FINREP98

COTTON RESEARCH AND DEVELOPMENT CORPORATION

PROJECT TITLE : USE OF BT FOR THE MANAGEMENT OF HELIOTHIS IN COTTON. PART 3: RESISTANCE MECHANISMS, SYNERGISM WITH AND COMPLEMENTATION OF BT TOXIN

PROJECT NUMBER : CSE52C

RESEARCH ORGANISATION : CSIRO ENTOMOLOGY

PROJECT SUPERVISOR : DR R.J. AKHURST
POSTAL ADDRESS CSIRO ENTOMOLOGY
GPO BOX 1700
CANBERRA ACT 2601
TELEPHONE (02) 6246 4123
FACSIMILE (02) 6246 4173

A FINAL REPORT PREPARED FOR THE COTTON R&D CORPORATION

PLAIN ENGLISH SUMMARY

This project aimed to estimate the capacity of *Helicoverpa armigera* to develop resistance to INGARD™ cotton, to examine the possibility of cross-resistance to other *Bacillus thuringiensis* (Bt) toxins and to elucidate the mechanism(s) underlying that resistance. We have used several approaches to selecting *H. armigera* for resistance to Cry1Ac, the toxin expressed by INGARD™ cotton. This involved presenting the toxin in two different formulations and selecting insects for the next generation on two different criteria. By the end of the current project we were seeing the beginning of resistance in at least two distinct selection lines. The slow rate at which *H. armigera* developed resistance is encouraging for the industry but has delayed our investigation of cross-resistance and mechanisms.

In parallel with the selection experiments we investigated the potential of various factors to increase the toxicity of Cry1Ac. We have tested the interactions between Cry1Ac and different Bt toxins, some proteinase inhibitors (to reduce the degradation of the toxin), and chitinase (to provide the toxin easier access to the midgut epithelium). There was no interaction between Cry1Ac and Cry1Ab or other Bt toxins that have very slight toxicity for *H. armigera*. Surprisingly, the two most potent toxins (Cry1Ac and Cry2Aa) proved to be antagonistic, although only slightly so and this antagonism is not expected to be evident in pyramided plants. Although the proteinase inhibitors and chitinase showed some effects on growth rate of *H. armigera*, the effects were not large enough to be useful.

Background

This project contributes to maintaining the value of Bt as a microbial insecticide by providing basic information and developing techniques that will be required for management of resistance to Bt in pest insects. The subject of the project is a major pest of cotton, *Helicoverpa (=heliothis) armigera*, that has developed resistance to chemical insecticides. The introduction of transgenic cotton expressing a single Bt toxin gene into cotton for the control of *H. armigera* has raised concerns about this pest developing resistance to Bt. This project seeks to estimate the capacity of *H. armigera* for developing resistance to a Bt toxin, to examine the possibility of cross-resistance to other Bt toxins, and to elucidate the mechanism(s) underlying resistance to Bt in *H. armigera.*

Bt is a Gram positive, spore-forming bacterium that produces large quantities of insecticidal toxin as a protein crystal. The protein nature of the Bt insecticidal toxins provides an alternative to spraying Bt for controlling pests because plants can be genetically engineered to produce a Bt toxin within the plant tissue (Perlak *et al.* 1990, Voisey *et al.* 1994). This technology has been used to clone the *cryIA(c)* gene into cotton cultivars used in to protect them against the major insect pests, *H. armigera* and *H. punctigera.* However, these pests are much less susceptible to Cry1Ac toxin than is *Heliothis virescens*, for which transgenic cotton was originally developed (Akhurst and Liao, 1996). Consequently, the loss of insecticidal activity in mid- to late season transgenic cotton presents a serious problem. This loss provides the basis for selection for resistance to CryIA(c) and the removal of Helix as an option for treating the survivors increases the probability of resistance very significantly unless suitably large refugia, which have a cost penalty for growers, are employed.

Toxin Specificity. There is a considerable variety of toxin genes among the >40 serotypes of Bt. Höfte and Whiteley (1989) classified the Bt toxins on the basis of their amino acid sequences and recognised 14 classes of toxin. Currently, 65 classes of toxin gene and over 120 individual Cry toxins have been recognised (Crickmore, 1998). As host specificity is determined by part of the amino acid sequence, the toxin classes vary in their effectiveness for particular hosts; Cry1, Cry2 and Cry9 toxins are effective against Lepidoptera, Cry2 and Cry4 against Diptera, and Cry3 against Coleoptera. Within these major groupings there are also some significant differences. For example, although Cry1Ab and Cry1Ac have comparable activities against *Manduca sexta*, their activities against *Mamestra brassicae* vary by an order of magnitude (Milne *et al.*, 1990). *Helicoverpa* species are generally less susceptible to Bt toxins than most caterpillar pests, including *Heliothis virescens*; only Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab are sufficiently toxic for *H. armigera* to be useful in control (Akhurst and Liao, 1996).

