Background

The formulation of strategies to manage the evolution of resistance to cotton containing Bt genes rely to a large extent on predictions made from models. Resistance models are invariably imperfect, as rarely are all parameters in the model known. Nevertheless they consistently indicate that when resistant genotypes are rare, the strongest influence on the rate of evolution of resistance is the degree of dominance of resistant genes. Dominance influences the survival of individuals that are heterozygous for a resistant allele (designated RS) relative to the survival of individuals that are homozygous for the susceptible allele (designated SS). As the degree of dominance of the resistant gene increases, so too does the range of concentrations of toxin that heterozygous individuals survive while homozygous susceptible individuals perish.

A Cry1Ac resistant colony (BX) has been isolated in *Helicoverpa armigera* by Ray Akhurst and colleagues in CSIRO Entomology. The allele that confers resistance to the toxin is incompletely recessive and essentially co-dominant at the LC_{50} value. Therefore, the potential exists for 'BX type' alleles to increase in frequency in cotton expressing Cry1Ac (Ingard®) through improved survival of RS individuals relative to the survival of SS individuals. Because the expression of Cry1Ac in Ingard® cotton declines as the plant matures, the advantage enjoyed by RS individuals relative to SS individuals is not fixed, but rather, should vary throughout the season. At the time the project started, we assumed (and subsequently confirm herein) that 'BX type' alleles, and thus homozygous resistant individuals (designated RR), were rare in the field. We therefore can ignore the contribution of RR individuals to the evolution of resistance. In this project we aimed to measure survival of the two genotypes, RS and SS, on leaf samples from field-grown Ingard® and conventional varieties of cotton. By performing repeated assays throughout the season, the occasions that favoured RS individuals, and thus the evolution of resistance to Cry1Ac, could be determined.

Our second aim was to examine the frequency of alleles that conferred resistance to Cry1Ac. During 2000-2002 Dr Ho Dang (NSW Dept. Ag.) detected changes throughout the season in susceptibility of *Helicoverpa armigera* as part of the Bt resistance monitoring program. During 2002-2004, similar results were also observed when the same program was continued by CSIRO Entomology. Of particular concern was an observed increase in frequency of survivors to a discriminatory dose of MVPII® (containing Cry1Ac) later in the season. Thus, initially we wished to establish if this change was due to the presence of major genes, and if possible, to capture these genes for further study. The proposed approach was to analyse survivors of discriminating doses obtained from the Bt resistance monitoring project (CSE102C). If present, potentially resistant alleles were to be isolated through crossing bioassay survivors (or their offspring) to a susceptible colony (GR). Sib-mating to produce F_2's were then planned to ensure that a proportion of individuals would be homozygous for potentially resistant alleles. Failure to "capture" such resistant alleles with this strategy would imply that the tolerance expressed is due to the contribution of multiple genes with additive effects.

However, when implementing this approach, survival and development to maturity of larvae following exposure to the MVPII® toxin was low. Consequently, this

approach was largely abandoned. Instead, we adopted an F_2 screen on field-collected material (Andow, 1998) that has several advantages over the discarded method, including the ability to provide precise information on the frequencies of rare resistant alleles.

Project objectives and summary of achievements

Objectives Year 1:

1. *Survivors of discriminating doses from a separate project submission to CRDC (Bt monitoring, CSE102C) will be further analysed and, if present, resistant alleles will be captured through the production of F_2's.*

   For the reasons outlined above this approach was abandoned in favour of F_2 screens. In this season, 34 *H. armigera* and two *H. punctigera* single pairs were processed. The BX form of Cry1Ac resistance was not found. The BX high-level resistance and its level of dominance would have been unmistakable. However, one isofemale line (SP15) exhibited marked resistance to Cry2Ab. Our preliminary evaluation of line indicated that it possessed 'high-level' resistance to Cry2Ab (with an LD_{50} in excess of 1000 fold that of susceptible lines). Importantly, the colony showed no cross-resistance to Cry1Ac

2. *Leaf samples from conventional and INGARD cotton to be collected from one location and used in bioassays (leaf disc). Both GR and F1's from the cross GR (susceptible) and BX (resistant) would be assayed on the leaf samples*

   This objective was completed successfully using Ingard® and other varieties of cotton grown at the Plant Breeding Institute, Narrabri. For Ingard® cotton varieties, it was evident that there were two distinct periods, the early and late parts of the season, where heterozygote individuals of the Cry1Ac resistant strain, BX, were favoured relative to susceptible individuals. Both periods would be important in causing an increase in frequency of the resistance allele in the population.

Objectives Year 2:

3. *Refine field sampling and assays, if necessary, based on experience gained in the previous year. Analyses of field population continued, with individuals carrying resistant alleles crossed to resistant strains already in culture to determine if resistant alleles are allelic with other forms of resistance.*

   F_2 screens were largely directed at detecting alleles that conferred resistance to Cry1Ac as for seven seasons the deployment of Ingard® may have caused such alleles to increase in frequency. Unexpectedly, from a small number of insects examined, an allele that conferred resistance to Cry2Ab was detected in 2002-03. An appreciation of the significance of this finding to the industry led to the provision of additional resources to this project (an additional technician for 6 months) by CRDC. The following modified objectives were proposed:-

a) *Obtain additional data from F_2's to improve our accuracy of the estimated frequency of this allele and any additional alleles that confer resistance to either Cry1Ac or Cry2Ab.*

   An additional 71 *H. armigera* and 15 *H. punctigera* pairs were examined. Again no alleles that conferred resistance to Cry1Ac were found, making a total of

104 *H. armigera* single pairs tested, representing 104 x 4 = 416 alleles. This confirmed that the field frequency of Cry1Ac resistance alleles was rare.

Two further isofemale lines that exhibited marked resistance to Cry2Ab were detected.

b) *Assay SP15 (Cry2Ab resistant strain identified during the first year) and F1's from the cross between GR (SS) x SP15 (RR), on leaf samples from conventional and Bollgard II® cotton collected from one location, using a leaf disc method.*

The resistance expressed by SP15 when grown on Bollgard II® appeared to be almost completely recessive but the Cry2Ab resistant homozygotes appeared to have a marked advantage over the susceptible strain on older Bollgard II®.

4. *Repeat leaf sampling and assays of individuals that are heterozygous for the BX resistance allele, and incorporate these findings into resistance models.*

The objective was completed successfully on Ingard® and other varieties of cotton grown at ACRI. The homozygous resistant genotype generally performed better on Ingard® than the heterozygote genotype that in turn performed better than the susceptible genotype.

Resistance models were developed that incorporate our findings on the frequency of resistance alleles to Cry1Ac and the relative fitness of individuals that are heterozygous for the BX allele that confers resistance to Cry1Ac. In collaboration with Dr Rick Roush, additional models are being developed to accommodate the unexpectedly high frequency of alleles that confer Cry2Ab resistance in *H. armigera* populations.

