HIGH LEVEL RESISTANCE TO INGARD® COTTON BY THE COTTON BOLLWORM *HELICOVERPA ARMIGERA*

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Introduction

Control of *Helicoverpa armigera* is being forced towards minimising reliance on broad spectrum insecticides. Serious environmental concerns have emerged over the extensive use of highly toxic, non-specific compounds to control this species. Pest resurgence associated with the destruction of natural enemies and the development of high levels of insecticide resistance have lead to the steady increase in the use of biopesticides and transgenic plants containing insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt).

Current Bt cotton cultivars based on INGARD® technology express a single insecticidal protein (Cry1Ac). Although this toxin provides good control of the lepidopteran pests *Pectinophora gossypiella* (Simmons et al. 1998) and *Heliothis virescens* (Parker et al. 2000) in the USA, *H. armigera* has a naturally higher tolerance to the Cry1Ac protein than these species (Akhurst and Liao 1996). Tolerance, combined with the decline of toxin expression in the latter part of the season (Fitt 1998), suggests that if not managed carefully, INGARD® could lose its ability to control field populations of this pest. Given the strong selection pressure applied in the field by INGARD®, the potential for rapid development of resistance is of major concern and remains the biggest threat to the sustainability of this technology in the Australian cotton industry.

There is no evidence to date that resistance to Cry1Ac exists in field populations of *H. armigera*. However the capacity for *H. armigera* to develop high levels of resistance to Cry1Ac has been demonstrated in the laboratory. We have previously reported that a laboratory strain of *H. armigera* (BX) reached a resistance ratio of 300-fold compared to a susceptible strain (Akhurst et al. 2000). Continued selection of a related strain (IS) has resulted in insects becoming significantly more resistant to Cry1Ac than the BX strain.

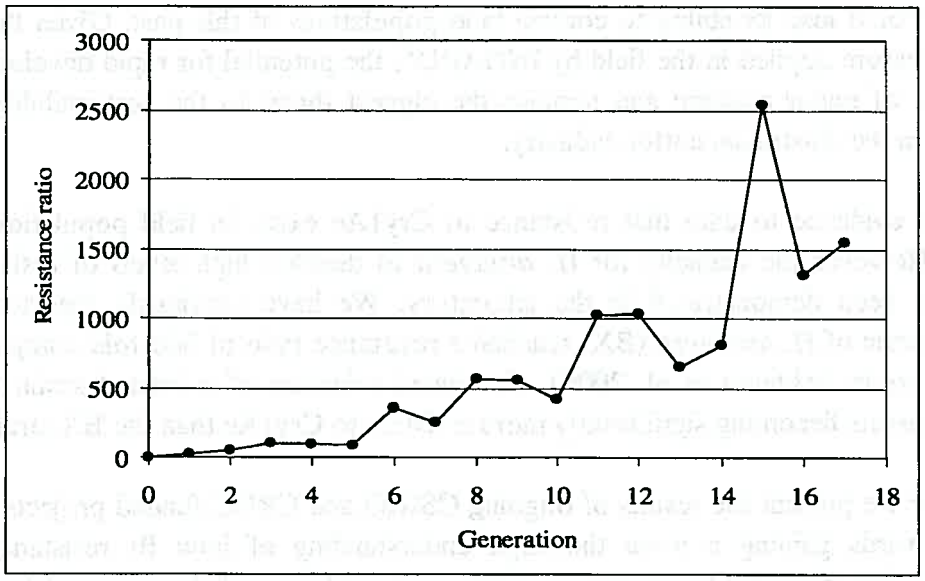
In this paper we present the results of ongoing CSIRO and CRDC funded projects that are working towards gaining a more thorough understanding of how Bt resistance in *H. armigera* has the potential to impact on cotton pest ecology and the expected benefits of resistance management strategies. The focus of our research is also directed toward estimating the capacity of other insecticidal proteins for the control of *H. armigera*. These include the second gene engineered into cotton, Cry2Ab, and the soluble protein, Vip3A.

