Australian Government

Cotton Research and Development Corporation

# Annual, Progress and Final Reports

## *Part 1 - Summary Details* **REPORTS**

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

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|---|---|---|
| **CRDC Project Number:** | | CSE102C |
| **Annual Report:** | ☐ | Due 30-September |
| **Progress Report:** | ☐ | Due 31-January |
| **Final Report:** | ☒ | Due 30-September |
| | | (or within 3 months of completion of project) |

**Project Title:** Monitoring Bt resistance

**Project Commencement Date:** Jul 2002 **Project Completion Date:** Jun 2005

**Research Program:** 3 Crop Protection

## *Part 2 – Contact Details*


**Administrator:** Mr Mark Hardwick, Finance Manager

**Organisation:** CSIRO Entomology

**Postal Address:** GPO Box 1700, Canberra ACT, 2601

**Ph:** 02-62464406 **Fax:** 02-62464095 **E-mail:** Mark.Hardwick@csiro.au

**Principal Researcher:** Dr Sharon Downes, Research Scientist

**Organisation:** CSIRO Entomology

**Postal Address:** ACRI, Locked Bag 59, Narrabri, NSW 2390

**Ph:** 02-67991576 **Fax:** 02-67931186 **E-mail:** Sharon.Downes@csiro.au

**Supervisor:** Dr Rod Mahon, Stream Leader

**Organisation:** CSIRO Entomology

**Postal Address:** GPO Box 1700, Canberra ACT, 2601

**Ph:** 02-62464082 **Fax:** 02-62464000 **E-mail:** R

**Researcher 2** (Name & position of additional researcher or supervisor).

**Organisation:**

**Postal Address:**

**Ph:** **Fax:** **E-mail:**


**Signature of Research Provider Representative:** _____

## 1. Background to the project

In the 1996/97 season the Australian industry adopted an insect-resistant variety of cotton (Ingard®) that is specific to the group of insects including the target *Helicoverpa* spp. but excluding predators and parasitoids of this pest. To prolong the efficacy of transgenic cotton against *Helicoverpa* spp., a resistance management plan (RMP) was implemented. This strategy was largely based on information from studies of the ecology and population genetics of *Helicoverpa* spp., and the outputs of computer simulation models that used biological information to predict the likelihood of resistance under different scenarios.

The industry adopted a necessarily conservative RMP for Ingard® due to the critical importance of preserving the Cry1Ac gene present in this variety until more robust two-gene transgenic cotton was available. A key component was to limit planting Ingard® to a maximum of 30% of the total area. Growers were also required to plant conventional crops alongside transgenic fields—these "refuges" harbour susceptible moths that should mate with potentially resistant individuals from the Ingard®, thereby diluting resistance in the population.

In the 2004/5 season Bollgard II® replaced Ingard® as the transgenic variety of cotton available to Australian growers. It improves on Ingard® by incorporating an additional insecticide protein (Cry2Ab) to combat Helicoverpa. Due to the perceived difficulty for *Helicoverpa* spp. to simultaneously evolve resistance to both proteins within Bollgard II®, the RMP for transgenic cotton was relaxed to allow growers to plant up to 95% of the total area to this product if appropriate refuge was also grown. Bollgard II® was well adopted, with an average of 70% planted area throughout the industry. Given the increased opportunity for moths to adapt to transgenic cotton, the mandatory RMP will be rigorously tested for the first time.

The cotton industry has sought to acquire early warnings of changes in sensitivity of insect populations to toxins that may signal the presence of resistance to transgenic varieties of cotton. The sensitivity of field-collected populations of *Helicoverpa* spp. to Bt products was assayed before and subsequent to the widespread deployment of Ingard® cotton expressing Cry1Ac in the mid-1990's. During the current project, baseline levels of susceptibility to Cry2Ab were established in preparation for replacement in the 2004/05 season of Ingard® with Bollgard II®. Preserving the efficacy of Cry1Ac and Cry2Ab is critical for the future of the industry, not only for the efficacy of Bollgard II® varieties, but also for the long-term future of varieties expressing Cry1Ac or Cry2Ab in combination with other effective toxins. In addition, if field resistance to Bollgard II® occurs, it will not only be more difficult to market new transgenic products for cotton, but other industries, growers and public perceptions could be affected.

CSIRO Entomology has worked on resistance by *H. armigera* to transgenic cotton for many years, and presently maintains strains that are resistant to Cry1Ac or Cry2Ab. In both cases the forms of genes (alleles) that confer resistance have been isolated from field populations (see report for CSE104C and below). Our work shows that resistant alleles in the field are rare for Cry1Ac but surprisingly common for Cry2Ab. *H. armigera* has an impressive track-record at evolving resistance to conventional insecticides. Therefore, while the combination of two genes in Bollgard II® should dramatically slow the evolution of resistance by a target pest, it is not an impossible task for *H. armigera* to adapt to this technology.

This project is an extension of the monitoring for Bt resistance in *Helicoverpa* spp. conducted since the mid 1990's by the New South Wales Department of Agriculture (now Primary Industries). The work completed during the first two years of this three-year program was performed at the CSIRO Black Mountain Laboratories in Canberra. The final year of the program was performed at the Australian Cotton Research Institute (ACRI) in Narrabri.

**2 & 3. Methods and findings**

**Aim one: Optimise the monitoring program by enhancing the sensitivity of the bioassays**

This objective was achieved in full. *From 1994/95 until 2002/03, a Bt spray (MVPII®) that contained formulation ingredients additional to Cry1Ac was used in the screens to detect the presence of resistance to Bt. The program also incorporated a Bt spray (DiPel®) with insecticidal proteins additional to Cry1Ac to test for resistance to combinations of Cry toxins. The program used only $F_0$ screens however this method cannot detect individuals that are heterozygous for a recessive form of resistance.*

*During this project we developed screens using a pure Cry1Ac spore/crystal mix as our source of toxin. In anticipation of Bollgard II® replacing Ingard® in 2004/05, we developed methods to screen for resistance to Cry2Ab. In addition to performing $F_0$ screens to detect major changes in gene frequencies, we incorporated an $F_2$ screen to detect and 'capture' any rare resistance alleles in natural populations. This method allowed us to simultaneously screen for resistance to Cry1Ac and Cry2Ab, hence making the screens with DiPel® redundant.*

Pre-amble

The standard method for Bt resistance monitoring tests the $F_0$ generation of insects collected directly from the field. Due to the recessive nature of most previous instances of resistance to Bt in Lepidoptera, these screens are likely to detect only individuals that have two resistance alleles (RR, homozygous resistant). In the early stages of the evolution of resistance such RR individuals are likely to be extremely rare. $F_2$ screens test the "grandchildren" of single pairs of insects collected from the field and can identify individuals that have one resistance allele and one susceptible allele (SR, heterozygotes).

From 1994/95 until 2002/03, the Bt formulations MVPII® and DiPel® were used in $F_0$ screens for the presence of resistant individuals of *Helicoverpa* spp. to Cry toxins found within Ingard® cotton. MVPII® and DiPel® were a readily available and cost-effective source of test material. However, both contain formulation ingredients as well as Cry1Ac, and DiPel® contains a variety of insecticidal proteins additional to Cry1Ac (i.e., Cry1Aa, Cry1Ab, Cry2Aa). DiPel® was used to screen for a theoretical form of resistance that provided protection to combinations of Cry toxins.

The presence of additional products in the formulated MVPII® rendered it imperfect for use in assays designed to monitor resistance to Cry1Ac. We therefore reviewed this protocol and developed sensitive resistance monitoring techniques based on discriminating doses of pure Cry1Ac. The pure toxin was obtained from a bacterial strain (HD73) that was originally cultured at CSIRO Entomology. With the greater demands associated with the monitoring program, toxin production was outsourced to a large fermentation facility that produced a culture designated GHD73. The product was similar to the parent culture in its toxicity to *H. armigera*. During the second year of this project we simultaneously employed $F_0$ screens with the single Cry1Ac protein and MVPII® to 'calibrate' the method. During the third year of the project, we ceased using MVPII® and the spore/crystal preparation was used in the $F_0$ screening program.

The introduction of Bollgard II® cotton in the 2003/04 season demanded that our techniques incorporated monitoring for resistance to Cry2Ab (the protein stacked with Cry1Ac in Bollgard II®). During 2003/04, we developed $F_0$ screens with a Cry2Ab spore/crystal preparation. This toxin was produced at CSIRO Entomology from a recombinant strain of Bt obtained from the Biotechnology Research Institute, Canada. This material was difficult to produce and required facilities that were not available at ACRI. Therefore, during the third year of the program, when the project moved from the CSIRO Black Mountain Laboratories to the ACRI, ground corn-stem material (supplied by Monsanto Australia) that contains Cry2Ab toxin was used in the $F_0$ screens.

During CSE104C, Dr Rod Mahon and his team developed $F_2$ assays to detect and 'capture' any rare resistance alleles in natural populations of *Helicoverpa* species. During 2002/03 homogenised leaf extract from cotton plants producing only Cry2Ab was employed. In 2003/04, Monsanto Australia made available ground corn-stem material grown in USA that contains Cry2Ab toxin. After calibration against the cotton leaf extract to ensure a similar dose of Cry2Ab was presented to the larvae, the corn material was used in all subsequent $F_2$ assays, including those performed during year 3 of this project.

In most cases with $F_2$ screens, large numbers of individuals from the same family are available for testing against both Cry1Ac and Cry2Ab. Therefore, in the third year of the project, we did not include screens for DiPel® because our $F_2$ screens using both toxins would identify any resistance to a combination of Cry1Ac and Cry2Ab toxins.

Developing resistance monitoring assays

Unless otherwise stated, all bioassays for developing discriminating doses were set up in 24-well trays containing treated diet to which newly moulted larvae were added, one larva per well. Assays were performed on three occasions for each strain using different cohorts. Candidate doses for field screening were tested using 72 larvae for each dose. At the chosen discriminating doses, some (presumably homozygous) susceptible survivors will be expected.

*$F_0$ screens*

Using early 3rd instars in the $F_0$ screening program is favoured because it is difficult to identify (non-destructively) larval *Helicoverpa* spp. before the late 2nd instar. However, it is not logistically possible to adopt this method when the toxin is difficult to produce and/or a relatively high dose of the toxin is required to kill 3rd instars. When this was the case, we developed a bioassay for neonates, and estimated the relative proportions of each species tested based on knowledge of the host preferences of the different species (i.e., *H. armigera* favour maize and predominate on late season cotton, whereas *H. punctigera* predominate on early season cotton). Further, during the final year of the study we estimated the relative proportions of each species used in neonatal assays based on the relative proportions of each species reared for the $F_2$ screens in a sub-sample of the same collection.

(i) Cry1Ac spore/crystal preparation (used 2003/04-2004/05):

Previous screening assays (using MVPII®) used mortality criteria for assessing susceptibility of 3rd instars. However, for 3rd instar larvae exposed to single Cry toxins the slope of the dose response line for development assays is greater than those for mortality assays and a steeper slope is favoured. Therefore, calibration of the GHD73 culture against *Helicoverpa* spp. was conducted using diet incorporation to assess development of early 3rd to 4th instar larvae over seven days. By incorporating GHD73 in diet at various concentrations we determined the dose at which 98% of larvae from field strains of Helicoverpa were unable to develop from the 3rd to 4th instar over a period of 7 days.

Fourteen field-derived strains of *H. armigera* and 13 field-derived strains of *H. punctigera* were tested against GHD73 (Table 1). From these data discriminating doses of GHD73 for *H. armigera* and *H. punctigera* were set at 80µg/ml of diet and 120µg/ml of diet, respectively.