Methodology

1. Comparison of resistant and susceptible genotypes on field-grown Ingard® cotton.

1.1 The strains and genotypes employed.

On three occasions during the course of the project, individuals from the BX strain that is highly resistant to Cry1Ac were progeny tested to determine the gene frequency of the resistant allele within the colony. While many of the individuals were homozygous resistant (RR), other genotypes were also detected (Table 1).

Table 1.
Results of progeny testing of BX individuals

	RR	RS	SS	Total	Frequency of R
November 2002	58	4		62	0.97
November 2003	14	13	1	28	0.73
April 2004	10	19	0	29	0.67

The frequency of the resistant allele in the colony was similar on the two latter occasions but clearly the colony had been more rigorously selected prior to the first test. This lack of homozygosity has significant implications for the present study, because in the second season, only approximately 70% of the offspring from a cross between BX and a susceptible strain (ANGR) will generate heterozygotes. Nevertheless for most of the following discussion, for the three genotypes examined, BX are presumed to be mostly RR, and crosses between BX and ANGR were presumed to produce mostly RS, while ANGR was presumed to be completely SS.

Importantly, through a series of outcrosses and re-selection performed by colleagues in CSIRO Entomology (Ray Akhurst and Lisa Bird), BX and ANGR were made near-isogenic. The availability of isogenic lines was useful for this study as there should be little hybrid vigour generated among the F_1 when the two colonies are crossed. This is important as the outcome of assays using *H. armigera* larvae can be strongly influenced by their fitness.

1.2 The cotton tested

Plants were grown at the Plant Breeding Institute, Narrabri in the first year and at ACRI in the second year. During both years, the project benefited through a major contribution by Ms Cheryl Mares in arranging planting and maintaining the crops. Two Ingard varieties, Siokra V16i and Sicot 289i; two conventional varieties, Sicot 189 and Siokra V16; and two Bollgard II varieties, Sicot 289ix and Siokra V16ix were grown in four plots of each variety with plots situated in a random arrangement within the field. To sample cotton, the first fully expanded leaf from 20-30 plants in each plot were removed, arranged on damp paper, and packed in insulated containers. The containers were transported in a refrigerator set at 6^0 C to our Canberra laboratory where the leaves were used in assays within 30 h of harvest.

1.3 Assays on survival of the genotypes

Presumptive homozygote, heterozygote and susceptible neonate larvae were fed on portions of leaves from field-grown cotton in standard leaf-disc assays. A portion of the leaf was placed onto a cooled and set mixture of 2% agar and 0.1% sorbic acid, 5 ml per well in a 32-well assay tray. Assays were repeated at approximately fortnightly intervals throughout both growing seasons.

2. Investigation of field resistance

2.1 F_2 screen

Adults reared from field-collected eggs were mated as single pairs (one female mated by one male), and the offspring (F_1's) from these partnerships were mated among themselves to yield F_2 larvae. Eggs were collected specifically for use in the F_2 analyses, but were supplemented with eggs obtained through the Bt resistance monitoring program. Separate samples of neonate F_2 larvae were challenged with either Cry1Ac or Cry2Ab toxin. Each of the parents in the original mating pair contribute two 'alleles' or copies of each gene to the F_2 line. The mating scheme ensures that if one of the four alleles from the original mating pair conferred resistance, then a proportion (1/16) of the F_2 larvae would be homozygous (RR) for that resistant allele. An important feature of this method is that even totally recessive 'resistant alleles' will be detected. As most forms of resistance to Bt toxins in other Lepidoptera are recessive, or nearly so, this capacity provides an important

advantage over the commonly used discriminating dose technique to detect resistance.

The initial proposal envisaged searching for alleles that confer resistance to Cry1Ac. However, the technique required to produce F_2's is labour intensive and for a small increase in effort it was possible to simultaneously screen for Cry2Ab resistance.

2.2 Discriminating assay to isolate resistant F_2 individuals

In both seasons pure toxin (+ spores) was available for Cry1Ac assays. In the first season (2002 - 2003) a leaf extract was employed for Cry2Ab assays. An experimental transgenic variety of cotton 'V2X' that produces the single toxin Cry2Ab was grown in the laboratory and the leaves were harvested, homogenised and frozen in aliquots. This leaf material provided a reliable and stable source of Cry2Ab toxin for use in the F_2 screens. In the second season (2003 – 2004), 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 assays.

The toxin or leaf homogenate was prepared at a concentration that would kill approximately 95% of susceptible larvae, and then dispersed over the surface of artificial diet contained within testing wells. Mortality and growth of neonate larvae fed the diet plus toxin was assessed after 7 days. Separate assays were performed on samples of larvae from each batch of F_2 neonates for each toxin. In addition, a 'control' batch of neonates was reared on diet without the toxin. These animals would be used for rearing if we suspected from the assays that one of the original mating pairs carried a resistant allele.

Results

1. Comparison of Cry1Ac resistant and susceptible genotypes on field-grown Ingard® cotton

The objective of this component of the project was to examine how and when individuals carrying the BX allele that confers resistance to Cry1Ac were favoured in field-grown cotton, particularly on Ingard®. BX-like and other forms of resistance to Cry1Ac are rare in field populations of *H. armigera,* (see results from F_2 tests below). It follows that individuals that carry BX-like resistance alleles will almost exclusively be heterozygous. Homozygous resistant individuals are clearly so rare that their effect on the evolution of resistance can be ignored.

Thus a measure of the advantage that heterozygotes possess relative to susceptible individuals in the presence of Cry1Ac (Ingard®) reflects the intensity of selection tending to favour resistant alleles that might ultimately lead to field resistance. Thus, in our assays, periods where RS genotypes survive and thrive whereas SS individuals fail to do so represent periods of selection.