Selection for Cry1Ac resistance

A starter population of *H. armigera* was established from field survivors of discriminating dose screens (Forrester 1998) combined with field insects collected by Dr Dave Murray (QDPI, Toowoomba) and an existing laboratory colony supplied by Dr Joanne Daly (CSIRO, Canberra). Intensive selection using Cry1Ac toxin resulted in a colony of *H. armigera* that is homozygous for a major resistance allele. Selection of this resistant colony, BX, resulted in a >300-fold increase in resistance after 16 generations compared to a susceptible strain (ANGR). Despite maintenance of a constant selection dose the resistance ratio declined, stabilising at 80-fold after generation 23 but returning to 200-fold after generation 38.

A second resistant strain, IS, has also been established. This strain originated from individuals from the BX colony that had survived a full larval cycle on glasshouse grown transgenic cotton. At this time the resistance ratio of BX was 100-fold. The surviving BX insects were then outcrossed to the susceptible strain ANGR. Subsequent selection at low, incremental doses restored the resistance ratio to 200-fold by generation 6. Continued selection at higher doses resulted in a resistance ratio of approximately 1500-fold at generation 17 (Fig . 1).

Figure 1. Selection of *H. armigera* (IS) for resistance to Cry1Ac.



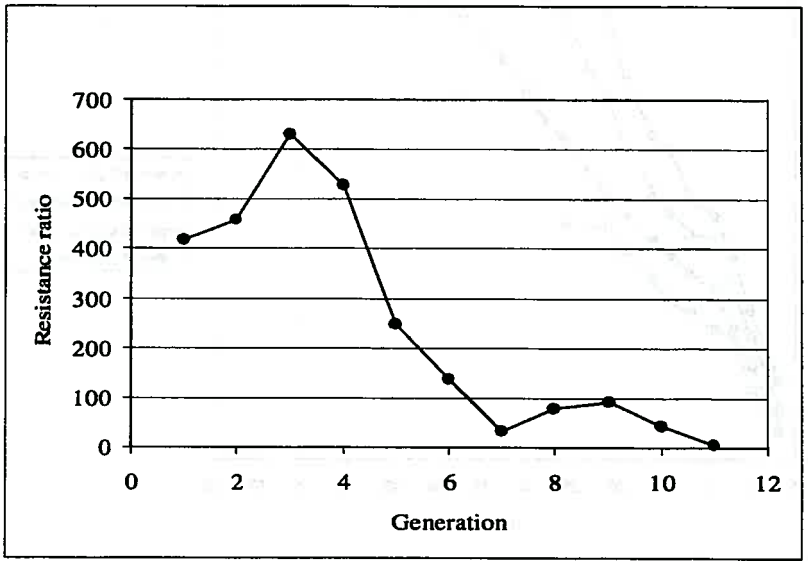
It seems unlikely that this high level resistance is merely the result of improved vigour in this colony. This is because the resistance ratio has continued to increase in response to increased selection pressure. Higher levels of resistance have been associated with more than one resistance gene (Ferré and Van Rie, 2002) and it is possible that a second gene conferring high levels of resistance is involved.

IS was tested for homozygosity by a series of single pair matings at generation 10. The progeny of these matings were screened with a discriminating dose of Cry1Ac. From this experiment it was estimated that this colony was between 60 and 80 percent homozygous for a major resistance allele.

Stability of Cry1Ac resistance

The stability of resistance is determined by measuring changes in the frequency of resistance genotypes in a population when exposure to insecticide ceases (Tabashnik et al. 1994). At generation 10 no further selection pressure was applied to half the IS population. At this time the resistance ratio of IS was 400-fold. An apparent initial increase to 600-fold resistance was observed following 2 generations of deselection. However, the LC_{50}s for generations 1 to 4 were not significantly different. The resistance ratio declined steadily over the next 8 generations to near baseline levels (Fig. 2).

Figure 2. Deselection of IS_{10}.



Reduced biotic fitness associated with resistance genes (or other genes at closely linked loci) are the most likely cause of instability (Tabashnik et al. 1994) and could be caused by effects on one or more parameters, including larval viability, fecundity and mating success. Understanding the instability of Bt resistance could help to explain why field resistance has been relatively rare.

