strain (SS). Resistance increased from 12.12 to 35.91 fold after 4 and 7 generations of selection in the AR strain, respectively (Table 1). However, resistance did not increase in the MR strain from 16.61 fold, even after selecting at higher concentrations for 3 more generations (Table 1 and Table 3).

The AR strain required only two generations of selection to obtain more than fifty per cent survivors for the first two increases in Cry1Ac concentration (Table 2) with each increase representing a 2.5-fold increase in concentration. On the other hand, the MR strain required a minimum of three generations of selection at a given concentration to obtain fifty per cent survivorship and the selection pressure was increased by only 2 times (Table 3).

Fitness costs

The percent egg hatch (84 - 87%) was not different between the SS and the AR strains. However, adult moths of the MR strain after the first generation of selection at 500 and 1,000 µg Cry1Ac/g diet laid eggs that had very poor (<30 %) hatching. Hatching percentage was severely affected after the 8th generation of selection (Table 3), in which less than one per cent hatching (only 62 larvae from over 7000 eggs) was observed.

The dissection of dead moths revealed that, 73.7 per cent of females were unmated in the MR strain; twice the level compared to the percentage of unmated females in both the SS and the AR strains (Table 4). Additionally, the sex ratio (females:male) for the MR strain (0.9) was substantially lower compared to the sex ratio of AR (1.18) and SS (1.19) (Table 4). These results suggest a reduction in the male's ability to find, mate and/or transfer the sperm packet to the female.

Table 1. Development of Cry1Ac resistance in cotton bollworm, *Helicoverpa zea*

Generations	LC_{50} (µg/g)	Fiducial limits	Slope ± SE	Resistance ratio*
AR Strain				
4	107.64 ± 10.71	75.37 – 155.56	1.42 ± 0.3	12.12
7	319.22 ± 17.60	Not detected#	1.89 ± 0.13	35.91
MR Strain				
4	384.30 ± 66.09	282.31 – 568.12	1.79 ± 0.27	16.61

7	291.40 ± 11.15	155.16 – 455.54	1.67 ± 0.41	12.60

*: Resistance ratio = ratio of LC_{50} of resistant strain to that of unselected strain (SS)
#: 95% confidence interval could not be determined as there were not many data points for prediction

Table 2. Selection details for AR strain selected using activated Cry1Ac

Generations	Concentration (µg/g)	Number of larvae selected	Per cent survivors
1	50	2000	5.45
2	80	3500	12.82
3	80	2000	60.29
4	200	1000	8.67
5	200	1000	62.71
6	500	950	8.96
7	500	1000	32.37
8	500	336	34.82
9	500		

Table 3. Selection details for MR strain selected using MVP II

Generations	Concentration (µg/g)		Number of larvae selected	Percent survivors	Remarks
	Cry1Ac	Inert material			
1	100	423.56	2000	10.13	
2	200	847.12	3500	4.64	
3	500	2117.8	4000	3.23	Poor hatching
4	500	2117.8	2000	20.71	
5	500	2117.8	1000	40.27	
6	1000	4235.6	2000	8.19	Poor hatching
7	1000	4235.6	1900	20.34	Reduced fecundity
8	1000	4235.6	2000	8.47	Poor hatching
9 - 11	No selection		Very few number of larvae		

Table 4. The sex ratio and mating status in different strains of cotton bollworm, *H. zea*

Strains	N	Sex ratio*	Number of spermatophores (% females)					
			0	1	2	3	4	5
SS	79	1.19	37.2	37.20	18.60	4.65	0.00	2.32
AR	85	1.18	39.13	36.96	21.17	2.17		
MR	40	0.90	73.68	10.50	15.78			

* : number of females for every male

Discussion

This research demonstrates that CBW can develop resistance to Cry1Ac insecticidal proteins quicker (3 times faster) when selected using activated toxin compared to MVP II. Thirty six-fold resistance in the AR strain achieved in just 7 generations of selection is considered as relatively rapid. Potential reasons for this relatively rapid rate of resistance development could be, 1) Selecting only larvae that had molted, thereby eliminating a higher percentage of susceptible insects in each generation and 2) Use of activated Cry1Ac toxin. A relatively rapid rate of resistance development has also been observed in *Spodoptera exigua* using Cry1C activated toxin (Moar et al., 1995).

Resistance development in the MR strain was slower and did not increase beyond 16-fold even after selecting for three more generations at higher concentrations. The fitness of this strain was adversely affected in terms of both fecundity and fertility. We believe that the 80.9% inert ingredients in the MVP II formulation might have an effect with the fitness of this strain, especially when selecting at 1 mg/g of Cry1Ac concentration. In addition, MVP II is quite different to the Cry1Ac insects ingest when feeding on Bollgard® which raises concerns about using MVP II as source for Cry1Ac for selection against CBW.

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