Resistance and Refuges

Sharon Downes[1], Rod Mahon[2], and Karen Olsen[2]
CSIRO Entomology, Cotton Catchment Communities CRC, ACRI, Narrabri[1] ,
CSIRO Entomology, Black Mountain, Canberra[2]

Introduction

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 pests *Helicoverpa armigera* and *H. punctigera* but excluding natural predators and parasitoids. To prolong the efficacy of transgenic cotton against Helicoverpa species, a resistance management plan (RMP) was implemented. This plan was largely based on information from studies of the ecology and population genetics of *H. armigera*, 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 value of 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, thereby restricting the opportunity for moths to adapt to the toxin. Growers were also required to plant conventional crops alongside transgenic fields—these "refuges" harbor susceptible moths that should mate with potentially resistant individuals from the INGARD®, thereby diluting resistance in the population.

In the 2004/05 season Bollgard II® replaced INGARD® as the transgenic variety of cotton available to Australian growers. It improves on INGARD® by incorporating an additional insecticide toxin (Cry2Ab) to combat Helicoverpa. Due to the perceived difficulty for this pest to evolve resistance to both toxins 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. Growing a dedicated refuge crop remained a critical requirement of the license for this Bt-crop. Bollgard II® has been well adopted, with an average of 70-80% planted area throughout the industry in the past two seasons. Given the increased opportunity for moths to adapt to transgenic cotton, the enforced RMP is being 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 *H. armigera* and *H. punctigera* to Bt products was assayed before and subsequent to the widespread deployment of INGARD® cotton expressing Cry1Ac in the mid-1990's. During 2002/03, 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 the Bollgard II® varieties of cotton, but also for the long-term future of cotton varieties expressing *cry1Ac* or *cry2Ab* in combination with other effective toxins.

An update on resistance monitoring for Bt

Most cases of resistance to Bt by Lepidoptera are at least partially recessive (Ferre and Van Rie 2002). A recessive resistance means that individuals carrying two resistance alleles (RR) are not killed but those carrying one resistance allele and one susceptible allele (RS) are killed. It is therefore possible for field populations to comprise individuals that carry resistance alleles as heterozygotes (RS) but are killed by Bt.

F_0 screens for Cry1Ac and Cry2Ab resistance

We use standard F_0 screens to detect major changes in resistance frequencies. This method tests the generation of insects collected directly from the field (Andow & Ives 2002). If resistance is recessive, F_0 screens will detect only individuals that have two resistance alleles (RR). This method is time effective which enables samples from many areas to be tested. Around 2% survival of susceptible insects is expected as a baseline for the doses of toxins used in the F_0 screens.

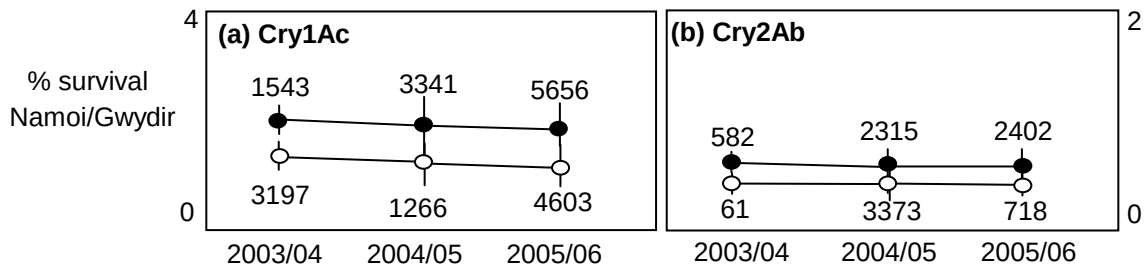
In assessing the data from the F_0 screens we are most interested in two features that would allow us to detect incipient resistance: (1) shifts in the survival of Helicoverpa among seasons and (2) shifts in the survival of Helicoverpa within seasons. The analyses on possible shifts are restricted to data from the Namoi/Gwydir region because it was only from this area that we have good samples of insects to enable a rigorous analysis for both issues.

Shifts in survivorship of Helicoverpa among seasons

Recently there have been changes to the methodology used in the F_0 program, including the products used in the screens. Thus, although data exists for 10 years on the proportion of individuals surviving F_0 screens, here we restricted our comparisons to the last 3 years.