*TABLE 1. Bioassay of GHD73 (Cry1Ac) spore/crystal mix and calibration of discriminating doses on* H. armigera *and* H. punctigera *tested as 3rd instars on diet incorporated Bt and assessed for development to the 4th instar at day 7. The laboratory strain was comprised of presumably homozygous susceptible individuals. Ppea = pigeon pea.*

| Site | Host | 95% CI | | Slope | % failure to reach 4th instar at 7 d by dose (µg/ml diet) | | |
|---|---|---|---|---|---|---|---|
| | | lower | upper | | | | |
| ***Helicoverpa armigera*** | | | | | **20** | **40** | **80** |
| Breeza NSW ($F_1$) '03 | maize | 2.0 | 4.3 | 2.6 | 100 | 100 | 100 |
| Goondiwindi QLD ($F_1$) '04 | cotton | 3.1 | 6.4 | 2.6 | 100 | 100 | 100 |
| Burren Junction NSW ($F_1$) | cotton | 2.8 | 7.2 | 2.0 | 93.8 | 97.9 | 100 |
| Wee Waa NSW ($F_1$) | cotton | 5.5 | 7.6 | 3.1 | 95.7 | 98.6 | 100 |
| Goondiwindi QLD ($F_1$) '03 | cotton | 5.3 | 8.8 | 2.2 | 87.5 | 96.8 | 100 |
| Moree NSW ($F_1$) | maize | 6.3 | 8.3 | 2.7 | 87.3 | 97.2 | 100 |
| Wee Waa NSW ($F_1$) | sorghum | 6.4 | 8.9 | 2.7 | 84.7 | 98.6 | 100 |
| Hillston NSW ($F_1$) | ppea | 6.2 | 10.0 | 2.3 | 75.0 | 97.9 | 97.9 |
| Piliga NSW ($F_1$) | cotton | 5.8 | 10.9 | 2.8 | 92.6 | 100 | 100 |
| Boggabri NSW ($F_1$) | cotton | 6.3 | 11.4 | 2.0 | 64.8 | 94.4 | 100 |
| Moree NSW ($F_1$) '04 | cotton | 8.1 | 10.6 | 2.8 | 84.7 | 93.1 | 100 |
| Warren NSW ($F_1$) | cotton | 5.6 | 13.4 | 1.8 | 72.2 | 87.0 | 97.6 |
| Breeza NSW ($F_1$) '04 | maize | 7.4 | 12.8 | 3.2 | 95.8 | 100 | 98.9 |
| St George QLD ($F_2$) | cotton | 8.6 | 15.1 | 2.0 | 70.8 | 81.9 | 97.2 |
| *Laboratory strain (ANGR)* | - | 2.4 | 3.3 | 1.9 | 94.4 | 100 | - |
| ***H. punctigera*** | | | | | **80** | **120** | **160** |
| Goondiwindi QLD ($F_1$) | cotton | 6.6 | 9.6 | 2.3 | 98.6 | 99.0 | 100 |
| Wee Waa NSW ($F_3$) | cotton | 6.0 | 12.4 | 2.8 | 96.9 | 99.3 | 100 |
| Breeza NSW ($F_1$) '04 | cotton | 8.4 | 12.6 | 2.4 | 93.1 | 100 | 100 |
| Moree NSW ($F_1$) | cotton | 9.4 | 12.2 | 3.3 | 100 | 100 | 100 |
| Myall Vale NSW ($F_2$) | cotton | 8.8 | 12.2 | 2.8 | 98.6 | 98.6 | 100 |
| St George QLD ($F_3$) | cotton | 9.4 | 15.1 | 2.3 | 96.4 | 97.5 | 100 |
| Dalby QLD ($F_1$) | cotton | 10.8 | 14.6 | 3.8 | 100 | 98.5 | 100 |
| Emerald QLD ($F_1$) | ppea | 11.2 | 15.4 | 2.4 | 94.4 | 99.3 | 100 |
| Burren Junction NSW ($F_1$) | cotton | 11.1 | 16.1 | 2.8 | 97.2 | 100 | 100 |
| Maules Creek NSW ($F_1$) | cotton | 11.6 | 15.6 | 2.5 | 98.6 | 98.9 | 100 |
| Maules Creek NSW ($F_1$) | ppea | 12.0 | 15.7 | 2.9 | 98.6 | 99.3 | 100 |
| Kingaroy QLD ($F_1$) | cotton | 11.8 | 18.6 | 2.2 | 97.2 | 98.6 | 97.2 |
| Warren NSW ($F_1$) | cotton | 16.6 | 25.3 | 2.5 | 94.4 | - | 100 |
| *Laboratory strain (LHp)* | - | 3.8 | 7.1 | 2.6 | 100 | 100 | 100 |

(ii) Cry2Ab spore/crystal preparation (used 2003/04):

The difficulty of production, combined with a need for relatively high doses to kill 3rd instar *H. armigera*, resulted in the adoption of a development-based $F_0$ assay for Cry2Ab with neonates. By incorporating Cry2Ab into diet at various concentrations, we determined the dose at which 98% of presumptive homozygous recessive *Helicoverpa* were prevented from developing from unfed neonates to the 3rd instar in 7 days.

A total of 13 field-derived strains of *H. armigera* and 12 field-derived strains of *H. punctigera* were tested against the Cry2Ab spore/crystal preparation (Table 2). These data were used to determine the discriminating dose of Cry2Ab at 5.92µg/ml of diet for both *H. armigera* and *H. punctigera*. During the 2003/04 season approximately 1500 additional *H. armigera* from 7 field-derived strains were tested against Cry2Ab at each of the doses considered as candidates for the field screening. While not included herein, these data confirmed the discriminating dose of Cry2Ab at 5.92µg/ml of diet (Lisa Bird, personal communication).

(iii) Cry2Ab milled corn powder (used 2004/05):

We used a discriminating dose of 1µg/cm$^2$ of Cry2Ab corn powder in the $F_0$ screens for both *H. armigera* and *H. punctigera*. During CSE104C this dose was determined to prevent 90% of presumptive homozygous recessive Helicoverpa from developing from unfed neonates to the 3rd instar in 7 days. The toxin was applied as a surface treatment and larvae were scored for both mortality and growth.

*$F_2$ screens*

Since the species of larvae used in the $F_2$ screens is known, the use of neonates is favoured for these assays. Fewer resources are required to rear insects to the neonate stage, and less of the toxin is required to kill them compared to that required to kill 3rd instar larvae. In addition, it could be argued that using neonates in a feeding bioassay is a more realistic approximation of what happens in nature compared to using 3rd instar larvae.

(i) Cry1Ac spore/crystal preparation (used 2004/05):

Mortality criteria gave the best dose response curves for assessing susceptibility of neonatal larvae to pure Cry1Ac. Therefore, for the $F_2$ screens, calibration of the pure Cry1Ac against *Helicoverpa* spp. was conducted using a surface treatment test to assess mortality of neonate larvae after seven days. By overlaying the toxin on diet at various concentrations we determined the dose at which 98% of neonate larvae from field strains were killed.

A total of 12 field-derived strains of *H. punctigera* were tested against the Cry1Ac spore/crystal preparation (Table 3). From these data a discriminating dose of HD73 for *H. punctigera* was set at 0.50µg /cm$^2$.

We used a discriminating dose of 0.25µg/cm$^2$ of Cry1Ac (GHD73) in the $F_0$ screens for *H. armigera*. During CSE104C this dose was determined to kill 98% of presumptive homozygous recessive *H. armigera* neonates after 7 days when applied as a surface treatment. This dose kills 50% of heterozygotes, and 10-20% of homozygotes of a Cry1Ac resistant laboratory strain (BX).

(ii) Cry2Ab milled corn powder

See details for "$F_0$ screens, (iii) Cry2Ab milled corn powder" above.

*TABLE 2. Bioassay of Cry2Ab spore/crystal mix and calibration of discriminating doses on* H. armigera *and* H. punctigera *tested as neonates on diet incorporated Bt and assessed for development to the 3rd instar at day 7. The laboratory strain was comprised of presumably homozygous susceptible individuals. Ppea = pigeon pea.*

| Site | Host | 95% CI | | Slope | % failure to reach 4th instar at 7 d by dose (µg/ml diet) | | |
|---|---|---|---|---|---|---|---|
| | | lower | upper | | **1.48** | **2.96** | **5.92** |
| ***Helicoverpa armigera*** | | | | | | | |
| Wee Waa ($F_1$) | sorghum | 0.07 | 0.22 | 0.2 | 94.4 | 91.2 | 100 |
| Moree NSW ($F_1$) '04 | cotton | 0.14 | 0.26 | 1.6 | 87.5 | 94.4 | 100 |
| Goondiwindi QLD ($F_1$) '04 | cotton | 0.18 | 0.28 | 2.2 | 94.4 | 100 | 100 |
| Katherine NT ($F_3$) | cotton | 0.19 | 0.28 | 1.8 | 100 | 99.6 | 100 |
| Hillston NSW ($F_1$) | ppea | 0.21 | 0.32 | 1.7 | 100 | 98.8 | 99.4 |
| Griffith NSW ($F_1$) | maize | 0.24 | 0.38 | 1.6 | 94.1 | 95.7 | 100 |
| Breeza NSW ($F_1$) '04 | maize | 0.28 | 0.39 | 2.7 | 97.2 | 100 | 100 |
| Goondiwindi QLD ($F_2$) | cotton | 0.27 | 0.42 | 1.9 | 98.5 | 99.1 | 100 |
| Wee Waa NSW ($F_3$) | cotton | 0.28 | 1.42 | 1.6 | 100 | 98.5 | 100 |
| St George QLD ($F_2$) | cotton | 0.12 | 0.61 | 1.6 | 83.3 | 100 | 100 |
| Warren NSW ($F_1$) | cotton | 0.36 | 0.54 | 2.1 | 94.3 | 93.0 | 98.6 |
| Emerald QLD ($F_2$) | cotton | 0.42 | 0.69 | 2.0 | 98.6 | 99.0 | 99.2 |
| Moree NSW ($F_1$) | maize | 0.47 | 0.79 | 2.8 | 98.6 | 99.1 | 100 |
| *Laboratory stain (ANGR)* | - | 0.24 | 0.38 | 1.8 | 99.6 | 100 | 100 |
| ***H. punctigera*** | | | | | | | |
| Dalby QLD ($F_1$) | cotton | 0.10 | 0.16 | 3.2 | 100 | 100 | 100 |
| Myall Vale NSW ($F_2$) | cotton | 0.18 | 0.32 | 2.3 | 93.0 | 100 | 100 |
| Goondiwindi QLD ($F_2$) | cotton | 0.18 | 0.27 | 2.0 | 95.7 | 100 | 100 |
| Breeza NSW ($F_1$) | cotton | 0.23 | 0.32 | 3.0 | 96.9 | 100 | 100 |
| Wee Waa NSW ($F_2$) | cotton | 0.22 | 0.35 | 2.4 | 91.7 | 100 | 100 |
| St George QLD ($F_3$) | cotton | 0.18 | 0.59 | 2.0 | 85.9 | 98.6 | 100 |
| Burren Junction NSW ($F_2$) | cotton | 0.24 | 0.52 | 2.3 | 93.0 | 100 | 100 |
| Maules Creek NSW ($F_2$) | cotton | 0.29 | 0.51 | 3.4 | 98.6 | 100 | 100 |
| Kingaroy QLD ($F_2$) | cotton | 0.28 | 0.65 | 2.0 | 91.6 | 91.3 | 100 |
| Moree NSW ($F_2$) | cotton | 0.41 | 0.70 | 2.4 | 88.7 | 95.8 | 98.6 |
| Emerald QLD ($F_1$) | ppea | 0.55 | 0.92 | 2.3 | 90.1 | 90.3 | 95.6 |
| Warren NSW ($F_1$) | cotton | 0.62 | 1.02 | 2.3 | 74.6 | 92.6 | 100 |