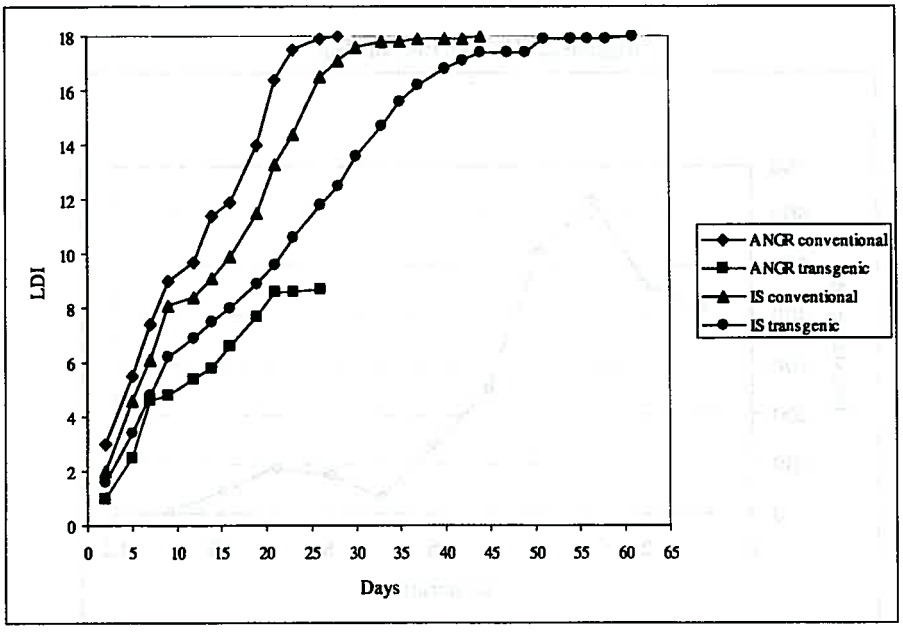
Fitness cost of Cry1Ac resistance

In order to delay the evolution of resistance, pre-emptive resistance management strategies based on the use of refuges have been implemented (Forrester and Bird, 1996). Refuges are designed to promote the survival of Bt susceptible insects so that they can mate with the few survivors carrying a resistance allele. Resistance in *H. armigera* is inherited as a

partially recessive trait (Daly and Olsen, 1998) and the hybrid progeny of these matings are functionally susceptible to Bt toxins. By controlling these "carriers" of resistance, the evolution of resistance in field populations can be substantially delayed (Roush 1996).

The refuge theory makes the assumption that mating between susceptible and resistant individuals is random. For this to be achieved resistant and susceptible adults must emerge synchronously (Liu et al. 1999). Observations of larval development of *H. armigera* on glasshouse grown plants showed that the development of resistant genotypes was significantly slower on conventional cotton (V15) than susceptible genotypes, and even slower on transgenic cotton (V15i) (Fig. 4).

Figure 4. Development time of *H. armigera* from 24 hour old neonate to pupa for susceptible strain (ANGR) and resistant strain (IS) of reared on conventional (V15) cotton or INGARD® (V15I) cotton. Larval development index (LDI) indicates average larval instar, ie. LDI 1=100% at 1st instar, LDI 18=100% pupae.



Analysis of these results indicate that, on conventional cotton, it takes 25 days for 90% of susceptible insects to pupate, whereas it takes 32 days for 90% of resistant insects to pupate The time taken for 90% of resistant insects reared on transgenic cotton to pupate was 47 days (Table 1). At the time when 90% of susceptible insects reared on conventional cotton had pupated, over 90% of the resistant insects reared on transgenic cotton were still in the larval stage. If this was replicated in the field, populations from refuge and transgenic crops would be unable to mate randomly.

Table 1. Effective time to pupation for susceptible strain (ANGR) and resistant strain (IS) of *H. armigera* reared on conventional (V15) cotton or INGARD® (V15I) cotton with 95% confidence intervals in brackets.