While the major focus of our research was the determination of the fitness of the three genotypes RR, RS and SS on Ingard®, other varieties of cotton (Bollgard II® and V2X) were available and included in the assays. However, during the course of the project a new Cry2Ab resistant colony (SP15) was isolated and with the imminent introduction of Bollgard II® it became clear that this form of resistance was of

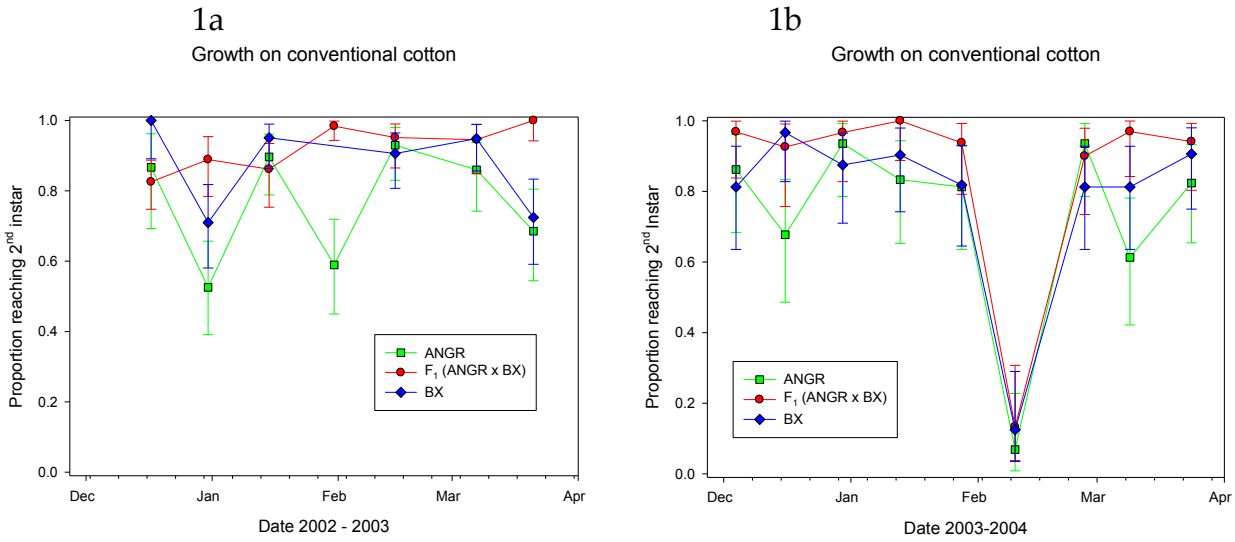
considerable significance to the Australian cotton industry. Thus it became important to assay the performance of this strain on varieties of cotton. During the second season both SP15 and 'heterozygotes' produced by crossing SP15 and ANGR were also tested in leaf-disc assays.

1.1 Selection for BX-like resistance

1.1.1 Growth of RR, RS, and SS on conventional cotton

In Figures 1a and 1b, and in subsequent figures, the proportion of test insects that reached at least the second instar when scored on day 7, and thus demonstrated survival and growth, are plotted with the asymmetric 95% confidence interval around that proportion. Differences in proportions are statistically significant wherever two confidence intervals do not overlap. This method is appropriate but is perhaps an overly rigorous test for differences. When more precise tests were required, proportions were compared in generalised linear models using the statistical package GLIM (Crawley, 1993) and binomial errors.

Figure 1a and 1b.
Performance of the three genotypes on conventional cotton for the 2002-03 (a) and 2003-04 (b) field seasons.



The two conventional varieties exhibited similar general trends and where valid throughout this report the results have been pooled to limit repetition. Support for combining data from varieties is provided by a GLIM analysis of the performance of ANGR during the 2003-04 season on the V16 series (V16, V16i and V16ix) and 189 series (189, 289i and 289ix). In this test we treated the genotypes (RR, RS and SS), series (V16 or 189), type of cotton (conventional, Ingard® or Bollgard II®) and sampling occasions as factors, and found that *type of cotton* is highly significant ($F_{(2,45)}$ = 40.66, P < 0.0001); and *occasion* was also highly significant ($F_{(7,36)}$ = 18.996, P < 0.0001), whereas *series* is not significant ($F_{(1,35)}$ = 1.724, P = 0.197).

The results of this same GLIM analyses were different for the first (2002 - 03) season. For these data pooling the V16 and 186 series is supported in a GLIM analysis for the conventional varieties ($F_{(1,6)}$ = 0.402, P = 0.549) however the factor *series* for both the Ingard® and Bollgard II® varieties exhibit significant differences ($F_{(1,6)}$ = 8.759, P =

0.025 and $F_{(1,6)} = 25.589$, P = 0.002, respectively). For this reason Ingard® and Bollgard II® varieties are shown separately for the 2002-03 season.

In the first year, on occasions ANGR exhibited diminished fitness (late December and late January), but generally all the genotypes thrived when fed conventional cotton. In the second season all the genotypes thrived with the marked exception of a single occasion (10th February 2004). Based on the clearly aberrant results presented in Figure 1b, and the failure of all genotypes to grow or survive on leaf discs of all varieties of cotton sampled on that occasion, it is probable that the whole plot had been contaminated through insecticidal spray drift. As a consequence, that sampling occasion has been deleted from all subsequent analyses and figures.

It is clear from Figures 1a and 1b that frequently (10 of 14 occasions where the comparison is possible) the performance of F_1's from the cross ANGR x BX (the presumptive heterozygous) exceeds that of either parental strain. Comparing the performance of the genotypes in GLIM models, it was necessary to treat the seasons separately, as the planting dates and sampling occasions did not align and there were clearly additional seasonal differences.

In the first season in a GLIM a model, the factor *occasion* was not significant ($F_{(6,11)} = 1.732$, P = 0.203) and nor was the factor describing *genotypes* ($F_{(2,11)} = 0.036$, P = 0.96). In the second year, in the model *occasion* was again not significant ($F_{(7,14)} = 0.703$, P = 0.669), however *genotype* showed significant levels of heterogeneity ($F_{(2,14)} = 7.072$, P = 0.008). As might be expected from Figure 1b, the heterogeneity was due to the presumptive heterozygotes that significantly outperformed both parental genotypes (a comparison of the heterozygote and ANGR [$F_{(1,7)} = 17.437$, P = 0.004], and between the heterozygote and BX [$F_{(1,7)} = 14.016$, P = 0.007]). Thus while BX and ANGR are near-isogenic, a measure of hybrid vigour is still present when the two strains are crossed. This subtly enhanced fitness must be taken into consideration when assessing subsequent analyses.

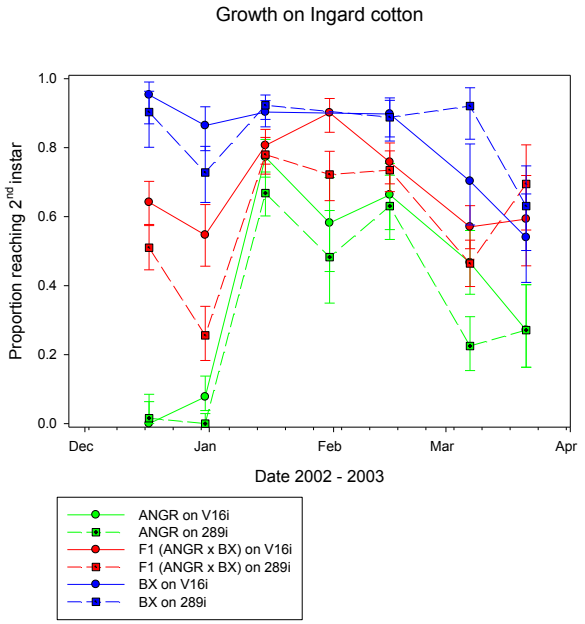
1.1.2 Growth of RR, RS and SS on Ingard® cotton

Few susceptible (ANGR) larvae thrived when fed leaves from Ingard® during the early part of each season (Figures 2a and 2b) but by January in both seasons, 40 – 60% of larvae grew to 2nd instar.