*TABLE 3. Bioassay of Cry1Ab spore/crystal mix (HD73) and calibration of discriminating doses on 12 strains of* H. punctigera *tested as neonates on a diet surface treated with Bt and assessed for mortality at day 7. Ppea = pigeon pea.*

| Site | Host | 95% CI | | Slope | % mortality at 7 d by dose (µg /cm$^2$) | | |
|---|---|---|---|---|---|---|---|
| | | lower | upper | | **0.125** | **0.250** | **0.500** |
| Emerald | ppea | 28.6 | 68.5 | 2.3 | 91.3 | 93.0 | 100.0 |
| Dalby | cotton | 18.2 | 28.3 | 2.1 | 90.1 | 98.6 | 100.0 |
| Goondiwindi | cotton | 28.2 | 67.6 | 2.8 | 86.5 | 100.0 | 100.0 |
| Moree | cotton | 29.9 | 111.9 | 1.7 | 70.8 | 78.9 | 95.7 |
| Maules Creek | cotton | 31.0 | 55.3 | 1.9 | 80.1 | 89.3 | 100.0 |
| Warren | cotton | 27.0 | 66.3 | 2.0 | 87.0 | 88.9 | 98.6 |
| Burren Jnct | cotton | 10.6 | 33.7 | 1.6 | 84.7 | 97.1 | 100.0 |
| Breeza | cotton | 33.8 | 65.6 | 1.9 | 72.2 | 94.4 | 97.2 |
| Myall Vale | cotton | 12.7 | 27.8 | 1.9 | 98.6 | 98.6 | 98.6 |
| Wee Waa | cotton | 19.9 | 31.2 | 2.3 | 98.6 | 98.6 | 100.0 |
| St George | cotton | 32.4 | 59.8 | 1.9 | 77.7 | 92.9 | 98.6 |
| Kingaroy | cotton | 28.8 | 61.9 | 1.9 | 83.3 | 91.4 | 98.5 |

**Aim two: Monitor the sensitivity of field-collected populations of *Helicoverpa* species**

This objective was achieved in full. *During each year of the project we monitored the sensitivity of field populations of* Helicoverpa spp. *to Cry1Ac and Cry2Ab. Variation in the numbers of insects tested among seasons during the program primarily reflects natural variation in insect abundance. The following account includes data obtained from $F_2$ screens of field-collected populations of* Helicoverpa spp. *obtained during CSE104C.*

Egg sampling and allocation to $F_0$ and $F_2$ screens

During the 2002/03 and 2003/04 season we received at the CSIRO Black Mountain laboratories field-collected *Helicoverpa* eggs on artificial diet from collaborators sampling the Namoi, Gwydir, Emerald, Darling Downs and Macintyre Valleys. During the 2004/05 season we collected eggs in the Namoi and Gwydir Valleys, and received at ACRI field-collected *Helicoverpa* eggs on leaves from collaborators sampling the Darling Downs, Emerald, Macquarie, Macintyre, and St George Valleys.

Eggs were collected from cotton plus all other crops present in a region that are hosts to Helicoverpa. Where possible at a site, eggs were taken from both conventional and transgenic cotton crops. Most collections were located after alerts from growers or consultants of the presence of high egg pressure, rather than by random sampling throughout a region. Variation in abundances of *H. armigera* and *H. punctigera* dictated the relative proportions and numbers of each species sampled at any point in time throughout the season. The number of eggs submitted to the Bt resistance monitoring program from each valley is summarised for each season in Table 4. We achieved better numbers of eggs for testing during 2004/05 when the program was conducted at ACRI.

*TABLE 4: The number of eggs allocated to the Bt resistance monitoring program during each year of the project. Collections were from cotton, sorghum, pigeon pea, maize, and mung beans. These values include eggs that successfully hatched and those that were non-viable.*

| Valley | 2002/03 | 2003/04 | 2004/05 |
|---|---|---|---|
| Namoi | 10584 | 8103 | 23730 |
| Gwydir | 4795 | 13657 | 6265 |
| Emerald | 3081 | 1985 | 148 |
| Darling Downs | 1024 | 2500 | 3636 |
| Macintyre | 3372 | 4766 | 6012 |
| Macquarie | - | - | 2340 |
| St George | - | - | 1269 |
| TOTAL | 22856 | 31011 | 43400 |

Any eggs received on leaves were transferred onto artificial diet. The eggs on diet were placed under standard larval rearing conditions until they reached the required stage for testing. To maximize the numbers of insects available for testing during normal working hours we (1) checked material twice daily during the week and daily on weekends and (2) cooled moulting 2$^{nd}$ instars and black eggs overnight to slow development.

In the first two seasons of the program, virtually all of the larvae were allocated to the $F_0$ screens. During 2004/05 we allocated around half of the larvae sampled per collection from each valley, up to 50 larvae for large samples, to the $F_2$ screens. The upper limit to the number of $F_2$'s was determined by the workload necessary to conduct $F_2$ screens. Approximately 50% of allocated larvae successfully completed the $F_2$ testing regime. Our aim was to sample around 150 single mated pairs of moths (600 alleles) of each species. In all years, if larvae initially destined for $F_0$ tests developed past the early 3$^{rd}$ instar (overnight or during the day on the weekends), we allocated them to the $F_2$ screening program (2002/03 and 2003/04: CSE104C; 2004/05: this project).

For both types of screen, larvae at the appropriate stage of development were placed into 24-well trays containing Bt treated diet. These trays were kept under standard larval rearing conditions until assessment. Each week throughout the season, a susceptible laboratory strain (ANGR for *H. armigera* and LHP for *H. punctigera*) was screened with all toxins to provide a control for the screening assays.

$F_0$ screens

*Methods*

Table 5 outlines the products and associated bioassay methods used in the $F_0$ program during each year of the project. For MVPII® and DiPel® we assessed survivorship of early 3$^{rd}$ instars. The MVPII® screen was assessed after 10 days (after Dang & Gunning 2001) while the DiPel® assay was assessed after 7 days (after Forrester 1995). For GHD73 we assessed development of early 3$^{rd}$ instars to 4$^{th}$ instars after 7 days. For the Cry2Ab spore/crystal preparation and the Cry2Ab milled corn powder we assessed development of neonates to 3$^{rd}$ instars after 7 days.

*TABLE 5: Protocols adopted for $F_0$ screens. Note that for GHD73 the discriminating dose is different for* H. armigera *and* H. punctigera. *Development assays score larvae that fail to develop to a required stage plus dead animals. Diet = diet incorporated, Surface = surface treatment.*

| Year | Product | Toxins and material | Discriminating dose | | Type | Stage | Criteria | Days |
|---|---|---|---|---|---|---|---|---|
| | | | *H. armigera* | *H. punctigera* | | | | |
| 02/03 | MVPII | Cry1Ac in dead *Pseudomonas* cells | 3µl/ml | 3µl/ml | diet | 3rd | kill | 7 |
| | DiPel | Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, spores | 2mg/ml | 2mg/ml | diet | 3rd | kill | 10 |
| 03/04 | MVPII | Cry1Ac in dead *Pseudomonas* cells | 3µl/ml | 3µl/ml | diet | 3rd | kill | 7 |
| | DiPel | Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, spores | 2mg/ml | 2mg/ml | diet | 3rd | kill | 10 |
| | GHD73 | Cry1Ac spores + crystals | 80µg/ml | 120µg/ml | diet | 3rd | develop | 7 |
| | Cry2Ab | Cry2Ab spores + crystals | 5.92µg/ml | 5.92µg/ml | diet | 1st | develop | 7 |
| 04/05 | GHD73 | Cry1Ac spores + crystals | 80µg/ml | 120µg/ml | diet | 3rd | develop | 7 |
| | Cry2Ab | Cry2Ab corn powder | 1µg/cm$^2$ | 1µg/cm$^2$ | surface | 1st | develop | 7 |

*Screening results*

During 2002/03, in all sampled valleys, *H. armigera* comprised at least 80% of the eggs available for testing from December until the end of the season. The 2003/04 and 2004/05 seasons were unusual in terms of the relative numbers of *H. armigera* versus *H. punctigera* available for testing. Unlike most years, *H. punctigera* were not only common early in the season but also dominated well into January. Consequently, we collected somewhat less data on *H. armigera* than normal, but good samples of *H. punctigera* were available.

In assessing the data, we were most interested in three features that would allow us to detect the development of resistance: (1) shifts in the survival of *Helicoverpa* among seasons; (2) shifts in the survival of *Helicoverpa* within seasons; and (3) shifts in the survival of *Helicoverpa* between the end of one season and the start of the next season. The analyses on possible shifts were restricted to data from the Namoi/Gwydir region because it was only from this area that we have good samples of insects. The analyses were conducted on the results from each source of toxin that spaned consecutive years and/or were processed at regular intervals throughout the season.

**Shifts in survivorship of *Helicoverpa* among seasons**

Data spanning 1999/00 until 2001/02 (Dr Ho Dang, NSW Department of Agriculture) and 2002/03 and 2003/04 (this project) is available for screens against MVPII® and DiPel®. During the course of the project we collected data over two consecutive seasons for pure Cry1Ac and Cry2Ab. We separated results for each screening product and divided the total number of individuals surviving screens by the total number of individuals tested. We plotted these "season totals" along with those collected in previous seasons.

(i) Findings:

DiPel® screens were employed to detect possible "super-resistance" to a suite of Cry toxins. Survivors from the DiPel® screen were rare during the 2003/04 season and this trend is consistent with data obtained since 1999/00 (Figure 1). This result is similar for both species of *Helicoverpa*.

Survivors from the MVPII® screen were relatively common during the 2003/04 season but this overall level was around 2% lower than that obtained in the previous season (Figure 2). This trend is similar for both species of *Helicoverpa* and represents a continuing decline in season totals since 2001/02.

There was no marked variation in the proportion of insects surviving screens with the pure Cry1Ac screens in 2003/04 versus 2004/05 (Figure 3). This trend was similar for both species.

In 2003/04 we used a pure Cry2Ab toxin obtained from Bt, whereas in 2004/05 we used a source of Cry2Ab from milled corn. Nevertheless, we compared these data because in both cases we used the same criteria for assessing the bioassay and determining the discriminating doses. For *H. armigera* and *H. punctigera* survivors from both types of Cry2Ab screens were relatively rare and there was no indication of an increase in survivorship between the two years (Figure 4).

(ii) Conclusions:

Our comparisons of survivorship across seasons provide no evidence that *H. armigera* or *H. punctigera* are evolving resistance to either DiPel® or MVPII®.

