CRDC

# FINAL REPORT

# *High level Cry1Ac resistance in Helicoverpa armigera*

## CSE 101C

**September 2004**


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**Australian Government**

**Cotton Research and Development Corporation**

Annual, Progress and Final Reports

# REPORTS

## *Part 1 - Summary Details*

*Please use your TAB key to complete Parts 1 & 2.*

**CRDC Project Number:** **CSE101C**

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**Project Title:** High level Cry1Ac resistance in *H. armigera*

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30/9/04

## 1. Background

The significance of transgenic cotton in the pest control strategy adopted by the Australian industry makes the management of resistance to the Cry1Ac toxin of *Bacillus thuringiensis* essential. Accordingly, the CRDC supported the selection of resistance in *H. armigera* so that the extent and nature of the threat could be estimated before it actually occurred in the field.

During selection of the original *H. armigera* strain (BX) in which Bt resistance was first detected, resistance rose to 300-fold and then declined to stabilise at approximately 80-fold. To assess whether the decline in resistance was due to a loss in general vigour associated with inbreeding or rather was the result of fitness costs associated with the resistance, the BX strain was outcrossed to a susceptible laboratory strain and re-selected as the IS strain. With increasing selection pressure, the IS strain reached a resistance ratio exceeding 800-fold. It was clear from this experiment that the decline in resistance of the BX strain was the result of inbreeding. However, it was not clear whether the very high resistance detected in the IS strain was merely the result of improved vigour. As higher levels of resistance in Cry1Ac-resistant diamondback moth can be associated with more than one resistance gene (Ferré and Van Rie, 2002, Ann. Rev. Entomol. 47, 501-503), the higher resistance in *H. armigera* might similarly be indicative of the presence of a secondary resistance gene (or genes). It is important that we understand all the resistance options available to *H. armigera* because we cannot be certain that the order in which resistance genes arise will be the same in the field as in the laboratory.

Fitness cost associated with resistance is an important factor in determining the parameters required for an effective refuge strategy. When the fitness of the BX strain was assessed against a susceptible laboratory strain, we recorded a significant delay in development that might impede the efficiency of the resistance strategy. However, it would not be reasonable to rely too heavily on these data because of the different genetic backgrounds of the resistant and susceptible lines used in that experiment. We backcrossed the resistance allele into the susceptible line so that we now have resistant (ISOC) and susceptible lines that share a common genetic background, to the extent that they are >93% genetically similar. We are, therefore, now in a much better position to assess the fitness cost associated with the lower level of resistance to Cry1Ac in *H. armigera*.

## 2. Objectives

The project sought to obtain a more accurate assessment of fitness cost associated with resistance in *H. armigera* and of its implications for the refuge model. Fitness costs were determined by comparing the growth and survival of homozygous susceptible and resistant, as well as heterozygous, larvae on young and maturing cotton and on some alternative refuge crops. These experiments showed that fitness was significantly affected by the developmental stage of the cotton and that there was no loss of fitness in the heterozygotes feeding on older cotton. These data suggest that conditions in late season cotton will favour the development of Cry1A resistance in *H. armigera*.

The project also sought to determine whether the higher level resistance in *H. armigera* was due to a second resistance gene and, if so, to determine the resistance mechanism encoded. Genetic analysis proved unsuitable for determining if there was a second resistance gene involved because the bioassays were insufficiently sensitive. With the reduction in CRDC funding for the second year of a two year project, we lacked the resources to complete this component of the current project. However, by collaborating with Professor Otto Schmidt, University of Adelaide, we have demonstrated that there is a second mechanism that apparently confers a degree of resistance to Cry1Ac in the ISOC strain and this mechanism has not previously been identified in any species.

## 3. Methods

*Plants.* Non-Bt (DeltaPearl®) and Bt (NuPearl®) cotton varieties, supplied by DeltaPine Australia Ltd, were planted into 20cm pots containing a sterile soil mix consisting of 60% garden soil and 40% perlite with a small amount of slow-release fertilizer. Plants were maintained in a clear-roofed glasshouse and maximum and minimum temperatures were recorded daily and ranged between 20 and 34°C.

The concentration of Cry1Ac in leaves collected was measured with an ELISA kit (Envirologix). For the high expression experiment, duplicate samples from plants at the six-leaf stage of growth were taken from each of six non-Bt and 12 Bt plants selected at random. For the low expression experiment, leaf samples were randomly collected on four dates at intervals of 3-4 weeks and freeze-dried. Duplicate samples were taken from each of 10 Cry1Ac plants collected on the first 3 sampling dates; due to the scarcity of leaf material on the final sampling date, only 5 Cry1Ac plants could be sampled.

*Toxins.* Cry1Ac protein was produced from *Bacillus thuringiensis* strain HD73, which was cultured as described in Akhurst *et al.* (2003, J. Econ. Entomol. 96: 1290-1299).

*Isogenic lines.* The resistance allele from BX was introgressed into the ANGR strain to produce near isogenic lines. ANGR males were mated with BX females and ANGR females with BX males. The $F_1$ progeny were then combined to create the $ISOC_1$ strain. The $ISOC_1$ strain was selected by rearing larvae on diet containing 1.4μg $ml^{-1}$ Cry1Ac from the $F_2$ and subsequent generations. When the selection regime restored the resistance ratio to 97-fold, the $ISOC_1$ strain was again crossed to ANGR. The $F_2$ of the resulting strain, $ISOC_2$, was selected at 4.2μg $ml^{-1}$ for a further four generations. A further two crosses to ANGR were carried out resulting in strains designated $ISOC_3$ and $ISOC_4$.

*Bioassays on diet.* The level of resistance in the ISOC lines was monitored by bioassay each generation using surface application of toxin onto artificial diet (Akhurst *et al.* 2003). A stock suspension of Cry1Ac spore/crystal mix was diluted with distilled water to produce six two-fold dilutions; distilled water was used as the control. Aliquots (50μl) of various dosages were pipetted onto artificial diet in 24-well plates (Falcon) and spread evenly across the surface. The trays were allowed to air dry before one neonate larva was placed in each well and the trays were sealed using a heat-sealing mylar membrane perforated to provide aeration. Each bioassay was done in triplicate and mortality was assessed at 7 days. Bioassays were performed on five generations of ANGR and the average $LC_{50}$ was used as the estimate of baseline susceptibility. Resistance ratios were expressed as the ratio of the $LC_{50}$ of the resistant strain to that of the susceptible strain.