Resistance to Bt. Despite expectations to the contrary, resistance to Bt has developed readily in field, as well as laboratory, strains of several important pests, including *Heliothis virescens* and *Plutella xylostella* (McGaughey, 1992). The development of resistance has been both rapid and substantial. McGaughey and Beeman (1988) reported 2- to 29-fold resistance in 3 generations of selection of a laboratory colony of *Plodia interpunctella* with >250-fold increase in one colony over 36 generations. Laboratory selection of a resistant field population of *Plu. xylostella* produced a

further 15- to 30-fold increase (LC_{50} was 430-820 times that for a susceptible strain) in 9 generations (Tabashnik *et al.*, 1991). Resistance in a lab-selected strain of *H. virescens* has reached more than 10000-fold (Gould *et al.*, 1995).

Studies of the mechanism of resistance to Bt show that resistance can develop by alterations at the binding site on the gut membrane. Resistance in a laboratory-selected population of *Plo. interpunctella* was shown to result from changes in the binding affinity of the toxin for the receptors on the brush border membrane (Van Rie *et al.*, 1990). For some chemical insecticide resistance, laboratory-selection results in a different resistance mechanism than field-selection does (Roush and McKenzie, 1987). However, a mechanism for resistance to Bt detected in laboratory-selected *Plo. interpunctella* was the same as that in field-selected *Plu. xylostella* (Ferré *et al.*, 1991).

Change in binding affinity may not be the only determinant of resistance. Wolfersberger (1990) found that the binding affinities of Cry1Ab and Cry1Ac were inversely related to their toxicities for gypsy moth, *Lymantria dispar*. Gould *et al.* (1991) found alterations in the feeding behaviour of *H. virescens* that could contribute to the development of resistance and Gould (1991) reported that there was no change in binding activity or receptor concentration in resistant *H. virescens*. Although resistance to Cry1Ab by *Plo. interpunctella* has been linked to changes in binding to gut membranes (Van Rie *et al.* (1990), Oppert *et al.* (1996) showed that resistance to Cry1Ac in some strains of this species resulted from loss of a major gut proteinase that activates the protoxin. Resistance in other lepidopteran species to Cry1A and to Cry1C have also been shown to arise independently of changes in binding site (Estada and Ferré, 1994; Moar *et al.* 1994).

Managing Resistance Cross-resistance can be a significant problem in managing insecticide resistance. When *Plo. interpunctella* was selected for resistance to DiPel, a commercial formulation of Bt toxins and spores, it did not increase resistance (it actually became less resistant) to Cry1C, which is not a component of DiPel and which recognises a different binding site (Van Rie *et al.*, 1990). However, when *H. virescens* was selected against a single toxin (Cry1Ac), it also developed resistance to the Cry1B, Cry1C and Cry2A toxins (Gould, 1991). It is uncertain whether this broader cross-resistance arises from the insect species selected or from selection against a single toxin rather than the multiple toxins used in the selection of *Plo. interpunctella*.

Although the lack of cross-resistance in *Plu. xylostella* might allow management of resistance by variation of the toxins, the slow restoration of susceptibility in *Plu. xylostella* after discontinuation of selection pressure indicates that rotation of toxins may not be effective for managing resistance in this pest (Tabashnik *et al.*, 1991; Akhurst *et al.*, unpublished data). Stability of resistance after relaxation of selection pressure was also recorded in *H. virescens* (Stone *et al.*, 1989).

Objectives

This project is a component of a tripartite program aimed at developing the basis for a management strategy for resistance to Bt toxins by *H. armigera*. In conjunction with projects run by Dr N. Forrester and Dr J. Daly, it sought to select *H. armigera* for

resistance to Cry1Ac. The specific objective of this project was to select a resistant line or lines of *H. armigera* and to determine the mechanisms of resistance to Cry1Ac that are available to *H. armigera* and the extent of cross-resistance to other biological control options. Knowledge of the resistance mechanism(s) would be used to develop very sensitive techniques for monitoring resistance in field populations. The project also aimed to search for synergists to Cry1Ac.

The mechanism(s) of resistance could not be determined because no resistance was evident until very late in the life of the project. However, this is being addressed in another project. Several classes of potential synergists for Cry1Ac were tested.

Methods

Selection Protocols

Several selection strategies were utilised. The parameters varied were the source of the insects under selection, the source of Bt protoxin, and the character selected for. In general, the selection protocol involved placing neonates on artificial diet into which the Bt preparation had been mixed; a control group was placed on uncontaminated diet. After the neonates had been exposed to the contaminated diet for 7d, survivors were transferred to uncontaminated diet to complete development to pupation and used to provide the neonates for the next generation of selection.