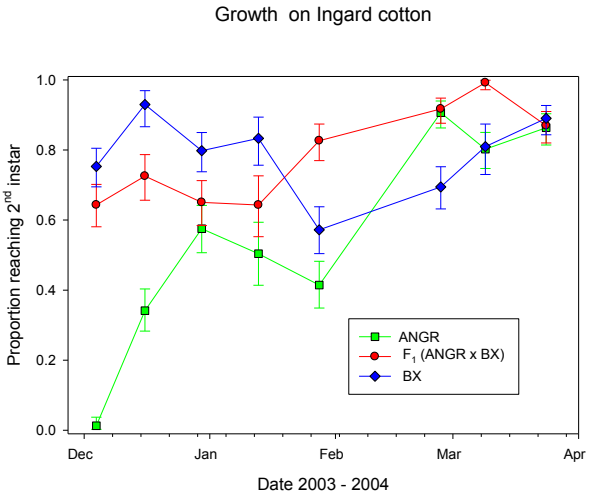
Figure 2a and 2b.

Performance of the three genotypes on Ingard® cotton for the 2002-03 and 2003-04 field seasons respectively.

2a.



2b.



In the first year, Ingard® regained efficacy later in the season. The recovery contrasts with the situation in the second season when efficacy of Ingard® continued to decline until ANGR survived at a similar rate as the two more resistant genotypes. The return to efficacy late in the season during the first year was unexpected. Perhaps in that year (but clearly not the second) additive effects of the intensifying plant secondary compounds in mature leaves, coupled with residual levels of Cry1Ac, was sufficient to affect survival of SS individuals. It would appear that the decline is neither a function of ANGR fitness nor of an increase in Cry1Ac titres as even the highly resistant RR genotypes also exhibited reduced levels of growth on the latter occasions (Figure 2a).

The low early season survival by ANGR was anticipated as many studies have found that Cry1Ac titres are maximal during this period. The extended efficacy in the first year (compare late December/January results for ANGR for both years) probably reflects the difference in planting dates and weather during the early months. In the first season, planting was delayed and in the second season, early growth was retarded due to a period of cold weather.

The presumptive heterozygote performed better than the susceptible homozygote (ANGR) throughout both seasons (Figures 2a and 2b). In 10 of the 15 occasions that direct comparisons are possible, the difference is significant (95% CI do not overlap) implying that the RS genotype exhibits improved growth on Ingard® for a large portion of the season. It should be noted that the magnitude of the improved performance of the heterozygote is far greater than can be attributed to improved fitness associated with hybrid vigour. If we assume that individuals exhibiting positive growth complete their larval stage and pupate, selection for increased frequency of the R allele should occur throughout the season. However, the rate of

selection will not be uniform throughout the season and intense selection would occur when the ratio of RS : SS survival is high. Clearly, in both years one such period occurs early in the season. While differences between the genotypes also occur at other times in the seasons, the extent of selection would be less than that occurring at the beginning of the seasons as the ratio begins to decline once significant numbers of SS survive.

The moderate period of differential survival late in the first season is likely to be particularly important, as this group of survivors, potentially enriched for RS genotypes, are likely to enter diapause and initiate populations the following spring. These results reinforce the importance of the resistance management strategy's requirement for the end-of-season 'pupae busting' and / or supplementary strategies to reduce the diapausing generation. Unfortunately, the strength of this message must be somewhat diluted, as in the second season the final generation of RS individuals would experience little or no advantage over SS individuals.

The presumptive homozygous resistant genotype generally performed better on Ingard than the RS genotype that in turn performed better than the SS genotypes (Figure 2a and 2b). This result confirms that the BX form of resistance is functionally partially dominant on Ingard®. However, as described above, the homozygous RR individuals are exceedingly rare, and for as long as they remain so, their influence in changing the frequency of the BX resistant allele in the field will be negligible.

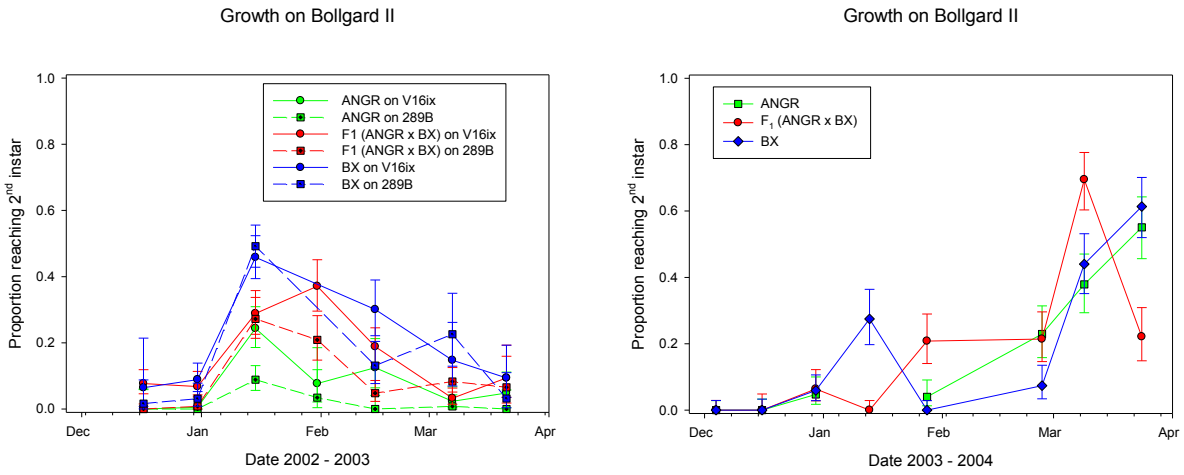
1.1.3 Growth of RR, RS and SS on Bollgard II® cotton

Now that Ingard® has been removed from the Australian environment, it is informative to determine if individuals carrying the BX form of resistance, especially as heterozygotes, are favoured in the more complex toxin environment found in Bollgard II®.

Figures 3a and 3b.

Summary of the performance of the three genotypes on 2 varieties of Bollgard II cotton for the 2002-03 (3a) and 2003-04 (3b) field seasons.

3a. 3b.



In both the 2002 - 03 and 2003 - 04 seasons all genotypes struggled on Bollgard II® (Figure 3a and 3b). In common with Ingard®, efficacy of Bollgard II® plants increased

late in the first season but declined in the second. Interestingly, both RS and RR individuals often exhibited better survival and growth than SS individuals, and on occasions the difference is significant. Clearly the advent of Bollgard II® should not diminish the respect that the Bt resistance management strategy pays to BX-like resistance. This result also emphasises the importance of monitoring changes in the incidence of both Cry1Ac and Cry2Ab resistance in *Helicoverpa* populations.