*Life history traits.* The relative performance of susceptible, resistant and $F_1$ heterozygote strains was tested on Bt and non-Bt cotton plants twice for each of the high expression (four weeks after germination, 5-6 leaf stage) and low expression experiments (16 weeks after germination). Comparisons were made between ANGR, $ISOC_4$ and the $F_1$ progeny of the two reciprocal crosses between the two strains.

Neonate larvae were allowed to feed on artificial diet for 24 h before being transferred onto the test plants to minimize mortality resulting from transfer of neonates onto plants. Larvae were constrained on plants using fabric mesh cages (one larva per plant). Because lower survival rates of the resistant strain were expected on Bt cotton, at least twice as many of this genotype/plant combination were set up. Larval development was measured using a development index where a value was assigned to each larval stage (e.g. first instar = 1, early second instar = 2, mid second instar = 3, early third instar = 4.... pupa = 18). Growth and mortality were recorded every 2-3 days until pupation.

Pre-pupae were removed from the mesh cages after they had dropped from the plants. They were then placed on artificial diet and allowed to pupate. Pupae were sexed and weighed. Adult eclosion was recorded daily. The frequency of successful eclosions within each sample was calculated as the number of moths that emerged as a proportion of the number of pupae. The rate of survival from neonate to reproductive adult was calculated as the number of fertile adults as a proportion of the total number of larvae tested.

On emergence each adult was paired with a single moth of opposite gender from diet-reared ANGR. The paired moths were provided with a 4% honey/sucrose solution in a 500 ml plastic container, lined with paper towel. A layer of muslin was placed between the container and the lid, covering the aeration hole. Both the muslin and the paper served as oviposition substrates; they were replaced every 2 days. Cage papers were incubated at 25°C overnight. Eggs that did not develop to the distinctive 'brown ring' stage within 24 hours were deemed to be infertile. Cage papers containing at least some eggs that showed signs of development were placed at 4°C to prevent hatching. The total numbers of fertile and infertile eggs laid by each female during her lifetime were counted. Realized fecundity was calculated as the mean number of fertile eggs laid per gravid female. Adults were maintained in cages until death, at which time the females were stored in 70% ethanol for later dissection to determine mating frequency.

The net replacement rate ($R_o = (n \times I_e \times I_a)/2$, where n is the mean number of eggs per female, $I_e$ is the proportion of fertile eggs, $I_a$ is the proportion of reproductive adults, and 2 is the sex ratio coefficient), which represents the mean number of female offspring produced by each female during its entire lifetime, was calculated for each genotype. The net replacement rate was then used to calculate the intrinsic rate of population increase $r_m = (\ln R_o)/T$, where $T$ is the development time from egg to adult eclosion).

*Survival of post-diapausing adults.* Resistant, susceptible and $F_1$ heterozygote strains were reared to the early fifth instar on artificial diet, at which time diapause was induced by incubating the larvae at 18°C with the photoperiod shortened to 11:13 (L:D) (Murray and Wilson, 1990, *In* Zalucki, M.P. (ed). *Heliothis*: Research Methods and Prospects. Springer-Verlag, New York). Pupae were maintained under these simulated overwintering conditions for another 3-4 weeks before being removed from the incubator to confirm that they were in a state of diapause (Cullen and Browning, 1978, J. Insect Physiol. 24:595-601). Diapausal

pupae were sexed and weighed. Pupal mortality and the emergence of non-diapausal adults were recorded. Surviving pupae were then placed in cold shock for 14 d at 4°C in order to break diapause before being restored to normal adult rearing conditions. Adult emergence was recorded.

*Significance of the ISOC strain.* The ISOC was created by crossing the BX strain developed by laboratory selection in CSE72C with the susceptible ANGR strain, in a series of backcrosses. The laboratory origin of this strain raises the possibility that it was merely an artefact. To test the significance of this strain for the field, we tested a survivor of the Cry1Ac screening in the monitoring program (CSE102C). The survivor was crossed with ANGR to establish a colony (G2) and then selected at 42 μg ml for three generations. The test for allelism was effected by crossing G2 with ANGR and BX and subjecting the $F_1$ progeny of the crosses, ANGR, BX and G2 to the standard surface contamination bioassay against *H. armigera*; mortality was scored at 7 d.

*Data analysis.* $LC_{50}$ and $ET_{10}$, $ET_{50}$ and $ET_{90}$ (effective time to pupation) were estimated by probit analysis. Samples for which the 95% confidence intervals did not overlap were considered to be significantly different. The mean fitness parameters were compared by analysis of variance.

## 4. Results.

The Cry1Ac concentration for the high expression experiments was 4.1 ppm and 3.2 ppm in the first and second experiments, respectively. For the low expression experiments, the Cry1Ac concentration was 5.6 ppm at 4 weeks and dropped to 1.5 ppm by 12 weeks (Fig. 1). The experiment was conducted at 16 weeks.

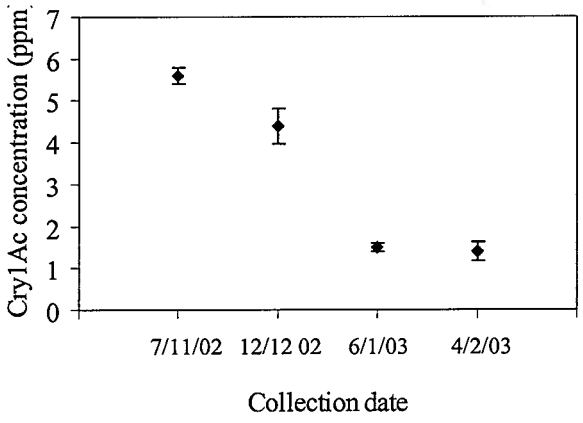


Figure 1. Changes in concentration (ppm) of Cry1Ac in transgenic cotton plants

After four serial backcrosses to the ANGR strain, the resistant ISOC strain was estimated to have nearly 95% genetic identity with the susceptible strain. The removal of background genetic variation by the use of near-isogenic resistant and susceptible enables a more precise evaluation of the fitness cost associated with resistance. This project sets a new standard for evaluation of fitness costs associated with resistance to Bt toxins.