Strain BA2 was established in Project NCQ 1C from field-collected insects supplied by Dr N. Forrester crossed with a lab strain (AN02) to ensure establishment. Prior to the commencement of the current project it had been selected against DiPel, a commercial formulation with three components toxic for *H. armigera* plus spores and synergists, for three generations and then against the HD73 strain (2.5 μg Cry1Ac/ml and spores) for another four generations. Although selection of BA2 against DiPel was made on the basis of survival, selection against HD73 was made on the basis of weight gain. Neonates were placed on artificial diet contaminated with a dosage of HD73 low enough to ensure impact on weight gain without significant effect on survival. After seven days the largest survivors (ca >50% larger than those rejected) were selected to produce the next generation; the proportion chosen varied according to the number of survivors. The survivors were transferred to artificial diet and reared to pupation in the absence of Bt. Due to the lingering effects of the toxin, many died during the rearing out, further reducing the number contributing to the next generation. Selection was continued to the 10th generation in this project.

Another option for selection against a single protoxin was the use of a commercial product (MVP®) that contains Cry1Ac protoxin encapsulated by killed cells of *Pseudomonas fluorescens* and has no spores. After four generations of selection against DiPel, *H. armigera* strain TO was selected for survival on diet contaminated with $1 \mu l \, ml^{-1}$ MVP® to the sixth generation in project NCQ 1C. Selection under the MVP® survival regime was continued to the 11th generation in this project.

BA8 was selected for weight gain, as for BA2, but against MVP® rather than HD73. In an effort to increase the number of insects selected, neonates were put onto diet

containing 0.1μl ml^{-1} MVP® in large trays (500 cm^2) rather than small cups (30 cm^2). The neonates were not counted into the trays but approximately 700 were placed in each tray. After 7d the largest larvae were transferred to Bt-free diet to complete development. After three generations of selection in project NCQ 1C it was selected to the 7th generation in this project.

When Dr Neil Forrester provided a sub-sample of his RATS and VicRATS strains, field collected *H. armigera* showing unusual tolerance to MVP®, we elected to combine the BA2, BA8 and TO selected lines by a series of mass crosses (eg BA2 males x TO females, TO males x BA8 females, etc.) and to continue selection against HD73 (2.5 μg ml^{-1} Cry1Ac and spores) using weight gain as the selection criterion. The resistance status of this new line (BX) was monitored by conducting bioassays against HD73 spore/crystals each third generation.

As there was some difficulty in establishing the RATS and VicRATS lines in Canberra, Dr David Heckel crossed them to the GR laboratory strain to establish strains RX and VX, respectively. As we were having difficulty maintaining the RX strain, we outcrossed it to the laboratory strain ANGR to aid its survival. Once the cultures were stabilised selection was commenced. RX was selected for survival on 2μl ml^{-1} MVP® and VX for survival on HD73 (25.0 μg ml^{-1} Cry1Ac and spores).

Evaluation of Resistance Levels

The BX strain was bioassayed at each third generation to monitor resistance development. Neonate larvae were exposed to seven dosages of HD73 (24 neonates/dosage) for 7d and mortality recorded. The bioassay was replicated up to three times, depending on the number of neonates available. The data were combined and LC_{50} estimated by probit analysis using the POLO-PC software.

The VX line was bioassayed against HD73 at the initial and fourth generations to estimate LC_{50}. Because this line had been selected and shown some enhanced tolerance to MVP® prior to the selection process, we did not assume that the LC_{50} for the initial generation represented the baseline for a susceptible population. Consequently we have maintained a sub-population of VX without selection to allow resistance to be selected out and susceptibility re-established so that we can make meaningful estimates of resistance ratio. The RX line has been more difficult to maintain and so no evaluations have been made at this time. However, a sub-population of RX is also being maintained without selection to allow us to establish the baseline LC_{50}.

The VicRATS population showed 10- to 40-fold resistance in bioassays with MVP® conducted by Dr Forrester and Dr Daly. We conducted a bioassay with HD73 to test the validity of MVP® assays because an additional step in the insects' processing of MVP® is required for its activation into a toxin and this step might give rise to a resistance mechanism that is not relevant in transgenic cotton or non-MVP® spray formulations.

EMS Mutagenesis

Examination of *H. armigera* larvae (AN02) showed that differentiation of the spermatogonia occurs in the fourth larval stage. Consequently the larvae were exposed to the mutagenic agent ethyl methane sulphonate (EMS) during the fourth larval stage. The protocol used to apply the EMS was an adaptation of the vacuum method described by Sega and Lee (1970). Batches of 50-75 fourth instar *H. armigera* were placed under vacuum and 250-300µl of EMS injected into the flask.