Strain	Cotton Variety	Effective time to pupation (days) (95% confidence intervals)		
		10%	50%	90%
ANGR	V15	20 (19 - 21)	22 (22 - 23)	25 (24 - 27)
IS	V15	22 (20 - 24)	27 (25-, 28)	32 (30 - 36)
IS	V15i	29 (27 - 30)	37 (36 - 38)	47 (46 - 50)

This apparent fitness cost favours asynchronous development between susceptible insects developing in refuges and resistant individuals surviving off transgenic cotton (and to a lesser extent resistant insects developing in refuges). Similar findings were described for larval development of *P. gossypiella* (Liu et al. 1999). Resistant larvae of this species reared on Bt cotton required an average of 5.7 days longer to develop than susceptible larvae on non-Bt cotton. Because 80% of these moths mate within 3 days of emergence it was postulated that non-random mating between resistant moths generated from Bt plants would produce a disproportionately high number of resistant homozygotes, thereby accelerating the evolution of resistance.

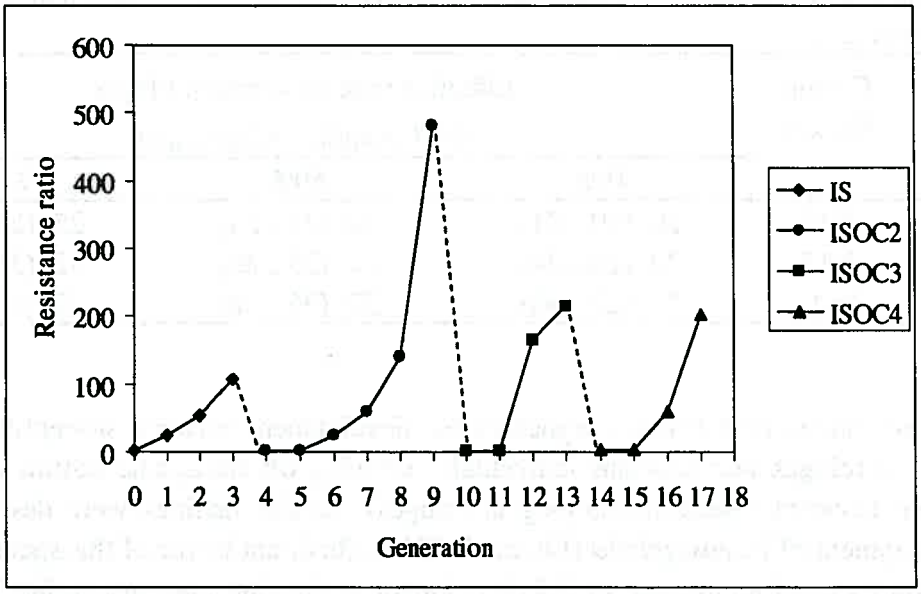
The results from fitness experiments described above indicate the potential for differential development of *H. armigera* to interfere with random mating and that all effort should be made to ensure that refuge plantings maintain a continuum of susceptible genotypes in the metapopulation.

Selection of Near Isogenic Lines of Resistant and Susceptible *H. armigera*

Fitness cost associated with resistance is an important factor in determining the parameters required for an effective refuge strategy. Valid fitness comparisons can only be made between strains of insects with similar genetic backgrounds. Fitness experiments to date have been conducted on non-isogenic strains of *H. armigera*. Isogenic strains have recently been developed by a series of outcrosses of the resistance allele into the parental strain. This has resulted in strains that are >95% genetically similar (Fig. 5).

Future work will include life history trait analyses on these isogenic lines reared on glasshouse grown cotton and alternative refuge crops. Of key importance is the response of heterozygotes as this is the most important influence on the number of generations required for resistance alleles to reach high frequency (Roush 1996).

Figure 5. Selection of near-isogenic lines of susceptible and resistant H. *armigera*. Broken lines indicate the generation at which outcrossing occurred.



Alternative toxins

The deployment of two (or more) insecticidal agents with different modes of action is considered to be one of the major options for managing resistance to Bt. *H. armigera* is significantly susceptible to only four Cry proteins (Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab) (Akhurst and Liao, 1996). Cross-resistance studies have shown that resistance to Cry1Ac extends to Cry1Ab but not to Cry2Aa or Cry2Ab (Table 2). This narrow spectrum of resistance indicates that Cry2 proteins are useful alternatives, either in transgenic plants or foliar sprays, to the toxin Cry1Ac currently used in INGARD®.