*Survival and development.* There were significant differences in survival among genotypes and between young and old plants. Survival on young non-Bt cotton was highest for the susceptible strain (98-100%) with 77-95% survival of the resistant (ISOC4) strain and $F_1$

heterozygotes (Fig. 1, Table 1). Only the resistant strain was able to complete development on Cry1Ac-cotton (30-32% survival). However on 16 week Bt cotton, while the susceptible strain was unable to complete larval development, both the resistant strain and heterozygotes did so (Fig. 2, Table 2). Survival to pupation on non-Bt cotton was lower for the $ISOC_4$ strain (74-86%) compared with the ANGR and heterozygote genotypes (93-96%) on the 16 week cotton (Table 2).

Larval development rate also varied with genotype and with age of the cotton plants. On both young and 16 week conventional cotton resistant larvae developed more slowly than either the susceptible or the $F_1$ heterozygotes (Table 3 and 4). Development of resistant larvae was further delayed on young transgenic cotton, though not on 16 week cotton. The ANGR strain did not develop past the early 2nd instar (LDI = 2) on young Bt cotton and mid-late 5th instar (LDI = 12-23) on 16 week cotton, whereas the heterozygotes reached LDI values of 9 and 12 on young Bt cotton and completed development on 16 week Bt cotton. When development was tested on artificial diet, the $ET_{50}$ for $ISOC_4$ was not significantly different from that for ANGR (Table 3 and 4).

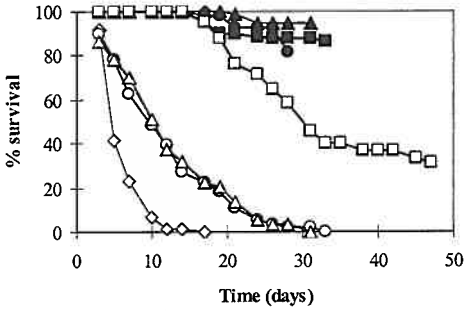


Fig. 1. Larval survival of ANGR (♦), $ISOC_4$ (■), $ISOC_4$ male x ANGR (●) and $ISOC_4$ female x ANGR (▲) on young non-Bt cotton (closed symbols) and Cry1Ac-cotton (open symbols). Data for one experiment.

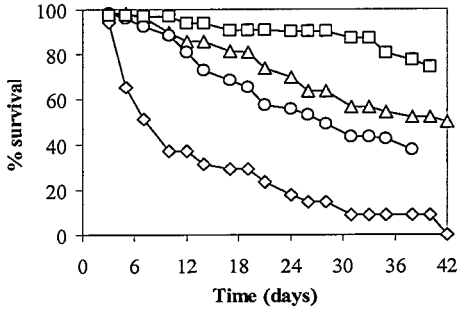


Figure 2. Larval survival of ANGR (◊), $ISOC_4$ (□), $ISOC_4$ male x ANGR (○) and $ISOC_4$ female x ANGR (△) on 16 week cotton. Data for one experiment.

Relative increases in the levels of larval, pupal and early adult mortality in the resistant strain contributed to a lower proportion of $ISOC_4$ surviving to reproductive maturity on young non-Bt cotton (35-64%) compared to the ANGR strain (93-95; Table 1). There was a difference between the relative survival to reproductive maturity of heterozygotes and of the susceptible strain in the two experiments on young cotton. Survival of heterozygotes to reproductive maturity was significantly lower in the second experiment despite the plants in this experiment having lower Cry1Ac content (3.2 ppm) than those of the first (4.1 ppm).

*Pupal weight and eclosion.* There were no consistent differences in pupal weights among genotypes on young conventional cotton (Table 1). However, pupal weights of resistant females that had developed on Cry1Ac cotton tended to be lower than those of the susceptible strain or heterozygotes on non-Bt cotton. On 16 week conventional cotton, there were no

Table 1. Mean fitness parameters for ANGR, $ISOC_4$, $ISOC_4$ x male and $ISOC_4$ x female strains of *H. armigera* on young cotton. Superscript letters after means within life history traits indicate significant ($P<0.05$) differences between means.