We divided the total number of individuals surviving screens by the total number of individuals tested in that season. For the past three seasons there has been no marked difference in the proportion of *H. armigera* individuals surviving the F_0 screens for Cry1Ac (Figure 1a) and Cry2Ab (Figure 1b), or in the proportion of *H. punctigera* individuals surviving the F_0 screens for Cry1Ac (Figure 1a) and Cry2Ab (Figure 1b). Therefore, our data from the F_0 screens do not indicate any major changes from previous seasons in survival rates to Cry1Ac or Cry2Ab.

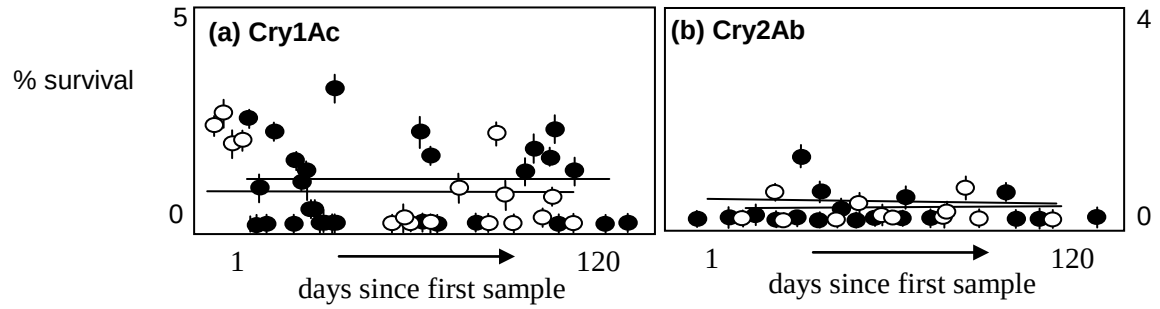
Figure 1: The proportion (expressed as a percentage) of individuals that survived the F_0 screens in the past 3 seasons. Data are presented separately for (a) Cry1Ac and (b) Cry2Ab. Data for *H. armigera* are in black, whereas those for *H. punctigera* are in white.



Shifts in survivorship of Helicoverpa within seasons

To look at trends within seasons, 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. We then calculated the median time period for that sample and plotted the data using the numbers of days from the first sample of the season as the measure of time. During 2005/06 there was no statistically significant change during the season in the proportion of *H. armigera* individuals surviving our screens to Cry 1Ac ($F_{1,28}$ = 1.5, P > 0.05: Figure 2a) and Cry2Ab ($F_{1,17}$ = 0.49, P > 0.05: Figure 2b), or in the proportion of *H. punctigera* individuals surviving Cry 1Ac ($F_{1,16}$ = 0.97, P > 0.05: Figure 2a) and Cry2Ab ($F_{1,12}$ = 1.0, P > 0.05: Figure 2b).

Figure 2: The proportion (expressed as a percentage) of individuals surviving the F_0 screens in 2005/06 plotted against days from first sample. Data are presented separately for (a) Cry1Ac and (b) Cry2Ab. Data for *H. armigera* (n = 60/sample) are in black, whereas those for *H. punctigera* (n = 40/sample) are in white.



F_2 screens for Cry1Ac and Cry2Ab resistance

We use F_2 screens to estimate the frequencies of rare resistance genes in field populations. This method screens the "grandchildren" (F_2 generation) of two field-derived parents. A proportion of the grandchildren will carry two copies of each gene present in their grandparents, which means that if either grandparent carries a single 'resistant gene' (a heterozygote, RS), a small proportion (1/16) of F_2's will be homozygous resistant (RR) and survive our screen (Andow and Ives 1998).

At the time that each F_2 family is tested, we set aside a subset of insects in a cool room. 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 at the CSIRO Entomology site at Black Mountain.

We incorporated F_2 screens into the monitoring program in 2002/03. Since then we have screened around 1700 alleles (forms of genes) of *H. armigera* against Cry1Ac and Cry2Ab, and 1600 alleles of *H. punctigera* against Cry1Ac and Cry2Ab (Tables 1 and 2).

Frequency of alleles conferring resistance to Cry1Ac

We have not isolated any alleles in *H. armigera* or *H. punctigera* that confer resistance to Cry1Ac (Table 1). Therefore the frequency of alleles conferring resistance to Cry1Ac remains low for both species, certainly at less than 1 in 1600.