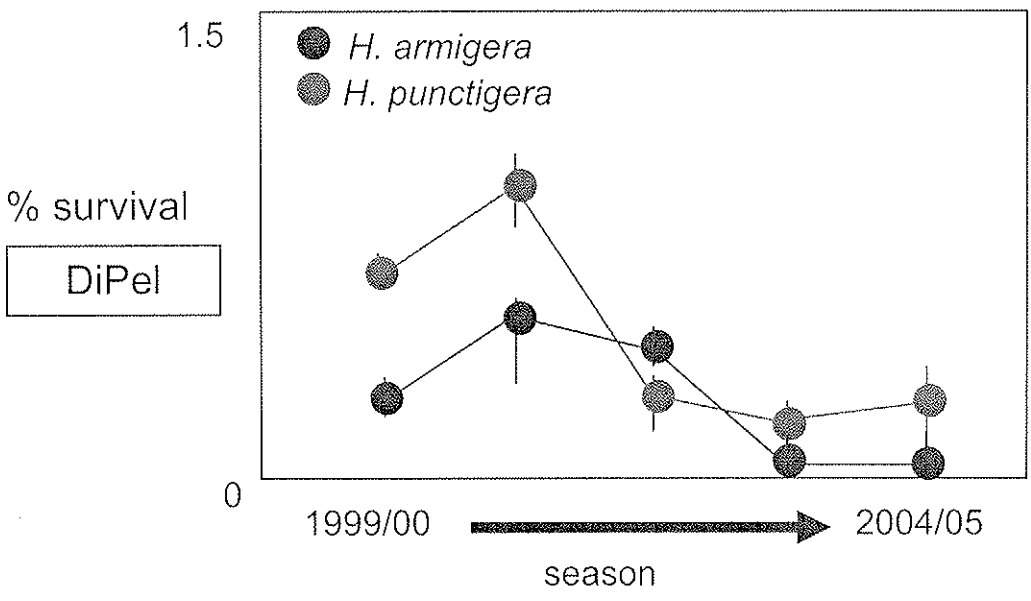


*FIGURE 1: The percentage of individuals that survived the DiPel screen for seasons 1999/00 to 2003/04. Data are presented separately for* H. armigera *(black dots) and* H. punctigera *(blue/grey dots). Error bars are 95% confidence intervals.*

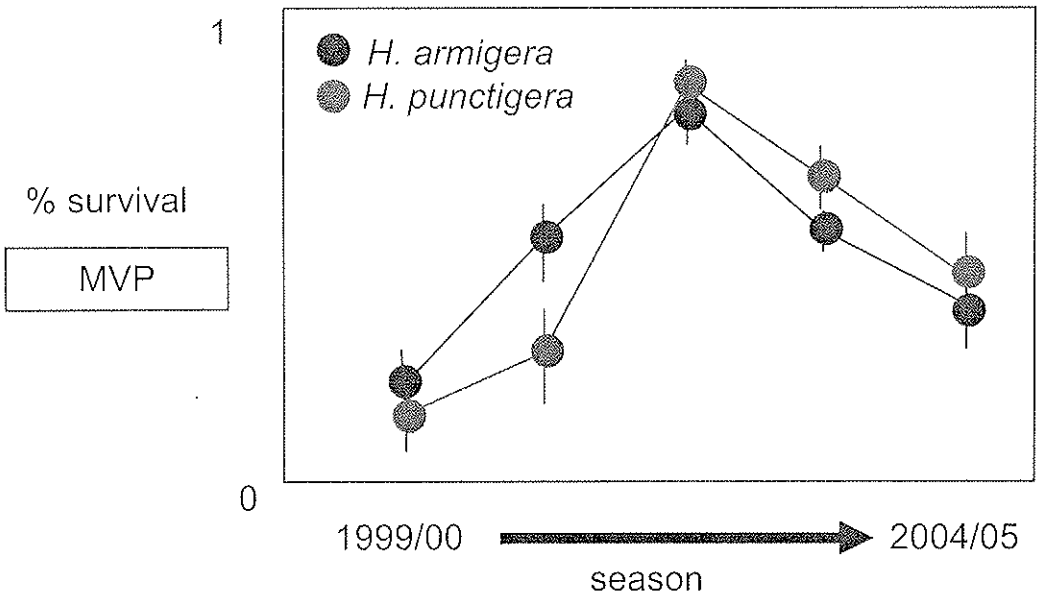


*FIGURE 2: The percentage of individuals that survived the MVPII screen for seasons 1999/00 to 2003/04. Data are presented separately for* H. armigera *(black dots) and* H. punctigera *(blue/grey dots). Error bars are 95% confidence intervals.*

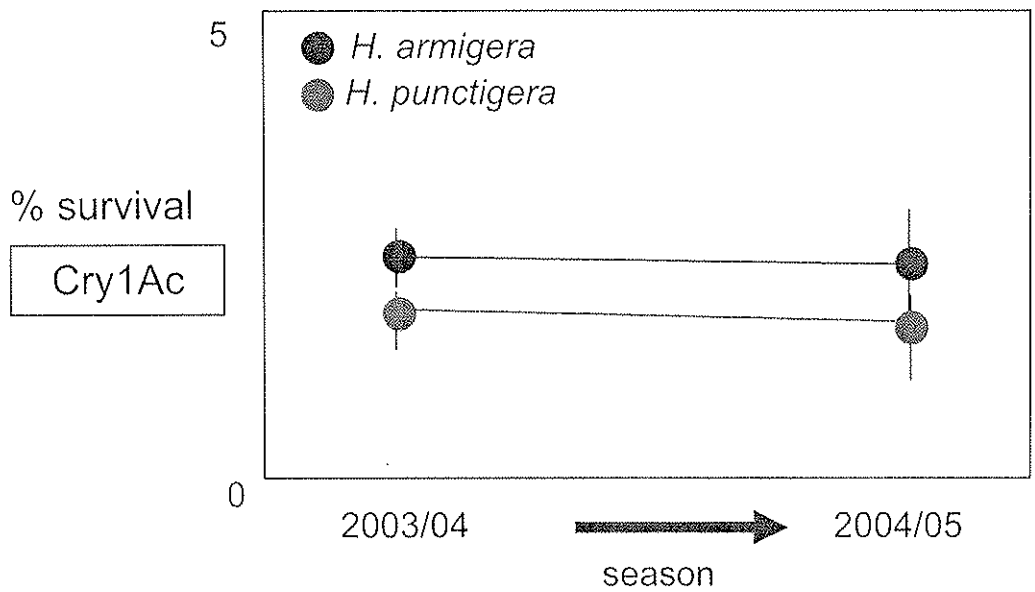


*FIGURE 3: The percentage of individuals that survived the pure Cry1Ac screen for seasons 2003/04 and 2004/05. Data are presented separately for* H. armigera *(black dots) and* H. punctigera *(blue/grey dots). Error bars are 95% confidence intervals.*

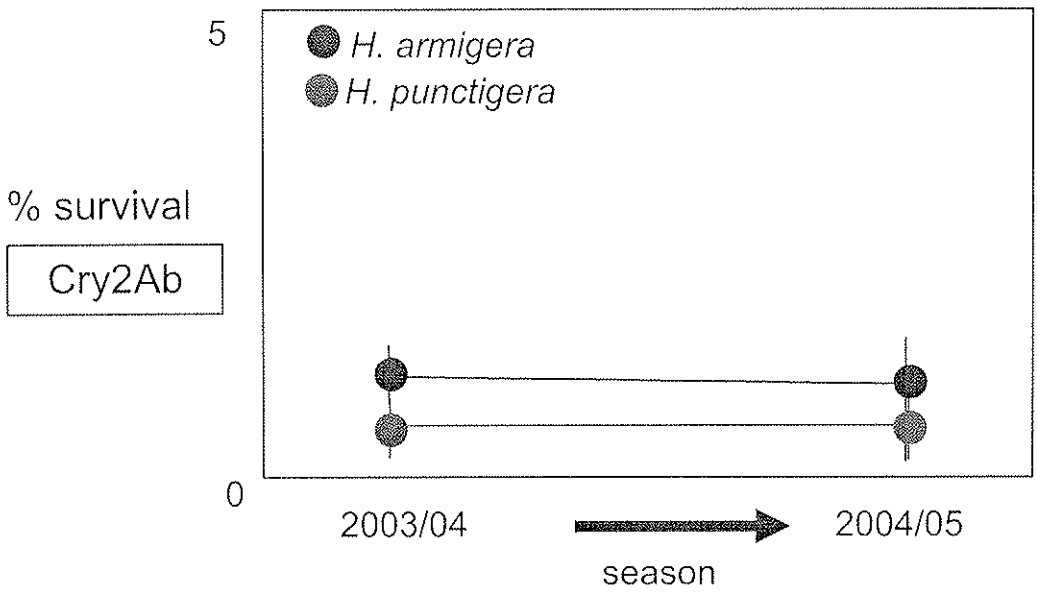


*FIGURE 4: The percentage of individuals that survived the pure Cry2Ab screen for seasons 2003/04 and 2004/05. Data are presented separately for* H. armigera *(black dots) and* H. punctigera *(blue/grey dots). Error bars are 95% confidence intervals.*

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**Shifts in survivorship of *Helicoverpa* within seasons**

Within the Namoi/Gwydir data, we analysed trends only for screens with MVPII® (2002/03 and 2003/04), pure Cry1Ac (2003/04 and 2004/05), and Cry2Ab corn powder (2004/05). We did not examine trends for screens with DiPel® because low survivorship for both species during the two years that this product was tested (2002/03 and 2003/04) prevents meaningful statistical analysis. We did not examine trends for screens with pure Cry2Ab (2003/04) because sample sizes for this product were small for both species.

Summary data for all regions and all toxins sampled during this project are presented for each season in Table 6. The number of individuals tested in each category should be considered when examining these data.

**2002/03 season**

(i) Findings:

When data from all crops were considered together, the frequency of *H. armigera* surviving MVPII® screens did not increase significantly throughout the season ($F_{(1,17)} = 0.02$; $P > 0.05$; Figure 5). Around 37% of the 3769 *H. armigera* tested against MVPII® during 2002/03 were from maize or pigeon pea, and 97 of these "non-cotton" larvae survived the screens representing 3% survival. There is no evidence of changes during the season in survival rates of *H. armigera* from non-cotton crops tested against MVPII® ($F_{(1,6)} = 0.01$; $P = 0.93$).

*TABLE 6: The number of larvae scored during the $F_0$ screens as positive expressed as a percentage of the number of individuals tested (the latter value is in parentheses to the right of survivorship). Data are provided separately for different regions, and for* Helicoverpa armigera *and* H. punctigera. *The available data have been divided into an "early season" sample (Dec-Jan) and "mid-late season" sample (Feb-April), as well as being summed across the entire season (Total). Discriminating doses for each toxin can be found in TABLE 5. NT = none tested. Dirr = Dirranbandi.*