| Life history trait | Experiment 1 | | | | | Experiment 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-Bt cotton | | | | Cry1Ac-cotton | Non-Bt cotton | | | | Cry1Ac-cotton |
| | ANGR | $F_1$ heterozygotes | | $ISOC_4$ | $ISOC_4$ | ANGR | $F_1$ heterozygotes | | $ISOC_4$ | $ISOC_4$ |
| | | $ISOC_4$ male x ANGR | $ISOC_4$ female x ANGR | | | | $ISOC_4$ male x ANGR | $ISOC_4$ female x ANGR | | |
| % survival to pupation | $100^a$ | $82^b$ | $95^b$ | $86^b$ | $32^c$ | $98^a$ | $85^b$ | $77^b$ | $77^b$ | $30^c$ |
| Mean female pupal weight (mg) | $302 \pm 8^a$ (n=24) | $307 \pm 10^a$ (n=22) | $320 \pm 10^a$ (n=21) | $264 \pm 10^b$ (n=22) | $267 \pm 25^b$ (n=9) | $286 \pm 12^{ab}$ (n=27) | $285 \pm 16^{ab}$ (n=20) | $305 \pm 11^a$ (n=15) | $290 \pm 7.4^{ab}$ (n=16) | $245 \pm 13^b$ (n=15) |
| Mean male pupal weight (mg) | $303 \pm 7^a$ (n=33) | $318 \pm 13^a$ (n=20) | $321 \pm 10^a$ (n=32) | $287 \pm 8^a$ (n=28) | $276 \pm 21^a$ (n=8) | $294 \pm 9^{ab}$ (n=32) | $292 \pm 10^{ab}$ (n=25) | $309 \pm 12^a$ (n=22) | $296 \pm 7^{ab}$ (n=20) | $260 \pm 10^b$ (n=18) |
| Time to emergence (d) | $31.7 \pm 0.4^b$ | $29.8 \pm 0.4^a$ | $30.7 \pm 0.4^{ab}$ | $38.1 \pm 0.4^c$ | NR | $31.4 \pm 0.3^a$ | $33.4 \pm 0.5^b$ | $32.4 \pm 0.6^{ab}$ | $39.4 \pm 0.9^c$ | $49.6 \pm 1.0^d$ |
| % eclosion | $98^a$ | $89^b$ | $96^{ab}$ | $74^c$ | NR | $95^a$ | $89^{ab}$ | $80^b$ | $59^{cd}$ | $52^d$ |
| % survival to reproductive adult | $95^a$ | $84^b$ | $86^{ab}$ | $64^c$ | NR | $93^a$ | $57^b$ | $54^b$ | $35^c$ | $15^d$ |
| % female fertility | $68^a$ | $78^a$ | $80^a$ | $91^a$ | NR | $84^a$ | $69^{ab}$ | $73^{ab}$ | $83^a$ | $33^b$ |
| % male fertility | $69^a$ | $70^a$ | $74^a$ | $62^a$ | NR | $80^a$ | $71^a$ | $67^a$ | $44^{ab}$ | $13^b$ |
| Realized fecundity | $1141 \pm 38^a$ (n=15) | $1043 \pm 61^a$ (n=14) | $1270 \pm 94^a$ (n=16) | $1067 \pm 53^a$ (n=19) | NR | $915 \pm 61^a$ (n=21) | $914 \pm 107^a$ (n=9) | $1004 \pm 155^a$ (n=8) | $1017 \pm 128^a$ (n=10) | $490 \pm 153^a$ (n=3) |

NR - not recorded

Table 2. Mean fitness parameters for ANGR, ISOC4, $ISOC_4$ x strains of *H. armigera* on 16 week Bt and non-Bt cotton. Subscript letters after means within life history traits indicate significant ($P<0.05$) differences.

| Life history trait | Experiment 1 | | | | | Experiment 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-Bt cotton | | | Cry1Ac cotton | | Non-Bt cotton | | | Cry1Ac cotton | |
| | ANGR | ANGR x $ISOC_4$ | $ISOC_4$ | ANGR x $ISOC_4$ | $ISOC_4$ | ANGR | ANGR x $ISOC_4$ | $ISOC_4$ | ANGR x $ISOC_4$ | $ISOC_4$ |
| % survival to pupation | $96^a$ | $95^a$ | $74^b$ | $64^b$ | $69^b$ | $93^a$ | $93^a$ | $86^{ab}$ | $42^c$ | $74^b$ |
| Mean time to pupation (d) | $17.7 \pm 0.2^a$ | $16.8 \pm 0.2^a$ | $24.4 \pm 0.6^c$ | $24.1 \pm 0.6^c$ | $22.1 \pm 0.6^b$ | $19.7 \pm 0.8^a$ | $19.5 \pm 0.6^a$ | $25.2 \pm 0.1^b$ | $27.3 \pm 1.3^b$ | $25.3 \pm 1.0^b$ |
| Mean female pupal weight (mg) | $343 \pm 11^a$ (n=20) | $317 \pm 7^a$ (n=52) | $322 \pm 12^a$ (23) | $287 \pm 9^b$ (n=22) | $324 \pm 15^a$ (n=11) | $278 \pm 8^a$ (n=12) | $314 \pm 10^a$ (n=31) | $291 \pm 16^a$ (n=15) | $234 \pm 16^b$ (n=16) | $290 \pm 14^a$ (n=12) |
| Mean male pupal weight (mg) | $334 \pm 7^a$ (n=21) | $320 \pm 5^a$ (n=52) | $321 \pm 15^a$ (n=13) | $282 \pm 8^b$ (n=26) | $321 \pm 16^a$ (n=9) | $299 \pm 7^a$ (n=15) | $314 \pm 11^a$ (n=20) | $240 \pm 23^b$ (n=10) | $239 \pm 15^b$ (n=17) | $269 \pm 17^{ab}$ (n=9) |
| % eclosion | $93^a$ | $98^a$ | $95^a$ | $78^b$ | $90^a$ | $96^a$ | $98^a$ | $88^{ab}$ | $70^b$ | $90^{ab}$ |
| % survival to adult | $89^a$ | $93^a$ | $70^b$ | $50^c$ | $62^{bc}$ | $90^a$ | $91^a$ | $76^{ab}$ | $29^c$ | $61^b$ |
| % female fertility | $95^a$ | $88^a$ | $90^a$ | $56^b$ | $77^{ab}$ | $73^a$ | $65^a$ | $71^a$ | $58^a$ | $67^a$ |
| % male fertility | $80^{ab}$ | $90^a$ | $89^{ab}$ | $63^b$ | $57^b$ | $67^a$ | $71^a$ | $50^a$ | $50^a$ | $43^a$ |
| Realized fecundity | $1297 \pm 74^a$ (n=19) | $1387 \pm 49^a$ (n=42) | $1272 \pm 81^a$ (n=15) | $794 \pm 81^c$ (n=12) | $959 \pm 41^b$ (n=10) | $1192 \pm 91^a$ (n=7) | $1287 \pm 74^a$ (n=20) | $1045 \pm 96^{ab}$ (n=10) | $542 \pm 128^c$ (n=7) | $861 \pm 131^b$ (n=8) |

Table 3. Effective time to pupation for ANGR, $ISOC_4$, $ISOC_4$ male x ANGR and $ISOC_4$ female x ANGR on Bt, non-Bt cotton and artificial diet with 95% confidence intervals in brackets. Different superscript letters after means within diet groups indicate significant ($P<0.05$) differences between the means.