2. Comparison of Cry2Ab resistant and susceptible genotypes on field-grown cotton

The advantage conferred to individuals carrying a Cry2Ab 'resistant allele' (designated R_2 to differentiate it from BX-type genotypes) can be examined in a similar fashion using the methodology employed above. Unfortunately the data set for SP15 is limited because information is only available for the second season, and even then, as the strain had only recently been isolated, its availability on the weeks the assays were performed was patchy.

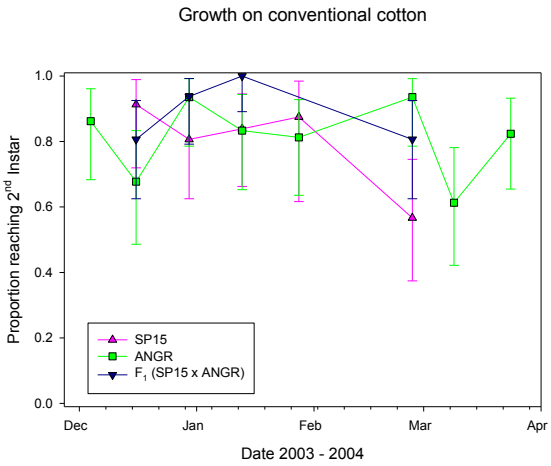
The SP15 colony was presumed to be homozygous for the resistant allele R_2. While this was not tested, it remains a reasonable assumption because the resistance appears to be largely recessive and therefore homozygosity was easy to establish after one or two generations of selection.

Unlike BX and ANGR that had been repeatedly crossed and were largely isogenic, SP15 and ANGR possessed different genetic backgrounds. As a consequence, the presumptive heterozygotes resulting from a cross between the two colonies may have generated even more hybrid vigour than was the case with ANGR and BX.

2.1.1 SP15 performance on conventional cotton

Survival and growth on conventional cotton to at least second instar of SP15, ANGR and the F_1 produced by crossing ANGR and SP15 were indistinguishable (Figure 4.)

Figure 4.
Growth of ANGR, SP15 and the F_1 of a cross between ANGR and SP15. The growth of ANGR on conventional cotton also appears in Figure 1b and is repeated here for comparison.

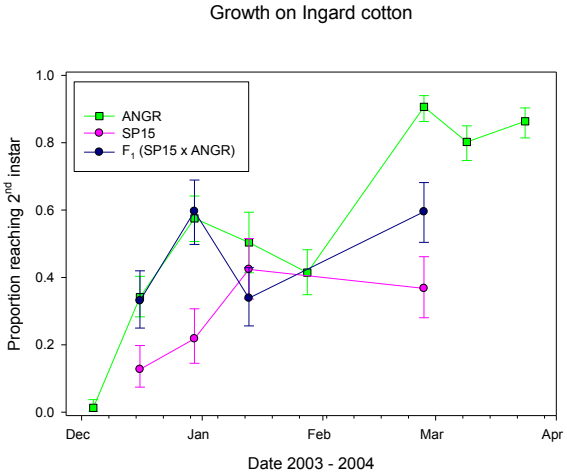


2.1.2 SP15 performance on Ingard® cotton

Laboratory analyses performed in CSE108C have shown that SP15 possesses no cross resistance to Cry1Ac. The results presented in Figure 5 support that conclusion as SP15 and the heterozygote exhibited no advantage relative to the susceptible insects (ANGR) when grown on Ingard®.

Figure 5.

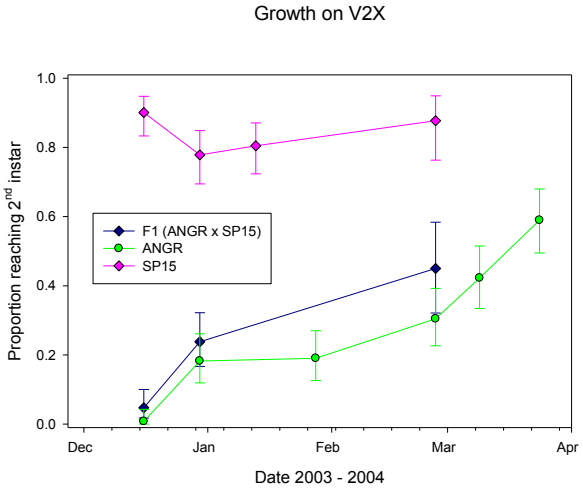
Growth of ANGR, SP15 and the F_1 of a cross between ANGR and SP15 on Ingard®. The growth of ANGR on Ingard® cotton also appears in Figure 2b and is repeated here for comparison.



2.1.3 SP15 performance onV2X cotton

The V2X transgenic cotton produces only Cry2Ab toxin. As SP15 in the laboratory can tolerate very high concentrations of Cry2Ab toxin it was not surprising that it thrived when larvae from that colony were fed leaves from V2X (Figure 6). Importantly, the heterozygote appeared only slightly more fit than the susceptible genotype and the difference was never significant. In a GLIM model comparing the performance of ANGR and the heterozygote over the three occasions the comparison is possible, the factor *occasion* was significant ($F_{(2,11)} = 38.362$, $P < 0.0001$) while the factor describing the two *genotypes* was not significant ($F_{(1,2)} = 5.600$, $P = 0.142$)

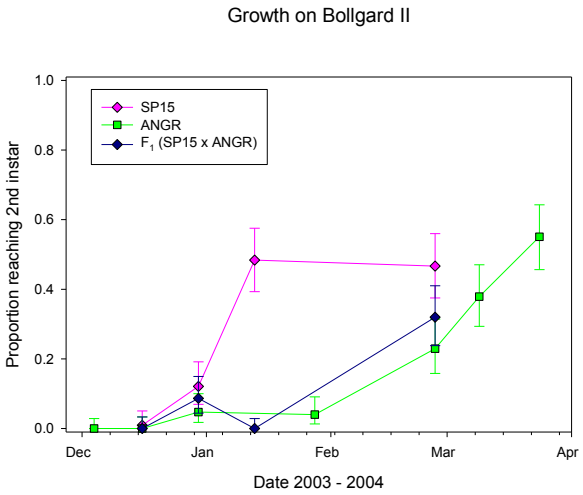
Figure 6.
Growth of ANGR, SP15 and the F_1 of a cross between ANGR and SP15 on V2X.