Table 1: Summary of results from F_2 screens for Cry1Ac resistance

Species	Season	No. alleles	No. resistant
Helicoverpa armigera	2002/03	136	0
	2003/04	280	0
	2004/05	364	0
	2005/06	900	0
	TOTAL	**1680**	**0**
Helicoverpa punctigera	2002/03	8	0
	2003/04	60	0
	2004/05	1012	0
	2005/06	468	0
	TOTAL	**1548**	**0**

Frequency of alleles conferring resistance to Cry2Ab

We have isolated 7 alleles from *H. armigera*, and 1 from *H. punctigera*, that confer resistance to Cry2Ab (Table 2). Of these 8 cases, none exhibited cross-resistance to Cry1Ac.

These data suggest that the frequency of alleles in *H. armigera* conferring resistance to Cry2Ab is 0.004 or 4 alleles out of every 1000 screened. There is no discernable trend between years, but the low frequencies limit our ability to identify slight changes. However, marked changes, or a sustained incremental change, will be detected using this technique.

The data suggest that the frequency of alleles in *H. punctigera* conferring resistance to Cry2Ab is low, at around 0.0006 or 1 in every 1560 screened.

Table 2: Summary of results from F_2 screens for Cry2Ab resistance

Species	Season	No. alleles	No. resistant
Helicoverpa armigera	2002/03	132	1
	2003/04	284	2
	2004/05	368	0
	2005/06	900	4
	TOTAL	**1684**	**7**
Helicoverpa punctigera	2002/03	8	0
	2003/04	60	0
	2004/05	1024	1
	2005/06	468	0
	TOTAL	**1560**	**1**

Late season Helicoverpa survival in Bollgard II® crops

During the 2005/06 season we received for testing 321 Helicoverpa larvae that were collected on Bollgard II®. Of these larvae, 44% were at least 'medium' in size. Bollgard II® may be less effective at killing Helicoverpa once they reach the medium stage. Thus, it is possible that up to 66% of the larvae submitted would have been killed by Bollgard II® had they not been removed.

Of the 'survivors' submitted, 146 (46%) were reared on artificial diet in the laboratory to healthy moths. All of the moths were assigned to the F_2 screen component of the monitoring program and 85 lines were screened against Cry1Ac and Cry2Ab. Two of the *H. armigera* lines scored positive for an allele conferring resistance to Cry2Ab and are included in the frequencies mentioned above.

There are several reasons why it is unlikely that the larvae found during the season on Bollgard II® were able to survive as a result of resistance. As mentioned above, an overwhelming proportion (98%) of the survivors carried no resistance alleles. The two individuals carrying one allele conferring resistance to Cry2Ab would also be fully susceptible to Cry2Ab as the resistance is recessive (i.e., insects need two copies to be functionally resistant; Mahon et al. 2006). Furthermore the Cry2Ab resistance does not confer cross-resistance to Cry1Ac (data presented above), thus the two insects 'carrying' the allele would have been as susceptible to a combination of both toxins as homozygous susceptible (SS) insects. We also know from associated work that the larvae contributing to these lines were collected off Bollgard II® plants that scored positive using qualitative ELISA tests for Cry1Ac and Cry2Ab (see also below). The remaining 'survivors' which carried no resistance alleles also managed to survive for some time on Bollgard II® plants that were scored as positive using qualitative ELISA tests for at least one of the toxins.

If the 'survivors' were not functionally resistant, then how did the larvae live on Bollgard II®? The most likely explanation is that at the time that larvae were found in Bollgard II® crops, especially when large larvae were present, the level of toxins in the plants was not sufficiently high to rapidly kill Helicoverpa larvae.

In the same project we screened the Bollgard II® plants associated with live larvae using a qualitative ELISA test for Cry1Ac and Cry2Ab. All of the Bollgard II® plants that were hosts, or nearby hosts, of surviving Helicoverpa larvae, scored positive for at least one of the two Bt toxins. In 72% of cases, these plants scored positive for both toxins within Bollgard II®.

We are planning work to examine another possible reason for the survival of larvae on Bollgard II® plants. Work on the behaviour of the pink bollworm (*Helicoverpa zea*) shows that first instar larvae are observed significantly more often in the white flowers of Bollgard II® cotton compared with the white flowers of conventional cotton (Gore et al. 2002). The pollen within Bollgard II® does not express Bt proteins. Therefore, it is possible that larvae are preferentially feeding on these areas until they reach a stage where they can survive a dose of Bt from the Bollgard II® plant.