Table 2. Relative susceptibility of Cry1Ac susceptible (ANGR) and resistant (IS) strains of *H. armigera* to Bt products. Cry1Ac, Cry1Ab and Cry2Ab were tested as a spore/crystal mix. Cry2Aa was tested as a solubilized crystal preparation.

	Cry1 and Cry2 Cross Resistance Study						
Toxin	ANGR			IS			Resistance ratio
	LC_{50} (ng/cm^2)	Slope	95% confidence intervals	LC_{50} (ng/cm^2)	Slope	95% confidence intervals	
Cry1Ac	49	1.5	34 - 68	28229	1.2	21303 - 37105	581
Cry1Ab	2885	0.8	1840 - 4704	2510282	1.2	1328330 - 11930119	870
Cry2Aa	174	1.9	129 - 224	282	1.8	222 - 355	2
Cry2Ab	306	1.4	239 - 391	502	2.1	410 - 606	2

We have previously reported that the resistant strain BX was not significantly cross-resistant to the sprayable formulation DiPel®, which contains Cry1Ab, Cry1Ac and Cry2A proteins (Akhurst et al. 2000). However, the resistant strain IS, with a resistance ratio of 1000-fold, is significantly less sensitive to Dipel® as well as to Xentari®, another commercial Bt formulation (Table 3). It appears that, elevated levels of resistance in IS confers cross-resistance to proteins contained in these commercial products. This may be further evidence of the existence of a secondary resistance gene in *H. armigera*.

Table 3. Relative susceptibility of Cry1Ac susceptible (ANGR) and resistant (IS) strains of *H. armigera* the commercial formulations DiPel® and Xentari®. LC_{50} is expressed as ng/cm^2 of spayable powder. The resistance ratio for Cry1Ac spore/crystal preparation was 1000-fold.

Toxin	DiPel® and Xentari® Cross Resistance Study						Resistance ratio
	ANGR			IS			
	LC_{50} (ng/cm^2)	Slope	95% confidence intervals	LC_{50} (ng/cm^2)	Slope	95% confidence intervals	
DiPel®	188	1.9	136 - 252	4447	1.9	2779 - 6261	24
Xentari®	654	2.7	442 - 848	9417	2.2	7705 - 11445	14

Investigations have begun into estimating the capacity of other insecticidal proteins to control *H. armigera*. One of these candidate proteins is Vip3A. This represents a new type of insecticidal agent because it is secreted as a soluble protein rather than forming a crystal inclusion inside a Bt cell (Donovan et al. 2001). Initial studies of Vip3A indicate that its potency is comparable to that of Cry1Ac on a susceptible strain of *H. armigera* (LC_{50} 155ng/cm^2). It also shows promise as an alternative control agent because it is sufficiently different from Bt crystal proteins as to suggest that it has a different mode of action from that of the Cry toxins and would, therefore, not be subject to cross-resistance.

Conclusion

The development of resistance to the insecticidal component of INGARD® cotton in a laboratory strain of *H. armigera* demonstrates that there is compelling need for care to be taken in managing transgenic technology. To date there is no evidence of resistance to the Cry1Ac toxin expressed by INGARD® cotton. However, our experiments have shown that *H. armigera* has the capacity to evolve elevated levels of resistance that would jeopardize the efficacy of both transgenic cotton and sprayable forms of Bt. Our research aims will be continually directed toward a better understanding of how Bt resistance impacts on the ecology of *H. armigera* as well as focusing on investigations into alternative toxins.

The introduction of a second Bt gene into cotton cultivars of the future will also require careful management. There is no indication that cross-resistance extends to the second gene engineered into cotton, Cry2Ab. However, there is the theoretical possibility of a resistance mechanism that would negate both Cry1Ac and Cry2Ab. We are currently developing discriminating dose bioassays for monitoring Cry2Ab resistance in field populations.

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