| Strain | Food source | Effective time to pupation (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Experiment 1 | | | Experiment 2 | | |
| | | 10% | 50% | 90% | 10% | 50% | 90% |
| ANGR | non-Bt cotton | 17.3 (16.7-17.7)$^{b}$ | 18.9 (18.6-19.3)$^{a}$ | 20.7 (20.3-21.4)$^{a}$ | 16.0 (14.0-17.0)$^{a}$ | 18.1 (17.1-19.0)$^{a}$ | 20.6 (19.6-22.8)$^{a}$ |
| $ISOC_4$ male x ANGR | non-Bt cotton | 17.2 (13.8-18.8)$^{ab}$ | 20.9 (19.1-22.4)$^{a}$ | 25.3 (23.4-30.2)$^{b}$ | 16.0 (13.7-17.3)$^{a}$ | 19.4 (18.2-20.6)$^{a}$ | 23.6 (22.0-27.0)$^{a}$ |
| $ISOC_4$ female x ANGR | non-Bt cotton | 14.5 (13.6-15.2)$^{a}$ | 18.8 (18.2-19.4)$^{a}$ | 24.6 (23.6-25.7)$^{b}$ | 15.214.2-15.9)$^{a}$ | 19.0 (18.4-19.6)$^{a}$ | 23.8 (22.8-25.2)$^{a}$ |
| $ISOC_4$ | non-Bt cotton | 21.6 (19.6-22.8)$^{c}$ | 24.4 (23.3-25.4)$^{b}$ | 27.6 (26.3-29.9)$^{c}$ | 20.3 (19.4-21.1)$^{b}$ | 24.9 (24.3-25.6)$^{b}$ | 30.5 (295-31.8)$^{b}$ |
| $ISOC_4$ | Cry1Ac-cotton | 33.4 (31.3-34.6)$^{d}$ | 38.3 (37.3-39.4)$^{c}$ | 44.0 (42.5-46.4)$^{d}$ | 33.7 (32.0-35.0)$^{c}$ | 42.7 (41.5-44.1)$^{c}$ | 54.1 (51.5-57.7)$^{c}$ |
| ANGR | artificial diet | 12.7 (11.8-13.6)$^{a}$ | 14.6 (13.7-15.5)$^{ab}$ | 16.6 (15.7-18.0)$^{b}$ | 11.0 (10.6-11.2)$^{a}$ | 12.1 (11.9-12.3)$^{a}$ | 13.4 (13.1-13.9)$^{a}$ |
| $ISOC_4$ male x ANGR | artificial diet | 13.0 (12.4-13.6)$^{a}$ | 15.1 (14.7-15.6)$^{b}$ | 17.5 (16.9-18.3)$^{b}$ | 12.1 (11.9-12.4)$^{b}$ | 13.2 (13.0-13.4)$^{b}$ | 14.3 (14.0-14.7)$^{b}$ |
| $ISOC_4$ female x ANGR | artificial diet | 12.9 (10.4-14.1)$^{a}$ | 15.0 (13.6-16.3)$^{ab}$ | 17.4 (16.1-20.8)$^{b}$ | 12.2 (11.1-13.0)$^{ab}$ | 13.6 (12.7-14.6)$^{b}$ | 15.2 (14.3-16.6)$^{b}$ |
| $ISOC_4$ | artificial diet | 12.6 (12.0-12.9)$^{a}$ | 13.6 (13.3-13.9)$^{a}$ | 14.7 (14.3-15.4)$^{a}$ | 11.0 (9.4-11.7)$^{a}$ | 12.5 (11.7-13.1)$^{ab}$ | 14.1 (13.3-16.2)$^{ab}$ |

Table 4. Effective time to eclosion for ANGR, $ISOC_4$ and $ISOC_4$ x ANGR on 16 week Bt and non-Bt cotton with 95% confidence intervals in brackets. Subscript letters after means within diet groups indicate significant ($P<0.05$) differences.

| Strain | Food source | Effective time to pupation (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Experiment 1 | | | Experiment 2 | | |
| | | 10% | 50% | 90% | 10% | 50% | 90% |
| ANGR | non-Bt cotton | 24.1 (22.7 , 25.1)$^{a}$ | 27.4 (26.7 , 28.0)$^{a}$ | 31.2 (30.2 , 32.7)$^{a}$ | 27.6 (26.7, 28.3)$^{a}$ | 30.6 (30.1, 31.0)$^{a}$ | 33.9 (33.2, 34.8)$^{a}$ |
| $ISOC_4$ x ANGR | non-Bt cotton | 25.4 (25.1 , 25.7)$^{a}$ | 28.2 (28.0 , 28.4)$^{a}$ | 31.4 (31.0 , 31.7)$^{a}$ | 26.7 (25.3, 27.8)$^{a}$ | 31.0 (30.2, 31.8)$^{a}$ | 36.1 (35.1, 37.6)$^{b}$ |
| $ISOC_4$ | non-Bt cotton | 32.1 (31.3 , 32.7)$^{c}$ | 35.5 (35.1 , 35.9)$^{b}$ | 39.3 (38.6 , 40.1)$^{c}$ | 29.4 (26.9, 31.0)$^{ab}$ | 34.6 (33.4, 35.6)$^{b}$ | 41.7 (39.3, 43.3)$^{c}$ |
| $ISOC_4$ x ANGR | Bt cotton | 30.9 (30.0 , 31.6)$^{bc}$ | 35.5 (35.0 , 36.0)$^{b}$ | 40.8 (39.9 , 41.9)$^{c}$ | 28.7 (27.0, 30.1)$^{ab}$ | 35.7 (34.7, 36.7)$^{b}$ | 44.5 (43.0, 46.4)$^{c}$ |
| $ISOC_4$ | Bt cotton | 30.3 (29.4 , 31.0)$^{b}$ | 34.6 (34.1 , 35.1)$^{b}$ | 36.0 (35.3 , 37.0)$^{b}$ | 31.3 (29.7, 32.5)$^{b}$ | 35.8 (34.9, 36.6)$^{b}$ | 40.9 (39.9, 42.4)$^{c}$ |
| ANGR | artificial diet | 23.4 (22.9 , 23.7)$^{a}$ | 24.7 (24.4 , 24.9)$^{ab}$ | 26.0 (25.0 , 26.2)$^{a}$ | 26.6 (25.8, 27.1)$^{ab}$ | 28.6 (28.1, 29.0)$^{ab}$ | 30.7 (30.2, 31.6)$^{b}$ |
| $ISOC_4$ x ANGR | artificial diet | 23.0 (22.6 , 23.2)$^{a}$ | 24.3 (24.1 , 24.6)$^{a}$ | 25.8 (25.5 , 26.1)$^{a}$ | 26.3 (25.7, 26.8)$^{a}$ | 27.8 (27.5, 28.2)$^{a}$ | 29.4 (29.0 , 30.0)$^{a}$ |
| $ISOC_4$ | artificial diet | 23.8 (23.1 , 24.2)$^{b}$ | 25.1 (24.8 , 25.5)$^{b}$ | 26.6 (26.1 , 27.2)$^{a}$ | 27.3 (27.1, 27.6)$^{b}$ | 28.7 (28.6, 28.8)$^{b}$ | 30.0 (29.8, 30.3)$^{ab}$ |