| | | | % Individuals surviving discriminating dose (number tested) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Helicoverpa armigera* | | | | | | *Helicoverpa punctigera* | | | | | |
| **Year** | **Toxin** | **Valley** | **Dec-Jan** | | **Feb-April** | | **Total** | | **Dec-Jan** | | **Feb-April** | | **Total** | |
| 02/03 | MVP | Namoi/Gwydir | 5.6 | (840) | 9.0 | (2929) | 8.3 | (3769) | 7.8 | (244) | 9.0 | (332) | 8.5 | (576) |
| | | McIntyre | 6.8 | (370) | 8.6 | (396) | 7.7 | (766) | 6.1 | (148) | NT | (0) | 6.1 | (148) |
| | | Emerald | 3.4 | (148) | 5.4 | (1058) | 5.2 | (1206) | 0 | (11) | NT | (0) | 0 | (11) |
| | | Darling Downs | 1.1 | (176) | 2.0 | (50) | 1.3 | (226) | NT | (0) | NT | (0) | NT | (0) |
| | DiPel | Namoi/Gwydir | 0 | (730) | 0.2 | (1011) | 0.1 | (1741) | NT | (0) | 0 | (255) | 0 | (255) |
| | | McIntyre | 0 | (192) | 2.2 | (89) | 0.7 | (281) | 0 | (75) | NT | (0) | 0 | (75) |
| | | Emerald | NT | (0) | 0 | (275) | 0 | (275) | NT | (0) | NT | (0) | NT | (0) |
| | | Darling Downs | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| 03/04 | MVPII | Darling Downs | 1.4 | (359) | 0.0 | (29) | 1.3 | (388) | 4.4 | (90) | 3.5 | (29) | 4.2 | (119) |
| | | Namoi/Gwydir | 0.9 | (226) | 5.9 | (1073) | 5.0 | (1299) | 5.1 | (2555) | 9.4 | (733) | 6.0 | (3288) |
| | | McIntyre | 0.0 | (34) | 4.6 | (194) | 4.0 | (228) | 7.4 | (458) | 1.7 | (117) | 6.3 | (575) |
| | | Emerald | 3.3 | (60) | NT | (0) | 3.3 | (60) | 8.0 | (25) | 8.7 | (104) | 8.5 | (129) |
| | DiPel | Darling Downs | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| | | Namoi/Gwydir | 0.0 | (92) | 0.4 | (254) | 0.3 | (346) | 0.0 | (310) | 0.0 | (40) | 0.0 | (350) |
| | | McIntyre | NT | (0) | NT | (0) | NT | (0) | 0.0 | (97) | NT | (0) | 0.0 | (97) |
| | | Emerald | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| | Cry1Ac | Darling Downs | 0.4 | (240) | 0.0 | (8) | 0.4 | (248) | 2.6 | (78) | 0.0 | (9) | 2.3 | (87) |
| | | Namoi/Gwydir | 0.8 | (254) | 1.6 | (1288) | 1.4 | (1542) | 0.9 | (2614) | 1.5 | (583) | 1.0 | (3197) |
| | | McIntyre | 2.4 | (42) | 0.5 | (199) | 0.8 | (241) | 1.2 | (608) | 0.0 | (140) | 0.9 | (748) |
| | | Emerald | 0.0 | (122) | NT | (0) | 0.0 | (122) | 1.1 | (264) | 0.0 | (120) | 0.8 | (384) |
| | Cry2Ab | Darling Downs | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| | | Namoi/Gwydir | 1.3 | (79) | 0.0 | (503) | 0.2 | (582) | NT | (0) | 0.0 | (61) | 0.0 | (61) |
| | | McIntyre | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| | | Emerald | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |

*TABLE 6 continued.....*

| | | | % Individuals surviving discriminating dose (number tested) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Helicoverpa armigera* | | | | | | *Helicoverpa punctigera* | | | | | |
| **Year** | **Toxin** | **Valley** | **Dec-Jan** | | **Feb-April** | | **Total** | | **Dec-Jan** | | **Feb-April** | | **Total** | |
| 04/05 | Cry1Ac | Darling Downs | NT | (0) | 3.2 | (312) | 3.2 | (312) | 0.2 | (429) | 1.6 | (306) | 0.8 | (735) |
| | | Namoi/Gwydir | NT | (0) | 1.0 | (1560) | 1.0 | (1560) | 0.3 | (4256) | 0.7 | (5270) | 0.5 | (9526) |
| | | McIntyre | NT | (0) | 0.7 | (137) | 0.7 | (137) | 0.0 | (792) | 0.9 | (752) | 0.5 | (1544) |
| | | Emerald | NT | (0) | 0.0 | (9) | 0.0 | (9) | NT | (0) | 0.0 | (23) | 0.0 | (23) |
| | | Macquarie | NT | (0) | 0.0 | (4) | 0.0 | (4) | 0.0 | (820) | 1.2 | (83) | 0.1 | (903) |
| | | Riverina | NT | (0) | NT | (0) | NT | (0) | NT | (0) | 0.0 | (7) | 0.0 | (7) |
| | | St George/Dirr | NT | (0) | 9.5 | (21) | 9.5 | (21) | 0.3 | (373) | 0.0 | (49) | 0.2 | (422) |
| | Cry2Ab | Darling Downs | NT | (0) | 0.4 | (140) | 0.4 | (140) | 0.0 | (74) | 0.0 | (92) | 0 | (166) |
| | | Namoi/Gwydir | 0.0 | (382) | NT | (0) | 0.0 | (382) | 0.0 | (1304) | NT | (0) | 0.0 | (1304) |
| | | McIntyre | 0.0 | (11) | 1.9 | (42) | 1.5 | (53) | 0.0 | (236) | 0.0 | (225) | 0.0 | (461) |
| | | Emerald | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) | NT | (0) |
| | | Macquarie | 0.0 | (4) | NT | (0) | 0.0 | (4) | 0.0 | (130) | NT | (0) | 0.0 | (130) |
| | | Riverina | 0.0 | (16) | NT | (0) | 0.0 | (16) | 0.0 | (16) | NT | (0) | 0.0 | (16) |
| | | St George/Dirr | NT | (0) | NT | (0) | NT | (0) | 0.0 | (72) | NT | (0) | 0.0 | (72) |

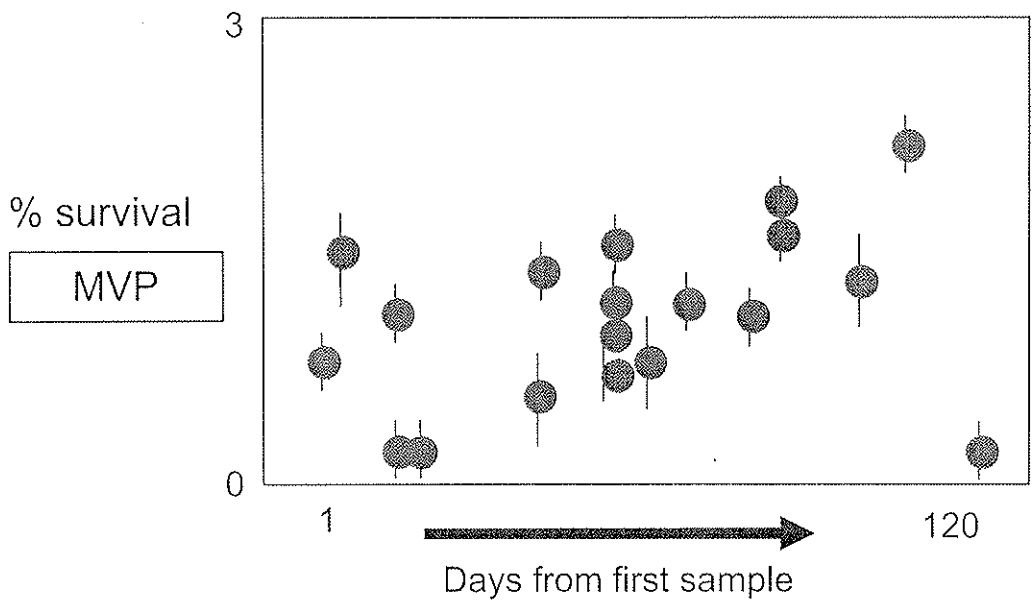


*FIGURE 5: The percentage of* H. armigera *collected from all crops that survived MVPII challenges throughout 2002/03. Error bars are 95% confidence intervals.*

In contrast, there is evidence of an increase in survival during the season from the 12 samples from cotton (Figure 6). An analysis of these data indicated there is a significant relationship between the date of collection and the proportion of larvae that survived the screen ($F_{(1,10)} = 13.45$; $P = 0.004$). We also partitioned the cotton samples into early and mid to late season: those taken during the early part of the season differ from those collected later, although the difference is barely significant at the 0.05 level ($F_{(1,10)} = 4.976$; $P = 0.049$).

When data from all crops were considered together, the frequency of *H. punctigera* surviving MVPII® screens did not increase significantly throughout the season ($F_{(1,9)} = 0.91$; $P = 0.37$). Around 21% of the 576 *H. punctigera* tested against MVPII® during 2002/03 were from maize or pigeon pea, and 104 of these "non-cotton" larvae survived the screens. This represented 18% survival. There is no evidence of changes during the season in survival rates of *H. punctigera* tested against MVPII® from cotton crops ($F_{(1,6)} = 0.20$; $P = 0.88$). We did not statistically analyse trends in other subsets of the data due to small sample sizes but a visual inspection indicates no marked changes in the proportion of survivors over time in non-cotton crops, conventional cotton crops, or transgenic cotton crops.

(ii) Conclusions:

There is evidence that *H. armigera* collected from cotton have increased survival against MVPII® as the season progresses. This phenomenon was first identified by Dr Ho Dang when he conducted the Bt monitoring program. The cause of this change is not understood. An obvious explanation is that selection for individuals resistant to Cry1Ac has occurred on Ingard® crops and is reflected in a valley-wide increase in resistant phenotypes. This hypothesis is not favoured as survivorship of samples from Ingard® was not particularly high. If significant levels of selection to Cry1Ac occurred on Ingard®, to elevate the proportion of survivors of the whole *H. armigera* population in the valley, populations in the neighbourhood of an Ingard® crop should be markedly enriched with resistant phenotypes. There is no evidence of such enrichment. However this argument would not apply if *H. armigera* from cotton dispersed widely between generations.

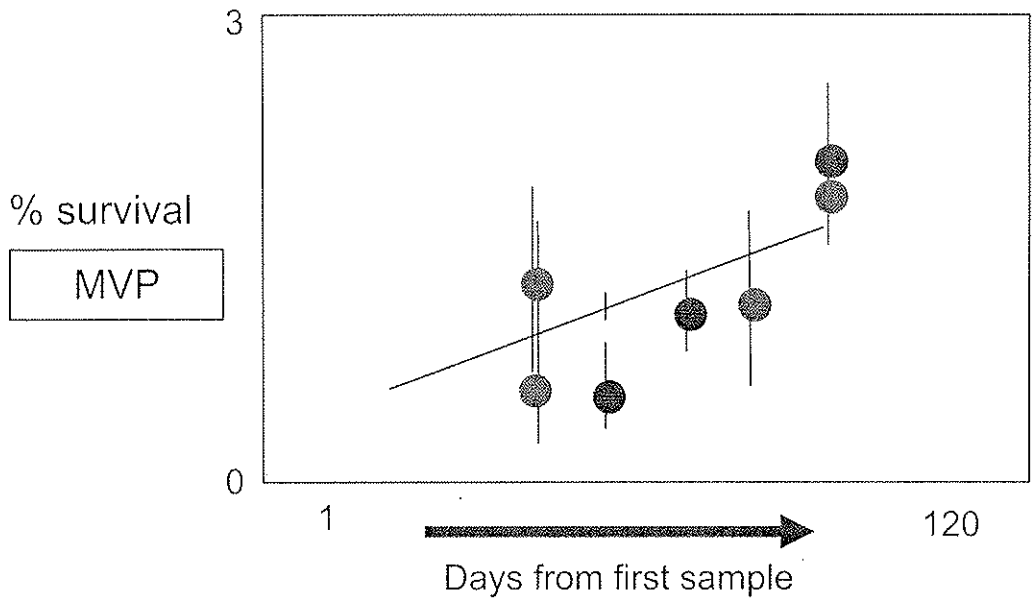


*FIGURE 6: The percentage of* H. armigera *collected from cotton that survived MVPII challenges throughout 2002/03. Eggs collected on conventional cotton are in light grey, Ingard are black and Bollgard II are blue/grey. Error bars are 95% confidence intervals.*

**2003/04 season**

An important finding in 2002/03 was that the proportion of *H. armigera* survivors from the MVPII® screen increased over the cotton season. These analyses included larvae from a variety of host types, with relatively few samples from transgenic cotton varieties being tested. The trend was interpreted as meaning that the incidence of tolerance to Cry1Ac among generations increased throughout the season. However, this explanation was tentative, as other interpretations were possible.

A major objective during 2003/04 was to re-examine seasonal changes, and compare these trends with those obtained during screens with the single Cry1Ac toxin. By sampling eggs from several varieties of cotton, we were able to perform analyses that considered seasonal changes among conventional versus transgenic crops. We were able to examine these trends in both *H. armigera* and *H. punctigera*. To perform analyses we summed data from properties that were sampled at the same time to reach test sizes of at least 40 individuals per time period throughout the season.