It is important to note that the ELISA work mentioned above was done only on plants that were associated with live larvae and therefore is likely to be biased towards plants that have reduced levels of the toxins. In addition, we do not know the total number of plants from which these parts were taken since most samples from the same property were received as a bulk package. We therefore cannot determine from our data the percentage of plants in the field that carried larvae or scored positive or negative for Bt toxins in the ELISA tests. The results from the ELISA tests are specific to this study and cannot be applied more generally.

Is our current RMP adequate?

To assess whether the current RMP is adequate we need to consider the characteristics of resistance in field populations as well as current information on the efficacy of refuges.

Resistance in field populations

Frequencies of resistance alleles in field populations

The duration of the period of efficacy of an insecticide product strongly depends on the initial frequency of resistance alleles: the lower the frequency, the more sustainable the strategy (Wenes et al. 2006).

The data from the resistance monitoring program show that the frequencies of alleles conferring resistance to Cry1Ac remains low in field populations. A frequency of 4 in 1000 for alleles conferring resistance to Cry2Ab in *H. armigera* is higher than expected. Importantly, the first isolation of a 'resistant gene' was made prior to the introduction of Bollgard II®. Presently we have no evidence that the frequency of alleles conferring resistance to Cry2Ab is increasing.

The isolation of two Cry2Ab resistant genes from larvae collected during what has been a 'high pressure' year for Helicoverpa is consistent with the frequency found previously. Nevertheless, any survivors on Bollgard II® are of concern. We will continue to pay close attention to the resistance status of 'survivors'.

Cross-resistance between Cry1Ac and Cry2Ab

If cross-resistance between Cry1Ac and Cry2Ab exists then the current pyramid in Bollgard II® will function as a single gene. Our research on SP15 indicates that neonate larvae exhibit significant cross resistance to the very similar Cry2Aa toxin but are as susceptible as a Bt-susceptible laboratory colony, or more so, to Cry1Ac toxin and to the mixture of Cry toxins in the formulated product DiPel (Mahon et al. 2006).

In our F_2 screens we are usually able to simultaneously screen families against Cry1Ac and Cry2Ab. All of the lines identified as carrying an allele conferring resistance to Cry2Ab were scored as negative for alleles conferring resistance to Cry1Ac.

Fitness costs associated with the resistance

Another factor influencing the evolution of resistance is the fitness cost – the decrease in fitness associated with the resistance gene in the absence of selective pressure – associated with some pesticide resistance alleles (reviewed in Wenes et al. 2006). If resistance entails a fitness cost, the spread of a recessive allele could be prevented by an appropriate RMP.

We are presently actively examining SP15 for the presence of fitness costs. Preliminary evidence suggests that in the laboratory the resistant colony is vigorous. Thus if present at all, fitness costs of resistant individuals are likely to be minor. If so, they are unlikely to have a major effect on the evolution of resistance.

Dominance of the resistance

The trajectory over time of the frequency of alleles that confer resistance under a selection regime is very strongly influenced by the degree of dominance, with resistance frequencies increasing rapidly if heterozygotes are advantaged (Roush 1997).

Our research on SP15 indicates that the form of Cry2Ab resistance present in this strain is completely recessive (Mahon et al. 2006). Our bioassay data indicate that the lines scored as positive in 2005/06 also show a recessive form of resistance. This means that the insects that we determined to carry an allele conferring resistance to Cry2Ab were as likely to be killed by fully-expressing Bt plants as Bt-susceptible larvae.

Efficacy of refuges

A critical assumption of the refuge strategy is that resistant insects selected on transgenic crops mate randomly, and hopefully, with susceptible insects produced on non-transgenic crops. These could be crops grown specifically as the mandated refuge, native vegetation or other crops in the area (e.g., grains). Since resistance to Bt is likely to be recessive, this strategy would act to dilute resistance by ensuring that functionally resistant RR individuals mate with a SS individual to produce heterozygote offspring (RS) that are functionally susceptible.

Data collected over the past four seasons, which analyse the origin of mating pairs of moths collected in Bollgard II® fields, suggest that moths from corn and sorghum refuges are mating up to 40% of the time with partners that do not originate from the same refuge crop (for more information on this study see the paper by Baker and Tann in this volume). These data indicate that moths are moving from refuge crops to mate. Further work is planned to test whether the partners of refuge moths originate from cotton.

