Australian Government
Cotton Research and Development Corporation

FINAL REPORT 2015

Part 1 - Summary Details

CRDC Project Number: **CSE 1304**

Project Title: Managing Bt resistance and induced tolerance with effective refuge crops in preparation for Bollgard III.

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CRDC Program: 2 Industry

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Part 3 – Final Report

(The points below are to be used as a guideline when completing your final report.)

Background

1. Outline the background to the project.

Moths of the genus *Helicoverpa* are the most destructive pests in Australian cotton. They have been also some of the most difficult to manage because *H. armigera* (in particular) has quickly developed resistance (within 5-8 years) to nearly every insecticide used in its control (Whitehouse et al. 2007). To hinder *H.armigera* developing resistance to Bt cotton, a Resistance Management Plan (RMP) was put in place when Bt cotton was first used commercially in Australia in 1996. As this was over 15 years ago, the RMP has been successful. Nevertheless, in light of the development of resistance to Bt cotton by *H. armigera* in other parts of the world (Tay et al. 2013) it is important to remain vigilant and keep testing the tools used in the RMP.

A key tool of the RMP is the use of refuges. Refuges help maintain the potency of Bt cotton by producing unselected *Helicoverpa* moths that mate with any resistant moths emerging from the Bt crop, thereby diluting their genetic contribution to the next generation and slowing the development of resistance.

Refuge governance is based on models with assumptions that are difficult to test on farms. The *Helicoverpa* Genome Project has mapped all of *Helicoverpa's* genes, making it easier to test two assumptions on the frequency of resistant (R) and susceptible (S) genes, and on the degree to which moths mix both within valleys and between Bt cotton and its refuges. If these assumptions are incorrect, then refuges may be underperforming.

Although refuges are designed to counter Bt resistance developing from genetic mutations, a recent CRDC project (03UA002) showed that under laboratory conditions, the exposure of *Helicoverpa* to low, non-lethal doses of Bt toxins over 12 generations can cause *H.armigera* to develop inducible tolerance to Bt toxins, to the extent that they are not killed by levels of Bt toxin fatal to susceptible *H.armigera*. As stressed Bt cotton plants may produce less toxin, and some parts of the plant produce low levels of toxin, inducible tolerance could be another pathway by which *Helicoverpa* could survive on Bt cotton. An aim of this project is to test the likelihood that inducible tolerance could occur in field crops of Bt cotton, and if so, if refuges could reduce that risk.

For refuges to counter genetic resistance and inducible tolerance to Bt toxins, they must be working optimally on farms and produce as many moths as possible. To do so refuges need to attract sufficient egglays, and then support as many of the resulting *Helicoverpa* larvae as possible until maturity. For many growers it isn't clear if their refuges are countering the development of resistance; how to improve the productivity of their refuges; or how to measure the effectiveness of their refuges in order to improve efficacy. Monitoring refuge productivity is a challenge, with current reporting often at odds with on farm realities. A remote method of checking refuges could be used to identify refuges facing difficulties, which could be then ground-truthed. The ultimate aim of this work is to incorporate best management practises into myBMP to improve refuge governance and also to develop better monitoring techniques to identify under-performing refuges which may need more assistance.

The overall aim of this project is to improve the ability of refuges to counter both the threat of resistance developing via genetic mutation, and the potential threat of crop failure via inducible tolerance. By accessing and countering these threats while concurrently developing better refuge management and benchmarking techniques to improve refuge governance, the ultimate aim is to avoid the cost of losing Bt cotton efficacy.

Objectives

2. List the project objectives and the extent to which these have been achieved.

Below is a summary of our findings against the milestones. The ultimate aim of our work is to publish our findings as a series of papers. To assist this process we are presenting our work as a series of sections (which we hope to quickly translate into one or more papers). Some sections may be relevant to more than one milestone, so this approach enhances linkages between the different aspects of our work. In these sections we will present our findings in more detail.

Below are the list of sections:

Section 1. Comparing field moth emergences from Bt cotton, its refuges (non-Bt cotton and pigeon pea crops) and the ramifications to IPM.

Section 2. Resistance and tolerance in field moth emergences and *Helicoverpa* larval survivors on Bt cotton, non-Bt cotton and refuges.

Section 3. The relatedness of *Helicoverpa* moths emerging from different crops in two locations.

Section 4. Characterization of tolerant colonies.

Section 5. Raising susceptible larvae on Bollgard II (Bt) cotton plants

Section 6: Larval movement between Bt cotton and its refuges.

Section 7. Comparing the attractiveness of commercially grown Bt cotton and its non-Bt cotton and pigeon pea refuges.

Section 8. Testing the ability of Satellite imagery to identify characteristics of Bt cotton and its refuges that make them attractive.