2.1.4 SP15 performance on Bollgard II®

Initially Bollgard II® prevented growth and survival of all genotypes (Figure 7). However, after January when presumably Cry1Ac titres had declined, significantly more homozygous R_2R_2 individuals survived and grew relative to ANGR larvae (Figure 7). The difference between the two genotypes remains significant in the late February sample ($F_{(1,13)}$ = 11.166, P = 0.005), although by then some growth was observed among ANGR larvae. In a similar fashion to growth and survival on V2X (Figure 6), the presumptive heterozygote exhibited a slight, but non-significant advantage relative to ANGR on Bollgard II® ($F_{(1,13)}$ = 11.937, P = 0.187). As discussed previously, this difference may reflect a fitness advantage acquired through hybrid vigour.

Figure 7.
Growth of ANGR, SP15 and the F_1 of a cross between ANGR and SP15 (R_2S_2) on Bollgard II®. The growth of ANGR on Bollgard II® cotton has been presented in Figure 4b and is repeated here to facilitate comparison with other genotypes.



2. Incidence of resistance alleles in field populations of *Helicoverpa*

In the 2002-03 season, 34 *H. armigera* and two *H. punctigera* single pairs were processed. In the 2003 - 04 season, a further 71 *H. armigera* and 15 *H. punctigera* pairs were examined. Of the 105 *H. armigera* pairs examined, 103 were screened for resistance to both toxins, but for the remaining two, only sufficient larvae were available to test a single toxin. One of these was tested for Cry2Ab, and the other was tested for Cry1Ac. All *H. punctigera* single pairs were screened for both toxins. The source of the eggs tested in both years is presented in Table 2.

Table 2. Number of single pairs examined and their source.

2002/03			
H. armigera		*H. punctigera*	
Location	No of pairs	Location	No of Pairs
Narrabri	8	Griffith	2
Wee Waa	7		
Goondiwindi	6		
Hilston	9		
Moree	1		
Griffith	3		
Total 02/03	34		2

2003/04			
H. armigera		*H. punctigera*	
Location	No of pairs	Location	no of pairs
Darlington Point	50	Moree	7
Narrabri	6	Wee Waa	3
Moree	15	Goondiwindi	3
		Emerald	2
Total 03/04	71		15

Three classes of isofemale lines were recognised when scoring F_2 larvae for resistance. The majority exhibited no exceptional levels of survival or growth and these were assumed to contain no 'resistant alleles'. Any surviving larvae present that had been subjected to toxin and the control larvae from this class were discarded.

A second class consisting of 20 isofemale *H. armigera* lines (12 in the first year, 8 in the second) exhibited slightly better survival and growth among larvae than was expected from SS individuals, and thus perhaps indicated the presence of low-level tolerance. For this group, the control larvae and the surviving larvae were retained and reared for subsequent generations and re-tested to isolate any 'resistance' present. One of these lines was tested on multiple occasions over a period of 12 months, but eventually it and all others in this class, was deemed to be susceptible and discarded.

The final class consisted of 3 isofemale *H. armigera* lines that contained a proportion of individuals that clearly thrived on their toxin-contaminated diet. For this class, all larvae including survivors of the screen and the 'control larvae' that had not been exposed to toxin from these lines were reared through to pupation and colonies established from the line.

No alleles that conferred resistance to either toxin were isolated among the 17 *H. punctigera* single pairs tested, representing 17 x 4 = 68 alleles.

No alleles that conferred resistance to Cry1Ac was found among the 104 *H. armigera* single pairs tested representing 104 x 4 = 416 alleles. Thus, 95% confidence interval around the estimated frequency of Cry1Ac resistance alleles would exclude frequencies more common than 0.0088.

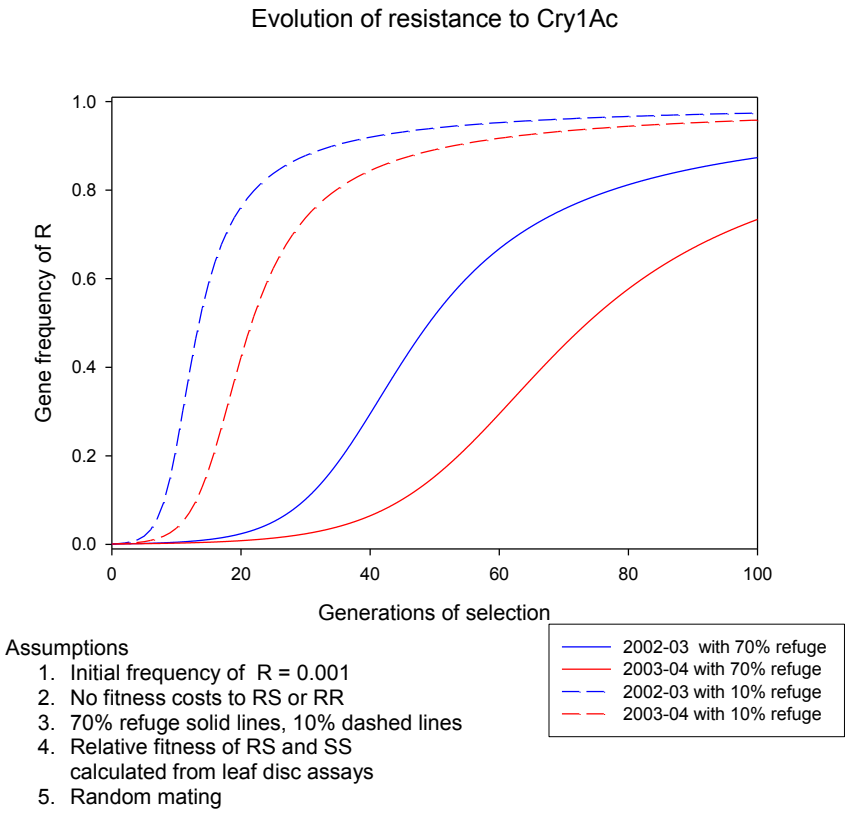
Three isofemale lines exhibited marked resistance to Cry2Ab. The first line (from the 15th single pair, called SP15) was identified in the 2002-03 season from eggs collected from maize near Griffith NSW. The second isolate (SP202) was established from eggs collected from Darlington Point/Coleambally in southern NSW. Analysis of this isolate is incomplete, but preliminary data indicate that it is allelic with SP15, and thus likely to share some of SP15's characteristics. The final resistant isolate, SP566 was derived from a sample of eggs collected at Moree. SP566 has only recently (August 2004) been established, and at present little information is available, however preliminary analyses also suggest that it is also allelic with SP15.