(i) Findings:

Less than 13% of the 1299 *H. armigera* tested against MVPII® during 2003/04 were from non-cotton crops, and none of these "non-cotton" larvae survived the screens. However, the frequency of *H. armigera* surviving MVPII® screens across all cotton varieties increased throughout the season ($F_{(1,21)} = 4.78$; $P = 0.042$). For conventional cotton there is no trend in the proportion of survivors against MVPII® as the season progresses ($F_{(1,11)} = 0.01$; $P = 0.932$: Figure 7). In contrast, for transgenic varieties there is a positive relationship between the proportion of larvae surviving MVPII® and date of collection ($F_{(1,10)} = 8.38$; $P = 0.018$). Within the two transgenic varieties there is a significant increase throughout the season in survival of larvae from Ingard® ($F_{(1,7)} = 29.01$; $P = 0.002$; Figure 8) but not from Bollgard II® ($F_{(1,7)} = 2.66$; $P = 0.16$).

Less than 10% of the 1542 *H. armigera* tested against GHD73 (Cry1Ac) during 2003/04 were from non-cotton crops, and only one of these "non-cotton" larvae survived the screens. In contrast to the situation where insects were challenged with MVPII®, there is no indication that the frequency of *H. armigera* surviving GHD73 screens increased during the season. This result holds true when considering data for all cotton varieties ($F_{(1,22)} = 0.47$; $P = 0.500$), conventional cotton ($F_{(1,11)} = 0.10$; $P = 0.774$: Figure 9), transgenic cotton ($F_{(1,12)} = 1.13$; $P = 0.312$), and Ingard® ($F_{(1,8)} = 2.01$; $P = 0.200$; Figure 10). The data for Bollgard II® were not statistically analysed due to small sample sizes but a visual inspection indicates no marked change in the proportion of survivors over time.

Less than 8% of the 3219 *H. punctigera* tested against MVPII® during 2003/04 were from non-cotton crops, and 18 of these 245 "non-cotton" larvae survived the screens. This represents 7% survival, which is slightly lower than the season average. The frequency of *H. punctigera* surviving MVPII® screens across all cotton varieties did not significantly increase throughout the season ($F_{(1,34)} = 3.88$; $P = 0.057$), and the same finding holds for conventional cotton ($F_{(1,16)} = 0.19$; $P = 0.674$). For transgenic varieties there is a positive relationship between the proportion of larvae surviving MVPII® and date of collection ($F_{(1,17)} = 5.77$; $P = 0.028$). However, this trend reflects the survival of 10 of 89 tested larvae from Bollgard II® during early March. The data for Bollgard II® were not statistically analysed because of small sample sizes but a visual inspection indicates no trends in the proportion of survivors over time. There is no marked seasonal trend in survivorship of larvae from Ingard® ($F_{(1,14)} = 1.34$; $P = 0.268$).

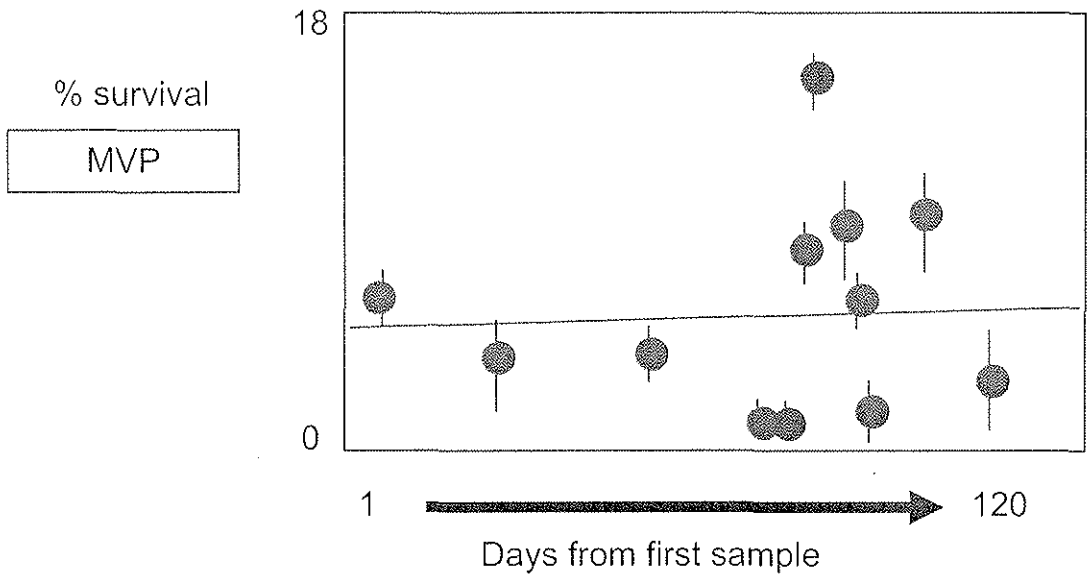


*FIGURE 7: The percentage of* H. armigera *collected from conventional cotton that survived MVPII challenges throughout 2003/04. We summed data from properties sampled at the same time to reach test sizes of at least 40 individuals per time period. Error bars are 95% confidence intervals.*

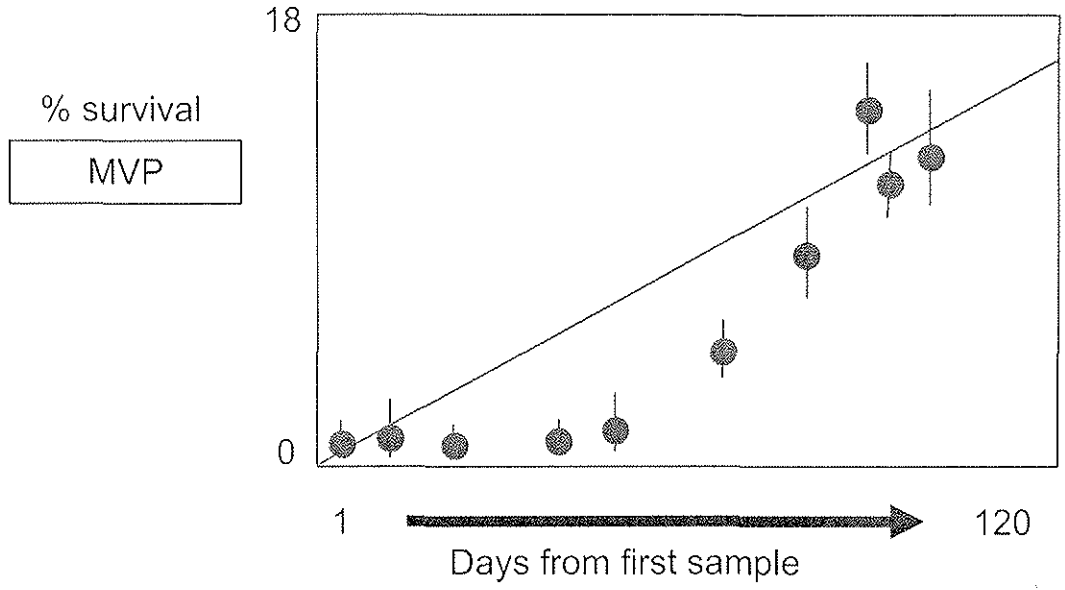


*FIGURE 8: The percentage of* H. armigera *collected from Ingard cotton that survived MVPII challenges throughout 2003/04. We summed data from properties sampled at the same time to reach test sizes of at least 40 individuals per time period. Error bars are 95% confidence intervals.*

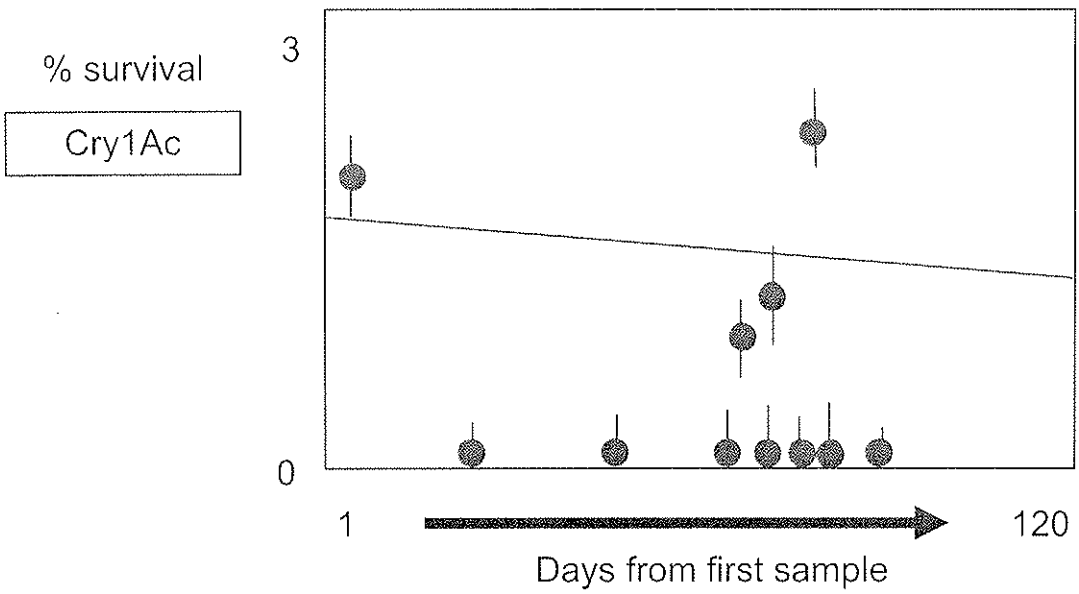


*FIGURE 9: The percentage of* H. armigera *collected from conventional cotton that survived GHD73 challenges throughout 2003/04. We summed data from properties sampled at the same time to reach test sizes of at least 40 individuals per time period. Error bars are 95% CI's.*

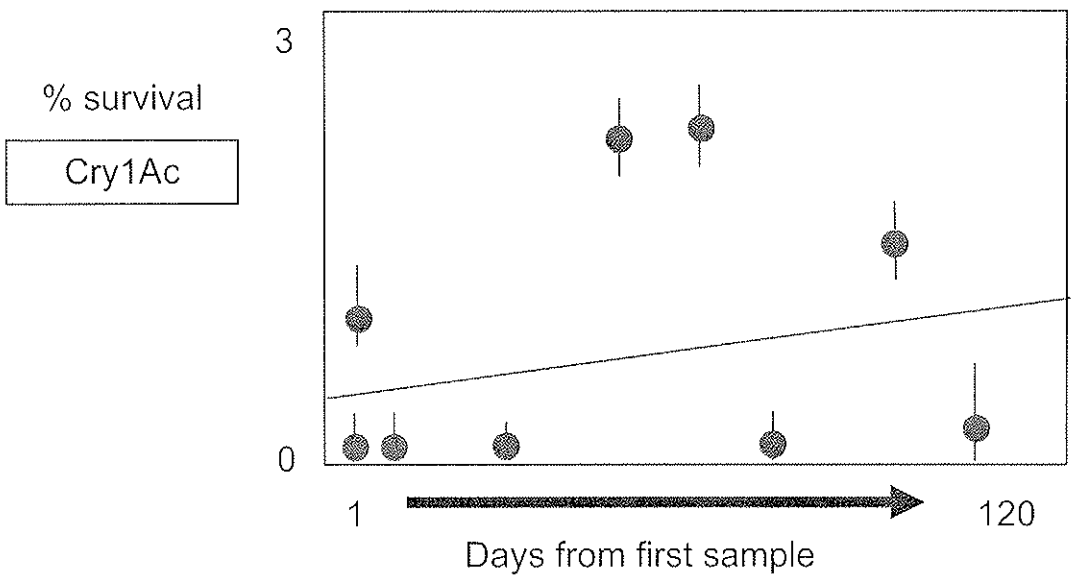


*FIGURE 10: The percentage of* H. armigera *collected from Ingard that survived GHD73 challenges during 2003/04. We summed data from properties sampled at the same time to reach test sizes of at least 40 individuals/period. Error bars are 95% confidence intervals.*

Less than 6% of the 3197 *H. punctigera* tested against GHD73 during 2003/04 were from non-cotton crops, and only one of these "non-cotton" larvae survived the screens. There is no indication that the frequency of *H. punctigera* surviving GHD73 screens increased throughout the season. This result holds true when considering data for all cotton varieties ($F_{(1,28)} = 1.50$; $P = 0.232$), conventional cotton ($F_{(1,13)} = 0.13$; $P = 0.724$), transgenic cotton ($F_{(1,15)} = 2.70$; $P = 0.122$), and Ingard® ($F_{(1,12)} = 3.85$; $P = 0.076$). The data for Bollgard II® were not statistically analysed because of small sample sizes but visual inspection indicates no marked seasonal trend in the proportion of survivors.