This work is a collaborative effort involving some people who were not part of the original project. At the beginning of each section we acknowledge the main contributors who wrote, analysed and produced the data for that section. These lists are not exhaustive, and the author lists on the final papers would probably be more extensive.

Below are the objectives and the summaries of our findings:

1. Are the same number of RR moths per metre found in Bt crops as in refuges, and is the ratio of RS to SS moths emerging from Bt crops and refuges the same?

Over the course of the project we collected 472 Helicoverpa moths about half of which were alive. Of those pairs we were able to develop 69 families through to the F2 bioassays. Of these, six families produced larvae that survived the discriminating dose of Cry2Ab and one family produced survivors on Cry1Ac toxin. There was no difference in the likelihood that the survivors came from refuges or Bt cotton.

Of the 472 Helicoverpa collected, 433 were tested using molecular tools for a known Cry2Ab resistance (none were tested for Cry1Ac alleles). Of these, 2 moths may be carrying a resistance gene to Cry2Ab. Again there was no bias to Bt crops.

Despite the lack of genetic resistance, much higher numbers of moths emerged out of Bt cotton than expected. The ratio per season at different sites of Helicoverpa moths emerging from Bt cotton and accompanying refuges varied between 4% and 22% from the Bt cotton. If these results are representative of the Namoi then about 50% of all moths in Bt/refuge complexes (excluding moths from unstructured refuges) in the Namoi would have originated from Bt cotton. This indicates a very high exposure level to Bt toxins, even though very few of these animals carry resistant genes.

For more information see Sections 1 and 2.

2. Do moths mix between nearby Bt crops and refuges, but less over larger distances?

We found evidence that moths from Bt cotton and its associated refuge, and moths from the two locations, were all closely related. There was no segregation of families which supports the view that moths from Bt cotton and non-Bt cotton readily mate, and that families mix over at least 40 km.

For more information see Section 3.

3. Can *Helicoverpa* develop tolerance to Cry2ab and Cry1Ac concurrently?

We initiated a colony (C1C2) which was exposed to low levels of Cry1Ac and Cry2Ab toxins to see if it could develop tolerance to both toxins concurrently. Unfortunately, after 16 generations of low level selection, a highly recessive resistance gene (HaR01) was detectable in the C1C2 colony. The most likely explanation is that the R01 resistant gene was in the original susceptible stock colony of *Helicoverpa armigera* at extremely low levels. However if R01 is as fully recessive as described, then it should not have spread under low toxin pressure. These results suggest that either HaR01 was not fully recessive, that the resistance gene was in the resistance colony at high enough levels that heterozygote resistant individuals could mate together to generate homozygous individuals that would have an advantage, or that tolerance could have enhanced any slight advantage of RS individuals, enabling more to survive.

For more information see Section 4

4. Can *Helicoverpa* develop tolerance on glasshouse grown Bt plants producing low levels of Bt toxin?

We raised larvae from neonates on Bt cotton material under laboratory conditions, but only 1% survived in the first generation. We were more successful raising 3rd instar larvae on Bt cotton material (following on from the work of our summer scholarship student, Sharna Holman, who found that 3rd instar larvae fed on Cry1Ac toxin survived better than neonates and produced offspring that were more tolerant). Ten percent of these larvae survived to moths on Bt cotton.

For more information see Section 5

5. Can tolerance be arrested by stopping exposure to Bt toxin for one or more generations?

We found that tolerance dropped when colonies were no longer exposed to Bt toxins.

For more information see Section 4

6. Do *Helicoverpa* larval field survivors have inducible tolerance to Bt toxins?

We found little evidence that the grandchildren of larvae of both species collected from Bt cotton fields have higher levels of tolerance to Cry1Ac or Cry2Ab.

For more information see Section 2

7. Do the offspring of moths emerging from Bt crops and refuges have different levels of tolerance to Bt toxins?

We found evidence that the grandchildren of *Helicoverpa punctigera* (but not *Helicoverpa armigera*) moths that emerged from Bt cotton fields have higher levels of tolerance to Cry1Ac, but not Cry2Ab.

For more information see Section 2

8. Are there genetic differences between laboratory and field tolerant *Helicoverpa?*

Unfortunately the susceptible *H. armigera* colony from which the tolerant colonies were derived may have been contaminated with undetectable levels of a common Cry2Ab resistant gene (HaR01). Thus genetic analysis of the tolerant colonies was not possible. Genetic analysis of the field moths whose F2 neonates survived the discriminating dose, revealed that none tested were carrying the common Cry2Ab resistance gene. However, at the loci where alleles that code for HaR01 resistance are found, we found a large amount of genetic diversity which could indicate that some of these moths were carrying unknown resistant alleles.