Conclusions

Selection for Cry1Ac resistance

We can use the information obtained during this project to improve our models of the evolution of resistance of *Helicoverpa* to Cry1Ac present in Ingard®. Firstly, based on our observations from F_2 screens, we can be confident that the frequency of alleles that confer resistance to Cry1Ac are rare, and we assign a nominal frequency of 0.001 to R. This value was previously unknown and because we have not encountered Cry1Ac resistance in the F_2 tests (see below) the value of 0.001 is likely to be conservative. We can also derive from the survival and growth of RS and SS summarised in Figures 2a and 2b, an estimate of the relative survival of each genotype throughout the season. The model requires a single value for the fitness of RS and SS that reflects an 'average' value of fitness that is applied to each generation. The simplest comparison of the genotypes would be to average the relative fitness for the two genotypes obtained on each occasion. Accordingly, the relative fitness on Ingard® of SS is 0.502 and 0.658 that of RS and in the first and second years respectively. Appling these values to a single gene model, the changes in frequency of R from a starting frequency of 0.001 can be seen in Figure 8.

Figure 8.
Model of the change in frequency over time of an allele that confers resistance to Cry1Ac using information from this project. Some of the various assumptions made in this model are detailed within the figure.



Modellers traditionally assume that resistance has evolved when the frequency of R reaches 0.5. Assuming the refuge of 70% (as has applied with Ingard®) from Figure 8, the model would predict that resistance would be reached in 51 generations under the selection regime on Ingard® encountered in 2002 – 2003 and 75 generations in 2003-2004. The importance of a refuge can be seen when the model is re-run after changing the size of the refuge to 10%. It must be appreciated that while the data generated in this study allows far greater precision in our models than was hitherto possible, there remain several unknown parameters and assumptions. One of the assumptions in the parameters used in the relative fitness of RS and SS genotypes is that insects that survived and grew to at least 2nd instar would, if continually fed, pupate and emerge as viable adult moths. We consider this a reasonable assumption because once the larvae have reached 2nd instar they would be capable of moving to less toxic portions of the cotton plant (e.g., undamaged leaves, flowers or the bolls).

Frequency of resistant alleles

Despite the deployment of Ingard® for the previous seven seasons, none of the 416 alleles scored from field-collected *H. armigera* in this study conferred resistance to Cry1Ac. The BX form of high-level resistance and its associated significant level of dominance would have rendered it unmistakable among the F_2 larvae. This result is especially significant given that we identified opportunities for individuals that are

heterozygous for the resistant allele to be favoured on Ingard®. The rarity of such alleles can be due to several causes including fitness costs and features of the ecology of the pest. However there can be little doubt that the rarity of Cry1Ac resistant genotypes (including the BX-form) is in part due to the efficacy of the resistance management strategy that has been in place since the introduction of Ingard®.

While the F_2 technique was expected to identify 'resistant alleles' if sufficient single pairs were tested, the detection of three separate instances of Cry2Ab resistance among the limited number of alleles examined was certainly unexpected. It was especially surprising as Bollgard II® has not been widely deployed. Indeed, the first isolate (SP15) was detected in 2002-03 when no commercial crops had been grown. From preliminary analyses, it would appear that all three isolates represent mutations (that may or may not be identical) at the same locus. From our data, the calculated frequency of mutant alleles at that locus in *H. armigera* is 3/416 = 0.007. Confidence intervals (95% CI) around that estimate would place the actual proportion in the range 0.0014 to 0.0209.

It is not yet possible to explain why resistance to Cry2Ab is common. In the absence of selection, the frequency of mutations in a natural population is a function of the mutation rate and the intensity of selection operating against the mutant allele, ie -

$$q \approx \frac{\mu}{s}$$

Where
q = frequency at equilibrium
u = mutation rate
S = selection coefficient against mutant state

Mutation rates at a single gene are generally assumed to exist at a frequency of one in 10^{-5} to 10^{-8}. Therefore, even in the total absence of fitness costs, a measure of positive selection would be required to bring about the observed frequency of 0.007. It has been speculated that perhaps natural soil-borne *Bacillus thuringiensis* that express Cry2Ab has been the source of such selection. This hypothesis suffers from a contradiction because in Australian soils far more *Bt* express Cry1Ac than express Cry2Ab (Ray Akhurst pers. com.), yet Cry1Ac resistance appears to be rare. Perhaps an explanation for the elevated frequency will become evident when we learn more about the mechanism that renders insects resistant and the fitness costs associated with resistant genotypes (to be examined in a new project funded by CRDC) or the genetic basis of the resistance (CSE108C).

Our current knowledge of the characteristics of SP15 will be presented in detail in our progress report for another project (CSE108C). In brief, it is clear that the strain exhibits 'high-level' resistance to Cry2Ab (with an LD_{50} in excess of 1000 fold that of susceptible lines). Importantly, the colony shows no cross-resistance to Cry1Ac. Further, the resistance appears to be due to a single gene, and in laboratory tests is almost recessive, or perhaps completely recessive.

The information available on Cry2Ab resistance indicates that it has the potential to reduce the useful lifespan of Bollgard II®. However further information is required in several areas to assess that threat. Apart from the initial detection of resistant alleles

and the determination of their frequency, two additional pieces of information were added in this project.

Firstly, the resistance expressed by SP15 when grown on Bollgard II® and V2X appeared to be almost completely recessive, and secondly, the Cry2Ab resistant homozygote (R_2R_2) appears to have a marked advantage over susceptible ANGR on older Bollgard II®. The recessive nature of the resistance is important, as given the existing frequency of the 'resistant' allele, any heterozygous advantage would ensure rapid increase in the frequency of the resistant form of the gene. This increase would occur even in the presence of significant fitness costs.

Clearly, estimating the costs and benefits to fitness will also be important; the greater the fitness cost to resistant genotypes, the slower the increase in the frequency of the resistant allele. Unfortunately, we hypothesise that without obvious selective forces for resistance to Cry2Ab in the field (at least prior to the widespread deployment of Bollgard II), fitness costs are likely to be minimal. Otherwise, we would not expect natural populations of *H. armigera* to sustain the observed frequency of 'resistant' alleles.

It is of prime importance is to confirm (or otherwise) the recessive nature of the resistance. The development of a near-isogenic susceptible strain will minimise any boost in fitness through hybrid vigour, and the necessary outcrossing and re-selection is underway. Our inference of near-recessive nature of the resistance on V2X and older Bollgard II® agrees with laboratory assays suggesting slight dominance, although in this latter case it is also difficult to rule out hybrid vigour.

Even if it proves to be completely recessive, the SP15 form of resistance may not be benign. We have shown that homozygous (R_2R_2) larvae possess a marked advantage over S_2S_2 larvae on Bollgard II® and the 'resistant' allele can perhaps increase in frequency through selection for the (R_2R_2) genotype. While the rarity of individuals that are heterozygous for Cry1Ac make it unlikely that they would drive an increase in frequency of that resistant allele, the same is not true for Cry2Ab resistance. Because heterozygotes are relatively common, homozygous Cry2Ab resistant individuals would be expected to occur when population sizes are very large. Their frequency can be estimated as the square of the gene frequency, i.e., $(0.007)^2$ = 0.000052 or 52 individuals in every million moths. While this frequency clearly is still rare, the incidence of these genotypes is of concern especially as we have shown that selection for this genotype appears to be possible on Bollgard II®.