Is our current RMP adequate?

When the data on resistance and mating ecology are considered together, we conclude that our current refuge options are adequate to delay the onset of resistance to Bt by Helicoverpa. However, while refuges are a critical component of the RMP for Bollgard II®, it is not a stand alone failsafe and must be employed in conjunction with the other elements of the strategy (as outlined in Farrell and Johnson 2005). The current areas for refuges are considered to be the minimum required for effective resistance management, and assume a well maintained crop that is attractive to moths. Computer models that try to mimic the evolution of resistance indicate that given our current RMP and if refuges are maintained, Bollgard II® should be effective for many years.

Is there potential for new refuge options or strategies?

Although the current refuge requirements are considered to be appropriate for delaying resistance to Bollgard II®, it incurs a significant financial cost to the grower. While undoubtedly unpalatable, this cost should be considered an insurance policy for the protection of Bt technology. At present, there is no reliable alternative in Australia for delaying resistance to Bt than refuges that produce large numbers of Bt-susceptible moths of Helicoverpa.

There is a move to eliminate mandated refuges in the USA. The argument is based on field evidence that relates to many of the cotton growing regions, which suggests that non-cotton crops and other 'natural' non-crop plants provide sufficient moths to replace the mandated refuge. It is important to recognise that we cannot assume that the US evidence is applicable to Australia. Our major Helicoverpa pests are inherently more tolerant of Cry toxins than the Heliothis species that pose the greatest resistance threat in the US. In addition, in many regions of Australia *H. armigera* is largely restricted to cotton, and the absence of a suitable refuge other than that currently required, would 'cycle' Bollgard II® survivors. Given these differences between US and Australia, we need appropriate research before we can contemplate removal of mandatory refuges.

The information used to determine the current refuge options was collected in the early 1990's in anticipation of INGARD® being commercially released. Undoubtedly, the environment within the cotton landscape has changed considerably since then. Research that evaluates whether there have been significant changes in the efficacy of refuges since the introduction of Bollgard II® is planned for the next three seasons (G. Baker and C. Tann). This work will provide up-to-date information on which to verify our current refuge requirements. The same project will examine the possibility of artificially increasing the density of egg lays on refuges to improve their efficacy, and the contribution of non-cotton refuges to the pool of Bt susceptible moths.

Acknowledgments

The work presented herein is funded by the CRDC and CCC CRC. The technical assistance of T. Parker, K. Stanford, L. Scott, T. Staines, S. Young and Kim Garsia is gratefully appreciated.

References:

Andow D and D Alstad 1998 F_2 screen for rare resistance alleles. Journal of Economic Entomology 91(3): 572-578

Andow D and A Ives 2002 Monitoring and adaptive resistance management. Ecological Applications 12(5): 1378-1390

Baker G and C Tann 2006 Mating of *Helicoverpa armigera* moths in Bollgard II cotton. ACGRA Cotton Conference, Gold Coast

Farrell T and A Johnson 2005 Cotton pest management guide 2005/06. New South Wales Department of Primary Industries.

Ferre J and J Van Rie 2002 Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annual Review of Entomology 47: 501-533

Fitt G 2004 Implementation and impact of transgenic Bt cottons in Australia. *In* Proceedings, World Cotton Research Conference 3, Cape Town, 9-13 March 2003. Agricultural Research Council, Institute for Industrial Crops, Pretoria, Republic of South Africa.

Gore J and J Adamczyk Jr 2004 Impact of bollworms [*Helicoverpa zea* (Boddie)] on maturity and yield of Bollgard cotton. The Journal of Cotton Science 8:223-229

Mahon R, Olsen K, Garsia K and S Young 2006 Resistance to the Bt toxin Cry2Ab in a strain of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). Journal of Economic Entomology In review

Roush R 1997 *Bt*-transgenic crops: just another pretty insecticide or a chance for a new start in resistance management? Pesticide Science 51:328-334

Wenes A, Bourguet D, Andow D, Courtin C, Carre G, Lorme P, Sanchez L and S Augustin 2006 Frequency and fitness cost of resistance to *Bacillus thuringiensis* in *Chrysomela tremulae* (Coleoptera: Chrysomelidae). Heredity advance online publication 17 May