After exposure to EMS the larvae were transferred artificial diet to complete development. The pupae were sexed and EMS-treated males were placed with untreated females in mating cages. The eggs were collected and neonates challenged with dosages of DiPel or MVP® that were expected to kill >95%. Any survivors were mated to each other and their progeny re-tested. Where deemed appropriate the challenge was applied to the F_2 generation.

Synergism

Interactions between Cry1Ac and various other toxins (Cry2Aa, Cry4/Cry11, Cyt1A), enzymes (chitinase) and other factors (proteinase inhibitors, spores, and Bt culture supernatant) were tested.

Cry1Ac and Cry2Aa, the most effective Bt toxins for *H. armigera*, were combined in various ratios and the LC_{50} for each combination estimated by replicated bioassays. The model of Tabashnik (1992) was used to test the nature of the interaction between the two Cry toxins. The crystals of Bt serotype *israelensis*, containing Cry4 and Cry11 which have no toxicity for *H. armigera* but cause a reduction in weight gain, were combined (3:1) with Cry1Ac and the LC_{50} estimated by replicated bioassay. Cyt1A is a different type of Bt toxin which has been considered as a peculiarly dipteran toxin until recently. It was known to synergise the Cry toxins when applied to mosquitoes but has recently been shown also to have some effect on beetle larvae. The toxicity of Cyt1A for *H. armigera* was assessed by bioassay and the interaction of Cyt1A and Cry1Ac tested by bioassaying various mixtures of the two toxins.

The binding of Cry1Ac to peritrophic membrane proteins in ligand blots suggested that the tolerance to Bt toxins by *Helicoverpa* spp. may be due to the peritrophic membrane acting as a sump, reducing access of the toxin to the gut epithelium. In conjunction with Dr Helen MacFadden, CSIRO Plant Industry, we tested the interaction of a chitinase and Cry1Ac in bioassays. Dr McFadden provided transgenic cotton leaves expressing a tobacco chitinase for which she had estimated the concentration of chitinase. The leaves were frozen in liquid nitrogen and ground to a fine powder. The leaf powder was mixed in various proportions with artificial diet and placed on wax paper resting on agar/sorbic acid. Neonate larvae were added to the diet and the wells covered with film to prevent desiccation and/or escape. Mortality and weight gain were assessed at 7d. The chitinase leaf powder and DiPel 2X, a Bt formulation containing Cry1Ac and Cry2Aa, were mixed in various combinations and tested against neonate larvae.

It has become evident to us that the "protease-resistant" toxin core of Cry1Ac is only relatively resistant and that the tolerance of *Helicoverpa* to Cry1Ac may be due to their ability to degrade the active toxin. Bioassays were conducted with Cry1Ac and various proteinase inhibitors such as the giant taro proteinase inhibitor (supplied by Dr Danny Llewellyn, CSIRO Plant Industry) and chymostatin. As most of the bioassays were conducted on a soybean-based artificial diet in which the soybean proteinase inhibitors may not be completely de-activated by heating we compared a chickpea-based diet with the soybean diet to assess the diet effect on toxicity of a HD73 spore/Cry1Ac crystal mix.

Cry1Ac was purified from the HD73 strain and separated from spores by solubilisation and centrifugation. The solubilised protoxin was recombined with spores from a strain of Bt that does not produce any Cry toxin and the mixture presented to neonates in a feeding bioassay.

The HD-1 strain is reported to produce Zwittermicin A, a synergist to Cry1A protoxins. The HD-1 culture supernatant was separated from the crystals and spores by centrifugation and tested in a feeding bioassay.

Binding Proteins

One of the key steps in the toxicity of Cry1Ac for *Helicoverpa* is its binding to a specific site on the midgut epithelium. As a change in the binding site is the only resistance mechanism so far detected in field populations resistant to Bt toxin, we sought to identify the specific binding sites in *H. armigera*. Ms Liao Chunyan, a PhD student, had previously demonstrated that Cry1Ac has two binding sites in *H. armigera*. She subsequently used Cry1Ac bound onto an affinity column to purify the binding proteins from midgut tissue of *H. armigera*. The N-terminal sequences of two major bands were determined and compared with sequences in protein databanks to obtain some idea of their nature. The purified proteins were tested for aminopeptidase and alkaline phosphatase activities by standard assay procedures.

Results

Selection for Resistance to Cry1Ac

i) BA2

Strain BA2 was selected for growth rate on HD73 (2.5μg ml^{-1} Cry1Ac) to the 10th generation at which time there was no evidence of resistance development (Table 1). This strain was merged with TO and BA8 to form the BX strain.