(ii) Conclusions

Our data do not suggest any significant change during the season in frequency of *H. punctigera* surviving MVPII® screens or GHD73 (Cry1Ac) screens.

As observed in 2002/03, the frequency of *H. armigera* surviving MVPII® screens across all cotton varieties increased throughout the season. An important insight from data collected during 2003/04 is that this trend primarily reflects an increase in survivorship during the season of *H. armigera* collected from Ingard® cotton and not from conventional varieties and Bollgard II®. Importantly, across all crops there was no indication of a seasonal increase in survivorship of *H. armigera* screened against the single Cry1Ac toxin. This result dismisses the possibility that the increased survivorship during the season is due to the BX form of resistance to Cry1Ac that has been detected in *H. armigera*. We therefore interpret the trend for MVPII® as reflecting an increase during the season in tolerance to a non-Cry1Ac component of this formulation.

Improved survivorship of larvae from Ingard® as the season progresses may be of little consequence to resistance management since this variety was completely replaced with Bollgard II® from 2004/05 onwards. However, it is worth noting that this finding indirectly suggests that *Helicoverpa* may be rather sedentary within a particular crop, and if so, this would reduce the efficacy of refuges.

**2004/05 season**

(i) Findings

Around 54% of the 1560 *H. armigera* tested against pure Cry1Ac during 2004/05 were from non-cotton crops, and four of these "non-cotton" larvae survived the screens. The frequency of *H. armigera* surviving Cry1Ac screens did not increase significantly throughout the season when data for all crops combined was considered ($F_{(1,19)} = 0.40$; $P = 0.85$). The same trend holds true when the data are divided into samples from all cotton combined ($F_{(1,17)} = 0.32$; $P = 0.58$), conventional crops ($F_{(1,9)} = 4.40$; $P = 0.07$), Bollgard II® crops ($F_{(1,7)} = 0.05$; $P = 0.83$), or non-cotton crops ($F_{(1,8)} = 0.33$; $P = 0.59$).

All of the 1382 *H. armigera* tested against Cry2Ab during 2004/05 were from cotton crops. The frequency of *H. armigera* surviving Cry2Ab screens did not increase significantly throughout the season when data for all crops combined was considered ($F_{(1,17)} = 0.41$; $P > 0.50$). The same trend holds true when the data from Bollgard II® samples are considered alone ($F_{(1,14)} = 0.23$; $P > 0.50$). Too few samples were collected from conventional cotton to explore whether there was a trend in survival throughout the season of insects from these crops.

Only 8% of the 9526 *H. punctigera* tested against pure Cry1Ac during 2004/05 were from non-cotton crops, and eight of these "non-cotton" larvae survived the screens. The frequency of *H. punctigera* surviving Cry1Ac screens did not increase significantly throughout the season when data for all crops was considered together ($F_{(1,32)} = 0.26$; $P = 0.62$). The same trend holds true when the data are divided into samples from all cotton combined ($F_{(1,28)} = 0.87$; $P = 0.36$) or conventional cotton ($F_{(1,13)} = 0.01$; $P = 0.96$). Too few samples were collected from non-cotton crops and Bollgard II® to statistically analyse trends but visual inspection indicates no changes in survival over time.

All of the 1304 *H. punctigera* tested against Cry2Ab during 2004/05 were from cotton crops. The frequency of *H. punctigera* surviving Cry2Ab screens did not increase significantly throughout the season when data for all crops combined was considered ($F_{(1,20)} = 0.15$; $P > 0.50$). The same trend holds true when the data from Bollgard II® samples are considered alone ($F_{(1,16)} = 0.24$; $P > 0.50$). Too few samples were collected from conventional cotton to explore whether there was a trend in survival throughout the season of insects from these crops.

(i) Conclusions

Our data do not suggest that a significant change occurred during the season in the frequency of *H. armigera* or *H. punctigera* surviving screens with either pure Cry1Ac or Cry2Ab corn powder.

**Does end of season survivorship "reset" before the following season?**

Given that, for at least two years, the proportion of *H. armigera* larvae that survived screens against MVPII® increased throughout the cotton season, it is important to determine whether survivorship "resets" between subsequent seasons. Figure 11 shows the proportion of larvae surviving MVPII® screens during the final month of one season and the first month of the subsequent season. Note that this analysis necessarily contains data from all crops, since different varieties were sampled to different degrees among seasons. Small sample sizes of eggs from transgenic cotton prior to the 2003/04 season prevent us from examining trends specific to Ingard® or Bollgard II®.

(i) Findings

During 2000 and 2001, there was a relatively small increase in survivorship against MVPII® between subsequent seasons. However, for the past two seasons there has been a considerable decline in the proportion of survivors at the end of one season and the start of the next season.

(ii) Conclusion

There is no evidence that the levels of survival of *H. armigera* at the beginning of the 2002/03 and 2003/04 season increased relative to frequencies in the early part of previous years. This is of considerable significance, as whatever phenomenon brought about seasonal changes they appear to "re-set" during the non-cotton growing season.

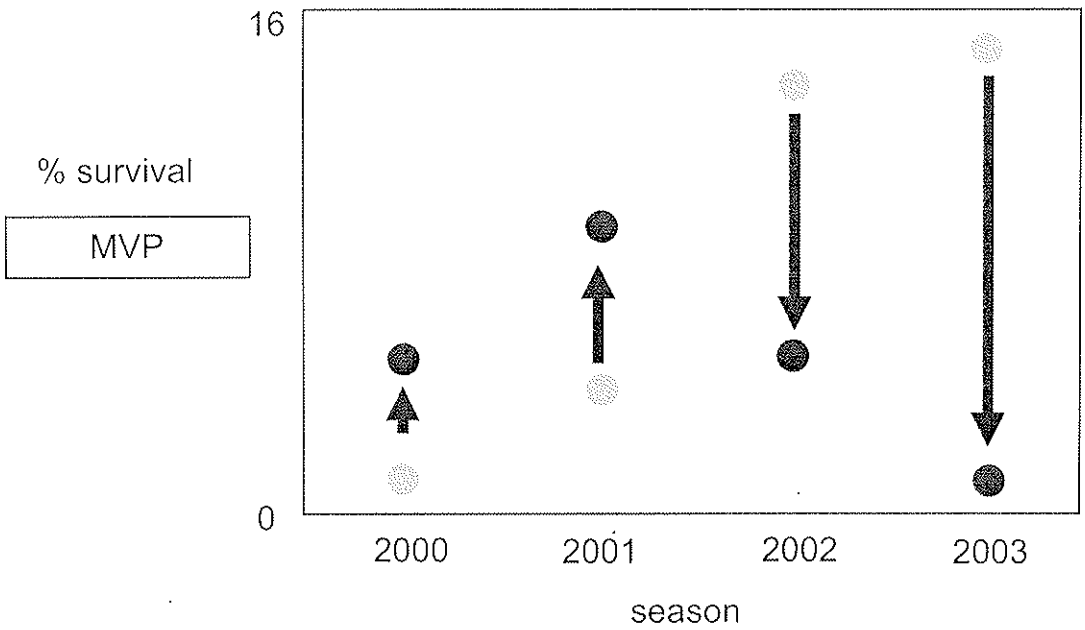


*FIGURE 11: The proportion of* Helicoverpa armigera *surviving MVP screens during the final month of one season (grey dots) and the beginning month of the subsequent season (black dots). All screens were diet incorporated mortality assays on early 3rd instars using a discriminating dose of 3µl/ml of diet. Arrows indicate the direction of change in survivorship.*

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$F_2$ screens

*Methods*

This method assayed the "grandchildren" of single pairs of insects collected from the field to identify individuals that have one resistance allele and one susceptible allele (SR, heterozygotes). In most lines, $F_2$ females laid enough eggs to enable screening against both Cry1Ac and Cry2Ab, thereby enabling the detection of resistance for either toxins to be determined by challenging different batches of larvae. At the time each $F_2$ family was tested, we set aside a subset of insects that were reared on regular diet. If our assessments indicated that a 'resistant gene' was present in the family, we attempted to 'capture' that gene by rearing the relevant subset of insects through to the next generation for further testing by colleagues at the CSIRO Entomology site at Black Mountain.

For the pure Cry1Ac screen we assessed the mortality of neonatal larvae after 7 days using diet covered with GHD73 at a dose of 0.25 µg/cm$^2$ for *H. armigera* and HD73 at a dose of 0.50 µg/cm$^2$ for *H. punctigera*. For the Cry2Ab milled corn powder we assessed mortality and development of larvae from neonates to 3rd instars after 7 days using diet covered with toxin at a dose of 1.0 µg/cm$^2$ for *H. armigera* and *H. punctigera*.

Table 6 summarizes the data from the $F_2$ screens for 2004/05 according to species and Bt toxin. Also included in this table are data collected as part of CSE104C during 2002/03 and 2003/04. Each $F_2$ family tested effectively screens for 4 alleles, two each from the male and female that comprised the single pair mating that generated the line.

*Findings*

Of the 780 alleles of *H. armigera*, and 1080 alleles of *H. punctigera*, which have been screened against Cry1Ac, none tested positive for resistance. However, one survivor from the 2002/03 $F_0$ screens with MVPII® was mated with a moth from a susceptible laboratory strain (ANGR). The offspring of this cross were mated to a Cry1Ac resistant strain of *H. armigera* (BX) and their offspring challenged with a dose of toxin that would identify homozygous individuals BX. This complementation test scored positive which indicates that the initial survivor of the MVPII® screen was carrying the BX form of resistance, probably as a heterozygote.

Of the 784 alleles of *H. armigera* which have been screened against Cry2Ab, three have tested positive for resistance. All were detected as part of CSE104C. Two originated from maize in Griffith and one originated from cotton in Moree. Based on these data our best estimate of the frequency of *H. armigera* moths with Cry2Ab resistant alleles in field populations is 0.004. We used statistics to assign an upper and lower value around this frequency that estimates the range with a 95% certainty, taking into account the number of alleles sampled. For the estimate the upper value is 0.011 and the lower value is 0.0008. All of these resistant lines were simultaneously screened against Cry1Ac and no cross-resistance was detected.

Of the 1092 alleles of *H. punctigera* which have been screened against Cry2Ab, one tested positive for resistance. It was detected as part of the current project, and originated from cotton in St George. Based on these data our best estimate of the frequency of *H. punctigera* moths with Cry2Ab resistant alleles in field populations is 0.009. For this frequency estimate the lower value is 0.0001 and the upper value is 0.0052. This resistant line was simultaneously screened against Cry2Ab and no cross-resistance was detected.