consistent differences among genotypes in pupal weight and the pupal weights for the resistant strain did not differ between conventional and Cry1Ac-cotton (Table 2). However, heterozygotes produced significantly ($P<0.05$) smaller pupae on Cry1Ac cotton than on non-Bt cotton.

The frequency of successful eclosions was significantly ($P<0.05$) higher in the ANGR strain compared with the $ISOC_4$ strain reared on young non-Bt cotton, and eclosion of the heterozygotes was perhaps marginally less than that of the ANGR strain (Table 1). In the 16 week cotton experiments, there was no significant difference ($P<0.05$) between the genotypes for pupal mortality on non-Bt cotton but eclosion of the heterozygotes on Cry1Ac cotton was significantly lower (Table 2).

*Fertility and fecundity.* There were no significant between-genotype differences in male or female fertility in *H. armigera* from non-Bt cotton, though in one experiment male fertility of the resistant strain was only 44% while that of the ANGR strain was 80% (Table 1). When reared on non-Bt cotton resistant females yielded comparable numbers of fertile eggs as the other genotypes in both experiments. However on young Cry1Ac-cotton only 15% of resistant insects survived to reproductive maturity and their fertility was low in comparison to those from non-Bt cotton (Table 2).

On young cotton, egg viability (number of fertile eggs / total number of eggs) was >90% for all genotypes and there were no significant ($P<0.05$) between-genotype differences in realised fecundity. The realised fecundity for $ISOC_4$ on young Cry1Ac cotton was much lower than for the same strain on non-Bt cotton, though not significantly, perhaps because of the very small number of individuals contributing. In the 16 week experiments, there were no significant ($P<0.05$) between-genotype differences in realised fecundity on non-Bt cotton but the fecundity of $ICOC_4$was reduced on Cry1Ac cotton and that of the heterozygotes was reduced even further (Table 2).

There was no correlation between the mating frequency of females (as determined by the numbers of spermatophores found by dissection) and fecundity.

*Intrinsic rate of increase.* On both young and 16 week non-Bt cotton, the intrinsic rate of increase ($r_m$) was comparable for the ANGR strain and the heterozygotes, and was appreciably reduced for the $ISOC_4$ strain (Table 5 and 6). While the $r_m$ for $ISOC_4$ on young Bt cotton was approximately half that of the same strain in non-Bt cotton, there was no appreciable difference between the $r_m$ for either $ISOC_4$ or the heterozygotes on 16 week non-Bt and Bt cotton (Table 6).

*Survival of post-diapausing adults.* The proportion of larvae that pupated under diapausing conditions was similar for all genotypes (Table 7). The pupal weights of ANGR were significantly greater ($P<0.05$) than those of $ISOC_4$ or the heterozygotes. The proportion of adults that emerged from pupae that had undergone diapause varied from 71 - 90% but was not consistently different across genotypes. However, there was greater overall survival of ANGR from the fifth instar to post-diapausal adult compared with either the heterozygote strains or the $ISOC_4$ strain.

Table 5. Intrinsic rate of population increase ($r_m$) of ANGR, $F_1$ (ISOC4 x ANGR) and $ISOC_4$ strains of *H. armigera* on young cotton

| Strain | Cotton Variety | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| | | Net replacement rate ($R_o$) | Intrinsic rate of population increase ($r_m$) | Net replacement rate ($R_o$) | Intrinsic rate of population increase ($r_m$) |
| ANGR | non-Bt | 541 | 0.199 | 427 | 0.193 |
| ISOC4 female x ANGR | non-Bt | 440 | 0.204 | 259 | 0.166 |
| ISOC4 male x ANGR | non-Bt | 546 | 0.206 | 267 | 0.172 |
| ISOC4 | non-Bt | 340 | 0.153 | 178 | 0.132 |
| ISOC4 | Bt | NR | NR | 37 | 0.073 |

NR – not recorded

Table 6. Intrinsic rate of population increase ($r_m$) of ANGR, $F_1$ ($ISOC_4$ x ANGR) and $ISOC_4$ strains of *H. armigera* on 16 week cotton.

| Strain | Cotton Variety | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| | | Net replacement rate ($R_o$) | Intrinsic rate of population increase ($r_m$) | Net replacement rate ($R_o$) | Intrinsic rate of population increase ($r_m$) |
| ANGR | non-Bt | 580 | 0.232 | 536 | 0.206 |
| $ISOC_4$ x ANGR | non-Bt | 646 | 0.229 | 587 | 0.206 |
| $ISOC_4$ | non-Bt | 447 | 0.172 | 399 | 0.166 |
| $ISOC_4$ x ANGR | Bt | 199 | 0.149 | 76 | 0.121 |
| $ISOC_4$ | Bt | 296 | 0.165 | 262 | 0.156 |

*Significance of the ISOC strain.*

Although the G2 x ANGR hybrids exhibited no significant resistance, the G2 x BX hybrids had resistance ratios comparable to that of BX (Table 8).. This test confirms that the allele that confers resistance to BX (and ISOC) occurs in the field.