Table 1. Selection data for BA2

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d selected	No. selected larvae pupating	% contribution to next generation
S4	0	3250	994	30.6	218	
	HD73	1620	1236	28.3	129	8.0
S5	0	3700	1856	50.2	529	
	HD73	7010	2526	12.8	196	2.8
S6	0	850	356	41.9	343	
	HD73	3950	1528	15.0	478	12.1
S7	0	3220	1750	54.4	608	
	HD73	10830	2762	14.8	503	4.6
S8	0	3590	1448	40.3	262	
	HD73	6100	1906	19.9	603	9.4
S9	0	7810	3286	42.1	1096	
	HD73	10110	2416	18.9	462	4.6
S10	0	3463	1213	35.0	469	
	HD73	2240	1044	28.5	419	18.7

ii) TO

Strain TO was selected for survival on 1µl ml^{-1} MVP® to the 11th generation without any indication of resistance (Table 2). At that time the strain was merged with BA2 and BA8 to form the BX strain.

Table 2. Selection data for TO

Generations of selection	Treatment	No. Tested	No. Survivors	% survivors at 7d	No. pupating	% contribution to next generation
S4	0	3460	1532	44.3	144	
	MVP	4320	613	14.2	150	3.5
S5	0	4880	2424	49.7	678	
	MVP	7530	1644	21.8	283	3.8
S6	0	2970	1776	59.8	767	
	MVP	8223	2212	26.9	413	5.0

Table 2 (ctd). Selection data for TO

Generations of selection	Treatment	No. Tested	No. Survivors	% survivors at 7d	No. pupating	% contribution to next generation
S7	0	1890	1061	56.1	356	
	MVP	6878	1911	27.8	687	10.0
S8	0	4370	1853	42.4	706	
	MVP	12946	1491	11.5	299	2.3
S9	0	7170	2522	35.2	812	
	MVP	5620	660	11.7	129	2.3
S10	0	3991	1196	30.0	335	
	MVP	2739	554	20.2	112	4.1
S11	0	1770	842	47.6	75	
	MVP	150	100	66.7	20	13.3

iii) BA8

Strain BA8 was selected for growth rate on 0.1μl ml^{-1} MVP® to the 7th generation (Table 3) at which time it was merged with BA2 and TO to form the BX strain.

Table 3. Selection data for BA8

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d selected	No. selected larvae pupating	% contribution to next generation
S3	0	1210	345	28.5	39	
	MVP	2200	216	9.8	89	4.0
S4	0	1860	873	46.9	321	
	MVP	2720	505	18.6	133	4.9
S5	0	6540	3154	48.2	639	
	MVP	1570	168	10.7	53	3.4

Table 3 (ctd). Selection data for BA8

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d selected	No. selected larvae pupating	% contribution to next generation
S6	0	200	110	55.0	43	
	MVP	300	100	33.3	12	4.0
S7	0	1350	747	55.3	356	
	MVP	100	91	91.0	1	1.0

iv) BX

The BX strain was selected for growth rate on HD73 (2.5μg ml^{-1} Cry1Ac). By generation 12 no resistance had been detected.[1] (Table 4).

Table 4. Selection data for BX strain

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d selected	No. selected larvae pupating	% contribution to next generation
S1	0	2270	1210	53.3	414	
	HD73	2660	1097	24.0	317	11.9
S2	0	1310	649	49.5	259	
	HD73	2510	1272	30.6	238	9.5
S3	0	1315	793	60.3	213	
	HD73	2040	1066	29.6	255	12.5
S4	0	1770	789	44.6	284	
	HD73	2100	770	21.5	164	7.8
S5	0	810	538	66.4	396	
	HD73	3090	1628	30.1	420	13.6
S6	0	447	287	64.2	147	
	HD73	3970	2179	33.1	753	19.0
S7	0	320	185	57.8	100	
	HD73	2540	1344	30.3	426	16.8

[1] At the 16th generation (August 1998) there was an indication of resistance but the LC_{50} was higher than the range of dosages used and so the resistance ratio could not be determined. Bioassays are currently being conducted on the 17th generation.

Table 4 (ctd). Selection data for BX strain

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d selected	No. selected larvae pupating	% contribution to next generation
S8	0	120	119	99.2	6	
	HD73	1470	694	25.6	211	14.4
S9	0	460	267	58.0	178	
	HD73	1080	724	40.3	367	34.0
S10	0	74	25	33.8	10	
	HD73	1080	725	38.5	230	21.3
S11	0	320	227	70.9	127	
	HD73	840	653	35.1	336	40.0
S12	0	420	240	57.1	175	
	HD73	1560	1023	33.5	384	24.6
S13	0	585	358	61.2	220	
	HD73	2000	1246	30.3	335	16.8
S14	0	380	255	67.1	159	
	HD73	1500	976	30.3	276	18.4
S15	0	300	200	66.7	125	
	HD73	540	426	36.7	122	22.6

Estimation of LC_{50} each third generation showed a large amount of variability and no consistent trend (Table 5). However, for each of the five generations tested, the LC_{50} for BX was consistently larger than that for a susceptible laboratory strain (ANGR).