For more information see Section 2

9. Develop refuge crop agronomy to enhance moth productivity.

Our work and the work of our students have shown that the best way to enhance productivity from refuges is to ensure that cotton refuges are well watered with sufficient nutrients, and that pigeon pea refuges flower at the optimal time, and are also well watered with sufficient nutrients. Although pigeon pea doesn't like too much water at key stages during its development, we have found for the past seven years at ACRI, that a very effective pigeon pea refuge is possible by managing its water schedule as if it were cotton.

For more information see Section 7

10. Benchmark the ability of refuges to attract and produce moths.

In order for refuges to be effective they need to attract egg lays and produce moths. Pigeon peas need to produce twice as many moths as would have been produced in Bt cotton if Bt cotton did not contain toxin. In this project we have continued work comparing the relative attractiveness of Bt cotton and their refuges, thereby benchmarking how refuges actually perform on commercial farms. We then used these findings as a basis for our exploratory work using satellite imagery to identify potentially underperforming cotton and pigeon pea refuges.

For more information see Sections 7 and 8.

11. Review and test the minimum optimum width for refuges.

Our work with the summer scholarship student Sharna Holman demonstrated that tolerance was significantly higher in the offspring of larvae exposed to Cry1Ac toxin as late instar larvae (3rd instar onwards) but not smaller larvae (neonates to 3rd instar). This indicates that there is less of a threat from larvae moving from Bt cotton to refuges or volunteers, but more of a threat to Bt toxin efficacy from larvae moving in field non-Bt volunteer plants to cotton plants, or moving into Bt cotton fields from deteriorated refuges.

Therefore our results suggest that refuges need to maintain the 24 row width and should be in a different field to Bt cotton to discourage movement of larvae between the refuge and the Bt cotton.

For more information see Appendix A and Section 6

12. Promote refuge governance

While we have tried to promote refuge governance, we have found it difficult to attract growers to listen to our message. Growers know that they need to produce refuges to maintain the efficacy of Bt cotton, but the threat is long term, and the success of the RMP to date may have made them a bit complacent.

The best way to promote refuge governance is to better monitor refuge productivity by identifying problematic refuges remotely, and then sending out monitors to ground truth efficacy and provide management suggestions to improve their potential productivity.

The second approach is to provide regular information on why maintaining refuges and the efficacy of Bt cotton is so important. Our work has demonstrated that this involves educating the growers that along with resistance, growers need to minimise the role of tolerance in reducing Bt cotton efficacy.

For more information see Section 8

13. Produce a simple refuge effectiveness calculator within the myBMP system

We have been working with members of the myBMP team to improve the information in myBMP on refuge maintenance, but results from this project indicate that our refuges may be operating in a manner different to that expected. For example, results from this project indicate that pigeon pea refuges, in many cases, may act more like a trap crop than a refuge (Section 7). Therefore destroying these crops as soon after harvest as possible may be more advantageous than leaving them in the ground, as would be the case if they were only refuges. Likewise, a big threat to Bt cotton efficacy is the movement of large larvae into Bt crops, where *H. punctigera* in particular can become more tolerant to Bt toxins, and produce more tolerant offspring (Sections 2,4,5,6, Appendix A). Therefore it would be wise to make sure that pigeon pea refuges are not in the same field as Bt cotton crops.
Because these are changes to current refuge approaches, we are cautious, and want to test our findings further before encouraging changes to the industry.

Methods

14. Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.

Results

15. Detail and discuss the results for each objective including the statistical analysis of results.

As mentioned above, we have written our methods and results as a series of sections. Each section has a short Introduction to explain how it relates to the milestones, Methods, Results, and a short Discussion to explain how it fits into the big picture and aims of this project. The aims are to improve the ability of refuges to counter the threat of *Helicoverpa* by: ensuring assumptions upon which refuge models are based are robust; establishing if inducible tolerance could flourish in the field and whether it can be countered by refuges; and encouraging on-farm refuges to perform optimally by improving refuge management.

SECTION 1

Comparing field moth emergences from Bt cotton, its refuges (non-Bt cotton and pigeon pea crops) and the ramifications to IPM.

Contributors: Mary Whitehouse, Abbey Johnston, Tom Walsh, Gail Spargo

INTRODUCTION

A major tool in the RMP against *Helicoverpa* spp is the use of refuges, whose aim is to produce moths that mate with any moths emerging from the Bt cotton, thereby diluting any resistant genes. Early modelling indicated that to delay resistance by 20 generations, 10% of the population should not be exposed to Bt toxins (Roush 1998). This equates to 10% or 5% of the cotton crop planted as a non-Bt cotton or pigeon pea refuge respectively.