Table 7. Survival of post-diapausing insects from ANGR, $ISOC_4$ and $F_1$ strains of *H. armigera*. Subscript letters after means within cohorts indicate significant ($P<0.05$) differences.

| Life history trait | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| | ANGR | $F_1$ | | $ISOC_4$ | ANGR | $F_1$ | | $ISOC_4$ |
| | | $ISOC_4$ x male ANGR | $ISOC_4$ x female ANGR | | | $ISOC_4$ x male ANGR | $ISOC_4$ x female ANGR | |
| % pupation | 88.4 (n=906) | 75.6 (n=319) | 82.9 (n=596) | 88.1 (n=646) | 87.5 (n=431) | 81 (n=673) | 75.8 (n=612) | 77.4 (n=301) |
| % pupae diapaused | 97.9 (801) | 80.6 (241) | 80.4 (494) | 84.5 (569) | 91.1 (368) | 98.7 (445) | 99.7 (359) | 100 (251) |
| % survival from 5th instar to emergence | 63.9 (n=785) | 37.7 (n=212) | 46.1 (n=425) | 38.0 (n=521) | 69.8 (n=431) | 46.8 (n=673) | 43.6 (n=612) | 46.3 (n=389) |
| Pupal weight (mg) | $405 \pm 2^a$ (n=514) | $360 \pm 5^b$ (n=112) | $368 \pm 3^b$ (n=259) | $360 \pm 2^b$ (n=250) | $406 \pm 3^a$ (n=331) | $389 \pm 2^b$ (n=439) | $382 \pm 3^b$ (n=358) | $352 \pm 3^b$ (n=251) |
| % emergence of post-diapausing pupae | 78.7 (n=639) | 73.4 (n=111) | 76.3 (n=258) | 79.3 (n=248) | 90.9 (n=331) | 71.6 (n=431) | 74.6 (n=358) | 72.0 (n=110) |

Table 8. Test for alleleism in the G2 and BX strain demonstrates that the resistance allele in the laboratory-selected BX strain occurs naturally in the field.

| Strain | LC50 ($ng/cm^2$) | 95% CI. | Slope | RR |
|---|---|---|---|---|
| ANGR | 75.3 | 49 - 105 | 2.1 | 1 |
| BX | 79353 | 566529 - 100706 | 2.1 | 1054 |
| G2 | 24990 | 16300 - 33832 | 1.9 | 332 |
| | | | | |
| G2♀/ANGR♂ | 229 | 154 - 358 | 1.8 | 3 |
| G2♂/ANGR♀ | 375 | 276 - 545 | 2.3 | 5 |
| Combined | 289 | 209 - 424 | 2.0 | 4 |
| | | | | |
| BX♀/G2♂ | 66438 | 48239 - 93823 | 1.4 | 882 |
| BX♂/G2♀ | 54980 | 30463 - 95369 | 1.3 | 730 |
| Combined | 57510 | 41305 - 82226 | 1.3 | 764 |

## 5. Conclusion

This study investigated the associations between the fitness of *H. armigera,* developing on Bt cotton and an allele that confers resistance to Cry1Ac. Fitness comparisons were made between near-isogenic strains of resistant and susceptible *H. armigera.* The resistant strain, $ISOC_4$, was 93% or more genetically identical to the susceptible strain, leading to a high probability that the fitness cost shown in $ISOC_4$ is directly associated with the allele that confers resistance to Cry1Ac rather than the result of effects from other loci.

The dominance or recessiveness of the resistance allele in the crop environment is a key factor in determining the rate at which resistance is likely to spread through a population. The resistance allele selected for in the $ISOC_4$ strain is functionally recessive on young transgenic cotton which expresses Cry1Ac at levels high enough to kill all homozygous susceptible genotypes as well as the heterozygote genotypes. This will mitigate against the development of resistance. However, on older cotton that has lost up to 70% of its original capacity to produce Cry1Ac, the effective dominance of the resistance increased, with many heterozygotes completing development on Bt cotton when all of homozygous susceptible insects were killed.

The refuge strategy is based on an assumption that sufficient mating will occur between resistant insects from Bt crops and susceptible insects from refuges to restrict matings between heterozygotes (Gould 1994, Tabashnik 1994). Asynchronous development of susceptible and resistant phenotypes decreases the likelihood that mating events will occur between these two sources of insects (Liu *et al.* 1999, Peck *et al.* 1999). This study has shown that in young cotton there is some potential for asynchrony between resistant individuals from the crop and susceptibles from the refuge when the cotton is young. However, that ceases to be a significant issue when the transgenic crop ages and toxin activity reduces to the point where heterozygotes can complete their development. In consequence, the issue of asynchrony is minimised at the time of greatest risk (i.e. the latter part of the season when heterozygotes could complete development in Cry1Ac cotton).

Fitness costs associated with resistance can reduce the risk of the development of resistance (Carrière et al., 2002, *In*: R.J. Akhurst, C.E. Beard and P.A. Hughes (eds), Proceedings of the 4th Pacific Rim Conference on the Biotechnology of *Bacillus thuringiensis* and Its

Environmental Impact. CSIRO Entomology, Canberra, Australia). In this project we have shown that the fitness cost associated with Bt resistance is largely recessive on young Cry1Ac cotton and completely recessive on older cotton. Apart from a reduction in survival to reproductive adulthood on young cotton, there were no significant differences in life history traits of the ANGR and heterozygote strains on non-Bt cotton. This resulted in similar intrinsic rates of population increase ($r_m$), demonstrating that the susceptible genotype has little or no selective advantage over the heterozygote genotype. The recessive fitness costs would induce only a weak selection differential for a decline in resistance in the refuge habitat.

This study has shown that there are important factors associated with fitness cost that will tend to promote the likelihood of resistance to Cry1Ac cotton (i.e. partial dominance on older cotton, recessive fitness cost). The introduction of BOLLGARD II cotton will be a major factor to minimise the risk. However, the frequency with which Cry2A resistance has been detected in field populations of *H. armigera*, presents a problem. Should Cry2A resistance become entrenched through selection in the latter part of the season when the activity of Cry1Ac is low, then BOLLGARD II would become effectively a single gene plant, and the risk of resistance to Cry1Ac as well as Cry2A would become significant.