Table 5. Estimation of LC_{50} of Cry1Ac for the BX strain

Generation of selection	LC_{50} (µg Cry1Ac ml^{-1})	95% Confidence Interval	Resistance ratio
S1	227	nd*	
S3	74	30-157	3.9
S6	560	nd	
S9	318	67-7278	16.7
S12	109	63-253	5.7
Unselected (ANGR strain)	19	10-33	

* nd - data are too heterogeneous; confidence interval could not be estimated.

v) VX

VX was selected for survival on HD73 (25.0 μg ml^{-1} Cry1Ac and spores) (Table 6).

Table 6. Selection data for VX strain

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d	No. pupating	% contribution to next generation
S1	0	1700	678	39.9	331	
	HD73	3160	609	19.3	218	6.9
S2	0	50	28	56.0	0	
	HD73	1060	312	29.4	154	14.5
S3	0	220	100	45.5	52	
	HD73	1390	429	30.9	235	16.9
S4	0	350	99	28.3	25	
	HD73	1140	375	32.9	78	6.8
S5	0	80	20	25.0	12	
	HD73	1020	331	32.5	78	7.6
S6	0	90	38	42.2	21	
	HD73	900	355	39.4	296	32.9

The estimations of LC_{50} at the first and third generations were not very reliable because the data were too heterogeneous - too few neonates were available for bioassay because they were required to establish the next generations.[2]

vi) RX

RX was selected for survival on 2μl ml^{-1} MVP® (Table 7). This strain is being maintained with some difficulty and no estimates of LC_{50} have been made.

[2] The bioassay data for the sixth generation (August 1998) were more satisfactory, estimating LC_{50} at 685 (405-2985) μg Cry1Ac ml^{-1}. The S_6 of VX is therefore significantly more tolerant of Cry1Ac than the S_{12} of BX.

Table 7. Selection data for the RX strain.

Generations of selection	Treatment	No. Tested	Survivors at 7d	% survivors at 7d	No. pupating	% contribution to next generation
S1	0	2692	1351	50.2	678	
	MVP	1696	349	20.9	247	14.6
S2	0	289	32	11.1	5	
	MVP	1178	530	45.0	319	27.1

vii) VicRATS

The LC_{50} of Cry1Ac crystals against VicRATS was 479 (342-643) ng cm^{-2}, which was significantly higher than that for the AN02 susceptible strain (150 [105-207]). However, the difference between the two strains was less than that between AN02 and BA8, a field-collected strain (LC_{50} = 848 [449-2873]). This suggests that resistance to MVP® may not be due to resistance to Cry1Ac but to the release of Cry1Ac from the capsule.

EMS Mutagenesis

EMS mutagenesis was applied to 285 males which were then crossed to untreated females and 8180 neonates resulting were screened for resistance to MVP®. No evidence for resistance could be found in any lines established from the 294 neonates surviving the screening. The mutagenesis approach was discontinued in September 1996.

Synergism

i) Bt toxins

We had previously demonstrated that Cry1Ac and Cry2Aa, the most toxic Bt proteins known for *H. armigera*, do not compete for binding sites in *H. armigera*. Nevertheless we have now found that they do interact in *H. armigera*. In every combination of Cry1Ac and Cry2Aa tested, the LC_{50} of the mixtures was greater than predicted from the LC_{50} values for the individual toxins (Table 8). The level of antagonism was small and would probably not be evident in Cry1Ac/Cry2Aa cotton unless the total Cry protoxin was only the same as that of single gene cotton.

Table 8. Interaction between Cry1Ac and Cry2Aa

Cry1Ac:Cry2Aa ratio	LC_{50} (ng cm^{-2})		Synergism ratio[#]
	Observed	Expected	
1:0	115 (82-159)[*]		
3:1	307 (203-630)	122	0.4
2:1	287 (204-483)	126	0.4
1:1	214 (122-420)	129	0.6
1:2	271 (167-507)	137	0.5
1:3	378 (276-622)	139	0.4
0:1	149 (117-189)		

[#] a synergism ratio >1 indicates synergism; <1 indicates antagonism.
[*]95% confidence interval

As Bti is not toxic for *H. armigera* it was not possible to calculate an expected LC_{50} for the Cry1Ac/Bti crystal mixture. However, a 1:3 mixture of Cry1Ac and Bti crystal yielded an LC_{50} of 590 (389-1037) ng cm^{-2} which is comparable with the value one would expect for Cry1Ac diluted with a non-toxic protein. There was, therefore, no evident interaction between Cry1Ac and Bti crystals.