Models of the evolution of resistance developed by Roush (1998) indicate that the longevity of a pyramid (e.g., Bollgard II® that posses two toxins) is sensitive to the frequency of resistance alleles at the time pyramids are introduced. To date, the thrust of the resistance management strategy concerning Bt cotton (Ingard®) has been to maintain Cry1Ac resistance alleles at low frequencies until a pyramid became available. Our data from the F_2 screens indicate that this objective has been achieved. It is unfortunate that resistance levels to the second component in the pyramid are unexpectedly high.

An undesirable scenario would be that at some time in the future, resistance to Cry2Ab became widespread in *H. armigera*. In that situation, in the absence of fitness

costs associated with Cry2Ab resistance, Bollgard II® would perhaps become equivalent to Ingard® in its effectiveness with only Cry1Ac controlling *H. armigera*. While the deployment of Ingard® for seven years has not brought about detectable levels of resistance, the Resistance Management Strategy has recently changed. Instead of a 70% refuge, the current RMS for Bollgard II® stipulates a 10% refuge. Using our current estimates of parameters in a single gene model (Figure 8), a 10% refuge might only provide protection for less than 20 generations or approximately 5 years. Clearly careful note should be made in the coming seasons to changes in frequencies of resistance to both Cry1Ac and Cry2Ab. It is also critical to establish the magnitude of fitness costs to Cry2Ab that may prevent this scenario from occurring.

Economic, Environmental and Social impacts

Transgenic cotton is expected to play an important role in ensuring the sustainability of the cotton industry through the reduction of insecticides and improved economic returns to cotton growers. The technology will fail if resistance evolves to the toxins present in transgenic cotton. These studies aimed to provide information on the frequencies of alleles that confer resistance to Cry toxins, and to improve our knowledge of possible routes that the processes associated with the evolution of resistance may take. An outcome will be the provision of information that will enable the refinement of the Resistance Management strategy in order to extend the lifespan of the technology.

Publications arising from the research project and publication plan

The results achieved during this project were presented to a Cotton Consultants meeting in Narrabri, August 2003. A paper entitled "Evolution of Resistance to Bt in *Helicoverpa*." by Rod Mahon, Karen Olsen, Su Young and Kim Garsia accompanied the presentation to the cotton consultants.

The results of the F_2 screens and the available data on the Cry2Ab resistant colony have been presented to the TIMS Bt tech panel and growers and consultants during the 'resistance roadshow'.

A poster on our F_2 work to detect resistance to Cry toxins was presented at the 2004 Cotton Conference, and at the International Congress of Entomology, 2004.

The results of research are being prepared for publication in international scientific journals.

Impact of the results and conclusions for the cotton industry

A major conclusion of this research is that significant numbers of *H. armigera* carry alleles that confer resistance to Cry2Ab present in Bollgard II®. While alleles that confer resistance to Cry1Ac appear rare, opportunities for both forms of resistance to increase in frequency have been identified.

On firm theoretical grounds, it was previously considered that a crop that expressed two insecticidal genes that had different modes of action against the pest would be

very resilient to the evolution of resistance by that pest. Given a gene frequency of 0.007 for alleles that confer resistance to Cry2Ab, the resilience of the pyramid might be tested. Early detection of the frequency of resistance in *H. armigera* populations with follow-up monitoring will enable the detection of changes in frequency. As Bollgard II® represents an extremely valuable asset to the Australian Cotton industry, if frequency changes are detected, modification to the resistance management plan may be appropriate if such changes are likely to extend the usefulness of the technology.

Executive Summary

This project examined how and when individual *Helicoverpa armigera* carrying a resistant allele (BX) were favoured in field-grown cotton, particularly on Ingard®. BX-like and other forms of resistance to Cry1Ac were found to be rare in field populations of *H. armigera*. It follows that individuals that carry BX-like resistance alleles will almost exclusively be heterozygous. We measured the survival and growth of heterozygous larvae and homozygous susceptible larvae on leaf samples from field-grown Ingard® and conventional varieties of cotton. By performing assays throughout the season, we were able to identify occasions that favoured heterozygotes and thus the evolution of resistance. Averaging relative survival rates of heterozygotes and homozygous susceptible genotypes over the 2002 - 03 and 2003 - 04 seasons we found that, over the first season, susceptible individuals would have survived and grown only half as frequently as the heterozygote. In the second season susceptible individuals fared slightly better surviving 0.68 as well as the heterozygote. Computer models were prepared that incorporated this information as well as new information on the frequency of Cry1Ac resistance in the field.

A second aim of the project was to detect and isolate alleles that conferred resistance to Cry1Ac and Cry2Ab in field populations of *Helicoverpa armigera* and *H. punctigera*. An F_2 screen technique was employed that enabled the calculation of the frequency of alleles that confer resistance, and importantly, enabled the detection of recessive alleles. No instances of resistance were detected among 68 alleles scored for Cry1Ac and Cry2Ab in *H. punctigera.* Similarly, no instances of resistance to Cry1Ac were detected among 416 alleles scored for *H. armigera.* For this species, 95% confidence intervals around the zero determine that Cry1Ac resistance alleles would not be more common than a frequency of 0.0088. Thus despite the deployment of Ingard® for seven seasons, Cry1Ac resistance remains rare.

However, a surprising result was that resistance to Cry2Ab, the second toxin present in Bollgard II®, is relatively common. For *H. armigera,* three instances of resistance to Cry2Ab were isolated from 416 alleles examined. Preliminary analyses suggest that all three isolates represent mutations at the same locus. While the F_2 technique was expected to identify 'resistant alleles' if a sufficient number were tested, the expectation was that prior to the deployment of Bollgard II®, Cry2Ab resistance would be at a frequency approaching the mutation rate, say 10^{-5} to 10^{-8}.

With the imminent wide-scale deployment of Bollgard II®, it was important to assess the magnitude of the threat posed to Bollgard II® by the previously unsuspected resistance to Cry2Ab. Unlike the situation for Cry1Ac resistance, where resistant alleles are rare, for the more common Cry2Ab resistance it was important to examine the fitness of both heterozygotes and homozygotes. Laboratory analyses performed in CSE108C have shown that Cry2Ab resistant insects are fully susceptible to Cry1Ac. Thus it is not surprising that early in the season all larvae struggled on Bollgard II®. However later in the season when titres of Cry1Ac presumably had declined, homozygous Cry2Ab resistant larvae (but perhaps not heterozygous larvae) exhibited enhanced growth on Bollgard II® relative to susceptible insects. Thus although the resistance appears to be functionally recessive on Bollgard II®, opportunities exist for the homozygote to be favoured. Together these results suggest that this form of resistance may inpact on the longevity of Bollgard II®.