The test for allelism confirmed that the allele that confers high level resistance to Cry1Ac in the laboratory selected BX (and ISOC) population occurs naturally in field insects and that the information generated form this and previous studies of Cry1A resistance in *H. armigera* are relevant to the real world.

## 6. How research has addressed the Corporation's three Outputs - Economic, Environmental and Social

This project has produced data on a key factor influencing the rate at which resistance to a Bt toxin can develop in *H. armigera.* It provides a strong argument for the need to maintain an effective resistance management strategy if the economic and environmental benefits of Bt cotton are to be sustained.

## 7. Summary

The use of near-isogenic lines set a new benchmark for evaluation of fitness costs associated with resistance in insects. In previous studies, resistant and susceptible populations that have been maintained in isolation over long periods with small population sizes have been employed. In such populations, even if the susceptible population was parental, there is scope for significant genetic diversity between the strains through founder effect and genetic drift. Consequently, it is not possible to definitively associate fitness cost with resistance in such comparisons. By removing most of the potential for genetic variability, we have been able to make a more meaningful evaluation of the fitness costs associated with resistance.

## 8. Future activities

The data generated here will be modelled to provide a better evaluation of the likely impact of the fitness costs associated with Cry1A resistance. The near isogenic lines have been further developed to the 7$^{th}$ backcross (>99% identical). It is proposed to use this line in a proteomic analysis to identify diagnostic proteins that can be used to develop a rapid diagnostic test for screening field populations.

## 9. Publications arising from the research

- Akhurst, R. (2002). Measuring heliothis resistance to Ingard cotton. The Australian Cottongrower 23 (3), 58-60.
- Akhurst, R., James, W. and Bird, L. (2002). Resistance to the Cry toxins of *Bacillus thuringiensis* in cotton bollworm *Helicoverpa armigera*. In: *Biotechnology of Bacillus thuringiensis and Its Environmental Impact*, Canberra. Akhurst, R.J., Beard. C.E., and Hughes, P.A. (eds). CSIRO, Canberra. pp.72-75.
- Akhurst, R.J., James, W., Bird, L.J. and Beard, C. (2003). Resistance to the Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 96: 1290-1299.
- Bird, L.J. and Akhurst, R.J. (2004) The relative fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* (Lepidoptera: Noctuidae) on conventional and transgenic cotton. *J. Econ. Entomol.* (in press).
- Bird, L.J. and Akhurst, R.J. (in prep.) The fitness of Cry1A-resistant and -susceptible *Helicoverpa armigera* on transgenic cotton expressing sub-optimal levels of Cry1Ac.
- Bird, L. James, W. and Akhurst, R. (2002). High level resistance to INGARD® cotton by the cotton bollworm *Helicoverpa armigera*. Proc. 11th Australian Cotton Conference, Brisbane (CD-ROM).
- Bird, L.J. and Akhurst, R.J. (2004). The relative fitness of Bt resistant and susceptible *Helicoverpa armigera* - implications for the refuge strategy. XXII Int. Congr. Entomol., Brisbane.

## 10. Online resources

None.

## 11. Assessment of the likely impact of the results and conclusions of the research project for the cotton industry.

The data generated in this project will be integrated in the planning of the resistance management strategy that is a key element of maintaining the sustainability of transgenic cotton, and by extension, the Australian cotton industry.

## *Final Report Executive Summary*

Fitness cost associated with resistance is an important factor in determining the design of an effective refuge strategy. When the fitness of the Cry1A-resistant BX strain of *H. armigera* was assessed against a susceptible laboratory strain in a previous CRDC project, we noted a significant delay in development that might impede the efficiency of the resistance strategy. However, it would not be reasonable to rely too heavily on these data because of the different genetic backgrounds of the resistant and susceptible lines used in that experiment. We overcame the difference problem by transferring the resistance allele into the susceptible line by a classical genetic method. This created resistant (ISOC) and susceptible lines that share a common genetic background, to the extent that they are >93% genetically similar. We were, therefore, now in a much better position to assess the fitness cost associated with the lower level of resistance to Cry1Ac in *H. armigera*

Fitness costs were determined on young cotton (four weeks) in which the activity of the Cry1Ac toxin was high and on 16 week cotton, in which the activity had dropped to the point where the susceptible strain could develop to mid-late $5^{th}$ instar, though not complete development. This approach revealed that the fitness costs will differ significantly through the season.

Although the study was conducted with a laboratory selected strain, we have shown that it is relevant to the field situation. By testing a survivor from the resistance screening program, we confirmed that the gene responsible for Cry1A resistance in the lab strain also occurs naturally.

The dominance or recessiveness of the resistance allele in the crop environment is a key factor in determining the rate at which resistance is likely to spread through a population. The resistance allele selected for in the $ISOC_4$ strain is functionally recessive on young transgenic cotton.. However, on older cotton, the effective dominance of the resistance increased, increasing the probability of resistance development in the absence of an adequate resistance management strategy.

The refuge strategy is based on an assumption that sufficient mating will occur between resistant insects from Bt crops and susceptible insects from refuges to restrict matings between heterozygote. This project showed that asynchrony is minimised at the time of greatest risk and is unlikely to pose a problem.

Fitness costs associated with resistance can reduce the risk of the development of resistance. In this project we have shown that the fitness cost associated with Bt resistance is largely recessive on young Cry1Ac cotton and completely recessive on older cotton. The recessive fitness costs would induce only a weak selection differential for a decline in resistance in the refuge habitat and are therefore unlikely to reduce the risk of resistance.

This study has shown that there are important factors associated with fitness cost that will tend to increase the risk of resistance to Cry1Ac cotton. The introduction of BOLLGARD II cotton will be a major factor in minimising the risk. However, the frequency with which Cry2A resistance has been detected in field populations of *H. armigera*, presents a problem. Should Cry2A resistance become entrenched through selection when the activity of Cry1Ac is low in the latter part of the season, then BOLLGARD II would become effectively a single gene plant, and the risk of resistance to Cry1Ac as well as Cry2A would become significant.