Cyt1Aa had no toxicity for *H. armigera*, as detected either by mortality or reduced weight gain, at dosages of 10-3000 ng cm^{-2}. No consistent interaction between Cry1Ac and Cyt1A affecting survival of or weight gain in *H. armigera* could be detected (Table 9).

Table 9. Feeding bioassays with combinations of Cry1Ac and Cyt1A

Treatment		Experiment 1		Experiment 2		Experiment 3	
Cry1Ac (ng cm^{-2})	Cyt1A (ng cm^{-2})	% mortality	Mean larval weight at 7d (mg)	% mortality	Mean larval weight at 7d (mg)	% mortality	Mean larval weight at 7d (mg)
0	0	4.2	41.18	0	111.79	4.2	22.42
90	0	45.8	2.05	41.7	0.01	54.2	1.14
90	10	37.5	5.17	37.5	0.01	70.1	2.73
90	300	41.7	5.39	54.2	0.56	33.3	1.38
90	1000	25.0	5.96	45.8	0.91	54.2	1.18
90	3000	16.7	5.23	62.5	0.69	50.0	2.83

ii) Chitinase

The transgenic leaf powder was toxic for *H. armigera* at a dosage of 700µg ml^{-1} diet, killing ca 50% of neonates and reducing the weight gain of the survivors by ca 80%. When DiPel and leaf powder were mixed there was no effect on the mortality of the insects; there was an inconsistent effect on growth rate. As the chitinase effect is only substantial at large dosages and there is no obvious synergism with Cry1Ac, no further experimentation was conducted.

iii) Proteinase inhibitors

No evidence for interaction between Cry1Ac and either the giant taro proteinase inhibitor (a trypsin inhibitor) or chymostatin (a chymotrypsin inhibitor) was detected.

There was a consistent, but not statistically significant, difference in the susceptibility of *H. armigera* to Cry1Ac on soybean and on chickpea diets. The LC_{50}s on soybean and chickpea diets were 12.8 (8.2-20.0) and 6.9 (1.2-14.4) µl HD73 ml^{-1}. The difference was not sufficiently large to have invalidated the negative findings from the proteinase inhibitor assays.

iv) Spores

There was no dose response when spores were added to a fixed concentration of solubilised Cry1Ac for bioassay (Table 10). However, the presence of spores did appear to enhance toxicity of Cry1Ac; further experimentation is required.

Table 10. Effect of Bt spores on toxicity to H. armigera *of solubilised Cry1Ac.*

Cry1Ac (ng cm^{-2})	No. Bt spores	% mortality at 7d	Mean wgt survivors(mg)
0	0	0	111.79
0	10^6	0	97.50
110	0	29.2	0.29
110	10^3	70.8	0.71
110	10^4	45.8	0.82
110	10^5	58.3	1.30
110	10^6	45.8	0.39

v) Bt culture supernatant.

The attempt to produce Zwittermicin A from HD-1 to test its synergism with Cry1Ac for *H. armigera* was unsuccessful. The supernatant of HD-1 contained sufficiently large quantities of soluble Cry1Ac to confuse the bioassay results. Further experimentation should be conducted if a suitable source of Zwittermicin A, uncontaminated by Cry protoxin, can be located.

Binding Proteins

Immunoblot analysis of the N-acetyl galactosamine fraction of *H. armigera* midgut epithelium proteins passed through a Cry1Ac-affinity purification column showed two strong bands at 124 and 162kDa and two weaker bands at 107 and 130kDa. N-terminal analysis of the two major bands showed that both have significant homology to aminopeptidase N sequences; we have been unable to get sequence data for the minor bands at this stage. The N-acetyl galactosamine fraction was shown to have significant aminopeptidase activity but no alkaline phosphatase. These data indicate that the binding proteins in *H. armigera* are similar, though distinct from, those of *H. virescens*.

Discussion

The major aim of the project, the determination of resistance mechanism(s), was frustrated by the long delay in obtaining *H. armigera* resistant to Cry1Ac. The culture of *H. armigera* is difficult and population crashes are not unusual, causing problems in maintaining selection lines. Nevertheless we have been able to maintain a selection line (BX) through 15 generations by combining three independent lines that had been selected for up to 11 generations. Although no resistance was detected by completion of this project, the 16th generation shows signs of very substantial resistance. In their complementary projects, Dr Neil Forrester and Dr Joanne Daly reported some success in obtaining Bt resistant *H. armigera*. The VX line, which was created by crossing Dr Forrester's VicRATS line with a susceptible laboratory strain, is also showing indications of a substantial resistance.