*TABLE 7: Summary information for the $F_2$ screens according to species (*H. armigera *versus* H. punctigera*), cotton season and Bt toxin.*

| Toxin | Species | Season | No. alleles | No. resistant | Frequency |
|---|---|---|---|---|---|
| Cry1Ac | *H. armigera* | 2002/03 | 136 | 0 | 0 |
| | | 2003/04 | 280 | 0 | 0 |
| | | 2004/05 | 364 | 0 | 0 |
| | | **TOTAL** | **780** | **0** | **0** |
| | *H. punctigera* | 2002/03 | 8 | 0 | 0 |
| | | 2003/04 | 60 | 0 | 0 |
| | | 2004/05 | 1012 | 0 | 0 |
| | | **TOTAL** | **1080** | **0** | **0** |
| Cry2Ab | *H. armigera* | 2002/03 | 132 | 1 | 0.008 |
| | | 2003/04 | 284 | 2 | 0.007 |
| | | 2004/05 | 368 | 0 | 0 |
| | | **TOTAL** | **784** | **3** | **0.004** |
| | *H. punctigera* | 2002/03 | 8 | 0 | 0 |
| | | 2003/04 | 60 | 0 | 0 |
| | | 2004/05 | 1024 | 1 | 0.009 |
| | | **TOTAL** | **1092** | **1** | **0.009** |

*Conclusions*

There have been no reported field failures of Bollgard II® due to resistance. However, a gene that confers high level resistance to Cry2Ab is present in field populations of *H. armigera*. This gene does not confer resistance to Cry1Ac. Our current best estimate based on CSIRO data is that it occurs at a high frequency of 0.004. Another gene (BX-like) is present in field populations of *H. armigera* that confers high-level resistance to Cry1Ac. This gene must be rare as such genes have not yet been detected in the 780 alleles examined. It is not cross-resistant to Cry2Ab.

The high frequencies of Cry2Ab resistant genes in natural populations are unexpected because to date there has been little opportunity for selection of *H. armigera* in cotton fields to increase the frequency of resistance genes above levels expected due to mutation. Indeed, the first case of Cry2Ab resistance was isolated before Bollgard II® was released commercially.

One possible explanation is that the moth has been exposed to Cry2Ab from naturally occurring Bt bacteria present in soils. While this is a reasonable theory, it is perhaps flawed, as though Cry2Ab toxin is produced by some Australian Bt bacteria, it is not a common element. The Cry1Ac toxin is far more common in native Bt's yet resistance to this toxin in *H. armigera* is rare. We simply do not know why the frequencies of Cry2Ab resistance genes are as common as they are. However, one theory that we are examining is that mutations which confer resistance to Cry2Ab may occur in natural populations of *H. armigera* at a very high rate.

Our current knowledge of the ecology and resistance profiles of Australian *H. armigera* populations suggest the RMP is adequate for preventing an increase in the frequency of this resistance. Computer models that incorporate our knowledge of resistance frequencies, fitness costs, form of dominance and refuge size, suggest that Bollgard II® should prove effective at managing *Helicoverpa* in the medium to long term.

**4. Provide a conclusion as to research outcomes compared with objectives. What are the "take home messages"?**

We achieved our main objective which was to look for early signs of the development of resistance by Helicoverpa to the Bt toxins within genetically modified cotton.

We have not detected field resistance to genetically modified cotton.

During the three year program we used $F_0$ screens to challenge around 1600 *H. armigera* and 2500 *H. punctigera* to various Bt products. During the final year of the program, and as part of CSE104C, we used $F_2$ screens to challenge a further 400 *H. armigera* and 500 *H. punctigera* to both Cry1Ac and Cry2Ab. Data from both of these sources suggest that there is no immediate cause for concern for the longevity of the extremely valuable transgenic technology currently available to Australian cotton growers.

Our data for the frequency of Cry2Ab resistance genes in natural populations is interesting because it is much higher than expected. Clearly future programs should carefully monitor any changes in this frequency.

**5. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?**

Bt crops have delivered enormous benefits to several aspects of the Australian cotton industry. They present an opportunity to significantly reduce the amount of insecticides applied as sprays to crops. In turn, this technology reduces both the amount of insecticides that run off into the environment and the cost of using insecticides to control a significant target pest. The consequences of these processes include a safer and cleaner environment for

rural people, support for an industry that is committed to being environmentally friendly, and an opportunity to maintain profitability and competitiveness in the world cotton market.

The monitoring program reported herein aims to provide an early warning of the advent of resistance by *Helicoverpa* spp. to this important technology. In doing so, it supplies information on which the industry can judge the effectiveness of its current RMP. This information is essential to prevent *Helicoverpa* spp. from developing field-scale resistance, and thereby prolong the important economic, environmental and social benefits outlined above.

**6. Provide a summary of the project ensuring the following areas are addressed:**

**a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.)**

This work is not of a technical nature.

**b) other information developed from research (eg discoveries in methodology, equipment design, etc.)**

Part of this project involved refining the protocols used to monitoring for resistance to Bt in Australian populations of *Helicoverpa* species. However, these improvements were based on standard protocols and did not involve discoveries in methodologies or equipment design.

**c) are changes to the Intellectual Property register required?**

No changes are required to the IP register.

**7. Detail a plan for the activities or other steps that may be taken:**

**(a) to further develop or to exploit the project technology.**

Not applicable (see response to 6a above).

**(b) for the future presentation and dissemination of the project outcomes.**

Approximately every month throughout the season, the findings from the Bt resistance monitoring program were distributed to industry via written reports to IDO's and other regional contacts. Informal talks were given at St George, and the Lower and Upper Namoi CCA/AWM group meetings.

The end of season results from the Bt resistance monitoring program were distributed to industry via written reports, and talks at the TIMS Roadshow, CCA Cotton Production Seminar and AGM, CRDC Resistance Forum, and CSD Science Review. They are summarised in the RMP section of the 2005 Cotton Pest Management Guide, and will be published in the Dec-Jan issue of *The Australian Cotton Grower* along with a reminder to industry about the importance of contributing eggs to the program. See also our response to question 8.

**(c) for future research.**

It is critical that this research continues in order to detect changes in frequencies of resistance genes as early as possible and thereby recommend changes in the RMS that would enhance the longevity of Bollgard II® and any other transgenic technology that makes use of either Cry1Ac or Cry2Ab. If additional transgenic varieties become available that employ new toxins the resistance monitoring program should be extended to assess frequencies of resistance genes for such toxins.

**8. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)**

**Downes SJ**, Rossiter L, 2004, Preventing *Helicoverpa* resistance: why we need your eggs. *The Australian Cotton Grower* Oct-Nov:14-15

**Downes SJ**, Rossiter L, Farrell T, Wilson L, Kauter G, 2005, Managing resistance: your IRMS and RMP questions answered. *The Australian Cotton Grower* Oct-Nov (in press)

Mahon RJ, Olsen KM, Garsia K, Young SR, 2005, Resistance to the Bt toxin Cry2Ab in a strain of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), In prep (target journal, J Econ Entomol)

Mahon RJ, Olsen KM, Downes S, Addison S, 2005, The frequency of resistance to Cry 1Ac and Cry2Ab toxins in Australian populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), In prep (target journal, J Econ Entomol.)

**9. Have you developed any online resources and what is the website address?**

A summary of the results from this project are given in the RMP section of the 2005 Cotton Pest Management Guide, a link to which will appear on the Cotton CRC Website.

**10. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry or the Australian community.**

The Resistance Management Plan (RMP) for Bollgard II® is designed to slow the rate that *H. armigera* and or *H. punctigera* develop field-scale resistance. This is important as the two insecticidal genes in Bollgard II® are likely to be the basis on which newer insecticidal genes are added. This means that protecting Bollgard II® also represents the protection of future investment in transgenic crops for the Australian cotton industry.

The output from the program is up-to-date information on possible changes in the frequency of resistant *Helicoverpa* spp. in natural populations. This information is used to evaluate the effectiveness of the Bt resistance management strategy, and to drive any changes to the RMP that might be required to maintain its success.

Bt-cotton is providing great financial environmental and OH&S benefits through a major reduction in the use of insecticides. A recent report (http://www.cotton.crc.org.au/Assets/PDFfiles/CRC/BDARpt.pdf) suggests that Ingard®, the initial Bt-cotton, provided a profit increase of $228 /ha. In a year where 80% of the 300,000 ha cotton crop is likely to be Bt varieties represents a profit increase of $54M to the Australian cotton industry.

## *Part 4 – Final Report Executive Summary*

Provide a one page summary of your research that is not commercial in confidence and that can be published on the World Wide Web. It explains the main outcomes of the research and provides contact details for more information. It is important that the Executive Summary highlights practical, the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

In the 1996/97 season the Australian cotton industry adopted an insect-resistant variety of cotton (Ingard®) that is specific to the group of insects including the target *Helicoverpa* spp. but excluding predators and parasitoids of this pest. To prolong the efficacy of transgenic cotton against *Helicoverpa* spp., a resistance management plan (RMP) that restricted the area grown to Ingard® was implemented due to the critical importance of preserving the efficacy of the Cry1Ac gene.

In the 2004/5 season Bollgard II® replaced Ingard® as the transgenic variety of cotton available to Australian growers. It improves on Ingard® by incorporating an additional insecticide protein (Cry2Ab) to combat Helicoverpa. Due to the perceived difficulty for *Helicoverpa* spp. to evolve resistance to both proteins simultaneously within Bollgard II®, the RMP for transgenic cotton was relaxed to allow growers to plant up to 95% of the total area to this product. Bollgard II® was well adopted, with up to 70% (200,000 hectares) planted area throughout the industry.

The sensitivity of field-collected populations of *Helicoverpa* spp. to Bt products was assayed before and subsequent to the widespread deployment of Ingard® cotton expressing Cry1Ac in the mid-1990's. From 1994/95 until 2002/03, a Bt spray (MVPII®) that contained formulation ingredients additional to Cry1Ac was used in the screens. The program also incorporated a Bt spray (DiPel®) with insecticidal proteins additional to Cry1Ac to test for resistance to combinations of Cry toxins. The program used only $F_0$ screens however this method cannot detect individuals that are heterozygous for a recessive form of resistance.

During this project we developed screens using a pure Cry1Ac spore/crystal mix as our source of toxin. In anticipation of Bollgard II® replacing Ingard® in 2004/05, we developed methods to screen for resistance to Cry2Ab. In addition to performing $F_0$ screens to detect major changes in gene frequencies, we incorporated an $F_2$ screen to detect and 'capture' any rare resistance alleles in natural populations. This method allowed us to simultaneously screen for resistance to Cry1Ac and Cry2Ab, hence making the screens using DiPel® redundant.

There have been no reported field failures of Bollgard II® due to resistance. Our work shows that alleles that confer high level resistance by field populations of *H. armigera* and *H. punctigera* are rare for Cry1Ac. However, resistance genes for Cry2Ab in field populations of moths are surprisingly common. Our current best estimate is that they occur for *H. armigera* at a frequency of 0.004 (upper limit, 0.011; lower limit, 0.0008) and for *H. punctigera* at a frequency of 0.009 (upper limit, 0.005; lower limit, 0.0001). Individuals that carry a resistance allele for Cry2Ab are killed by Cry1Ac.

Our current knowledge of the ecology and resistance profiles of Australian Helicoverpa populations suggests the RMP is adequate to retard increases in the frequency of resistance. Computer models that incorporate our present knowledge of resistance frequencies, fitness costs, form of dominance and refuge size, suggest that Bollgard II® should prove effective at managing *Helicoverpa* in the medium to long term. However, it should be emphasised that these models assume that refuges are well maintained in order to produce large numbers of susceptible moths.