Cry1Ac toxin was presented in two different formulations in this project: a mixture of Cry1Ac crystals and Bt spores and MVP®, a commercial product in which the Cry1Ac toxin is encapsulated in bacterial proteins and lipids. MVP® has the advantage of being easily and cheaply available whereas tbe crystal/spore mix must be produced in-house and is therefore more demanding of personnel resources. However, we were concerned that the need for the insect to degrade the capsule to release the Cry1Ac toxin may lead to a resistance mechanism that is not relevant to transgenic cotton. This concern appears to be borne out by our analysis of the VicRATS line. This line, established by Dr Forrester from field-collected *H. armigera* which survived screening against MVP® and was subsequently selected against MVP®, was reported to be 10- to 40-fold resistant to MVP®. However, the VicRATS line was no more tolerant of the Cry1Ac crystals than BA8, another line established from field-collected *H. armigera* but which was not selected for resistance. This suggests that resistance to MVP® may be more a matter of resistance to the formulation (ie encapsulation) than to the toxin itself. It is worth noting that there are unpublished reports that the YHD2 line of *H. virescens*, which is >10,000-fold resistant to MVP®, is killed by BOLLGARD™ cotton.

Two selection protocols were adopted in this study. Some lines were selected for survival and some for growth rate. The survival lines are difficult to maintain because the lingering effects of intoxication cause many *H. armigera* to die during larval and pupal development. The growth rate selection protocol, using a much lower dosage of Bt, allows much better survival and facilitates maintenance of selection lines. However, we have some data that suggests that this selects the most vigorous members of the population and may produce a resistance that has little meaning in field populations. Until we have a clearer view of which protocol is ultimately more useful, we have chosen to run both.

Sprayable Bt formulations contain a number of synergists, many of which are commercial secrets, that potentiate the toxin. We have tried to identify some synergists that might be applied through or over transgenic cotton to make it more effective for killing *H. armigera* and reduce the risk of resistance development. The discovery that resistance to Bt toxins can be mediated by altered gut proteinase activity

resulting in a reduced half-life for the activated toxin (Oppert *et al.*, 1996, 1997) and our observation that Cry1Ac appears to be quickly degraded in the *H. armigera* gut, suggested that proteinase inhibitors might be used to enhance Cry1Ac toxicity for *Helicoverpa* spp. Although we were unable to demonstrate any effect from the use of the giant taro proteinase inhibitor or chymostatin, Shao *et al.* (1998) recently reported that tomato protease inhibitor and potato protease inhibitor at 0.1 mg ml^{-1} increased the toxicity of Cry1Ab and Cry1Ac protoxins by five-fold. Further investigation of other proteinase inhibitors is warranted.

Before a Bt toxin can exert its toxicity it must pass through the peritrophic membrane, a chitin-protein matrix. Since Cry1Ac binds strongly to *H. armigera* peritrophic membrane (Akhurst and Liao, 1996) and the potentiation of Bt toxins by chitinase has been reported for several species (Regev et al., 1996, Ding *et al.* 1998), we investigated with Dr Helen McFadden, CSIRO Plant Industry, the use of a tobacco chitinase in conjunction with Cry1Ac. Although there was no effect on mortality and only a variable effect on growth rate with the tobacco chitinase, the testing of some alternative chitinases (bacterial, insect) might be considered.

The most likely resistance mechanism for *H. armigera* involves a change in the Cry1Ac binding sites. The purification and identification of the Cry1Ac binding proteins of susceptible *H. armigera* will be useful in testing the hypothesis that resistance is mediated by a change in binding site and in determining the nature of that change. With that information in hand, we hope to be able to generate a protocol for assessing the frequency of resistance alleles in field populations. If the frequency of resistance alleles is known, the resistance management strategy can be monitored and management protocols defined with greater precision than currently.

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Assessment of Impact of Results and Conclusions on the Cotton Industry

This project has provided the basis for investigation of the resistance mechanism(s) that *H. armigera* may develop in response to the deployment of INGARD™ cotton. By the completion of this project we could see the first signs of resistance to Bt appearing in *H. armigera*. In the next phase we expect to compare the binding characteristics of Cry1Ac in susceptible and resistant *H. armigera* and to evaluate the role of proteases in resistance. The results of this will enable us to assess the advisability of the two Bt gene strategy; for example if the resistance is due to altered proteases, resistance to Cry1Ac and Cry2Aa may develop simultaneously. We may be able to use our understanding of the resistance mechanism to assist our search for resistance breaking synergists and to develop molecular methods for monitoring the level of resistance in field populations.

Project technology

No technology was developed in this project.

Technical Summary

The discovery that insects apparently resistant to MVP® are not resistant to purified Cry1Ac crystals needs to be considered. The use of MVP® as a source of Cry1Ac for studies on *H. armigera* and transgenic cotton may not be meaningful.

Recommendations

Simultaneous bioassays of MVP® and purified Cry1Ac toxin or transgenic cotton for which the Cry1Ac content has been determined should be conducted on the VicRATS population to assess the usefulness of MVP® in these studies.

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