An assumption in the refuge model (Roush 1998) is that the resistant gene is recessive, allele frequency of resistance is initially low (<0.001) and that Bt cotton is high dosage killing 95% of heterozygous resistant individuals. Extensive monitoring work (using F2 and F1 screens) has shown that in Australian Bollgard II cotton, the allele frequency for Cry1Ac and Cry2Ab toxins range from below 0.001 for Cry1Ac for both *H.armigera* and *H.punctigera* (F2 screens) to 0.027 for Cry2Ab for *H.armigera* (F1 screens) (Downes: 2014 end of season resistance monitoring report).

If both Cry proteins in Bollgard II cotton are high dosage and kill 95% of Heterozygotes, using the most conservative estimates and following a simple Hardy-Weinberg equation ($a^2+2ab+b^2$) we would expect about one moth emerging out of Bt cotton for every one million moths emerging out of the same area of non-Bt cotton (see Appendix B). If only Cry2Ab toxin was expressed, we would still expect only three in a thousand *H.armigera* moths and two in a thousand *H.punctigera* moths to have emerged from Bt cotton.

However recent work has indicated that Cry1Ac is not as efficacious as Cry2Ab when killing Helicoverpa (Downes CSE1202). In addition, some parts of Bt cotton are known to express lower levels of Bt toxin; levels of Cry2Ab are thought to drop during the season; and stressed plants will express less toxin. Given these factors, the number of moths emerging from Bt cotton could be substantially higher than assumed in the models. Because the ratio between moths exposed to Bt toxins and moths not exposed to Bt toxins is critical for refuges to provide a sufficient diluting effect, we need to measure how many moths emerge from Bt crops in relation to non-Bt crops.

While most of the focus in Bt cotton has been on its efficacy against *Helicoverpa*, there may be other moths that are not so well controlled by Bt toxins. Documenting how Bt cotton alters the lepidopteran community would identify moth species that could become problematic.

The aim of this section is to report the relative proportions of *Helicoverpa* and other moths emerging from Bt cotton and its associated refuges.

METHODS

To collect moths emerging from Bt cotton and its associated refuges, we used White Cages (Fig. 1.1). White cages were developed and described in the previous project (1.01.65 CRC1005). Each cage sampled $1m^2$ of soil surface at its base (*Helicoverpa* pupate in the soil). They were sealed to the ground with pegs and soil. The collecting vial at the apex of the dome had an inverted mesh funnel which allowed moths to enter but not leave. Cages were arranged in rows 20-35 cages long, with 1 metre between the cages and an un-sampled row of cotton between each row of cages. The placement of the white cages in the field was staggered, with a row of 20 cages moved to a new row every 5-7 days. Each cage was left in

place for 2-3 weeks (cotton plants within the cage were removed ensuring that only *Helicoverpa* pupating before the cage was put in place were counted as emerging moths). Cages were checked daily for moths. For each moth, we recorded its species and the location in the field where it emerged.

Fig 1.1 The white cages (inset) and their field layout for the 2013/14 season. Cages in the Bt cotton (A2 and B2) were always paired with cages in a refuge (A1=pigeon pea, B1= non-Bt cotton).

Cages in Bt cotton were always paired with cages in a refuge. In all three seasons we worked at two sites, ACRI and on a commercial farm in the Namoi, and in the third season we also included cages on a commercial farm at Emerald.

Season	Bt	Conv	Pigeon pea	TOTAL
2012/13	710	710		1420
2013/14	740	560	180	1480
2014/15	630	210	420	1260

Table 1.1. The number of cages put out per season in the Namoi in the different crops. Another 240 cages were set up in Emerald in the 2014/15 season.

In the 2012/13 season, cages were set up from the 17th December to the 25th March, with 228 cages open at any one time. A total of 1420 metres were sampled over the season. Only Bt cotton and non-Bt cotton were sampled (Table 1.1.).

In the 2013/14 season, cages were set up from The 18th December to the 12th March, with 320 cages open at any one time. A total of 1480 metres were sampled over the season. Cotton refuges and their corresponding Bt crops were sampled at both ACRI and the commercial farm for 14 weeks, while Pigeon pea and its corresponding Bt crop was only sampled at ACRI.

In the 2014/15 season, as well as the cages in the Namoi we set up cages at Emerald from the 8th December to the 9th March, with 40 cages open at any one time (a total of 240 metres were sampled). In the Namoi 360 cages were set up at any one time from the 15 of December to the 16th of March. These were open for 3 weeks rather than two weeks to aid the logistics of the operation. We assumed that the majority of the moths would emerge in the first 2 weeks that the cages were in position. At ACRI there were two reps of both non-Bt cotton /Bt cotton and pigeon pea /Bt cotton (Fig. 1.2); and at the commercial farm there were two reps of pigeon pea /Bt cotton. In total 1260 metres were sampled in the Namoi, 1500 metres all together.

Therefore over the three seasons we sampled 4400 metres of cotton.

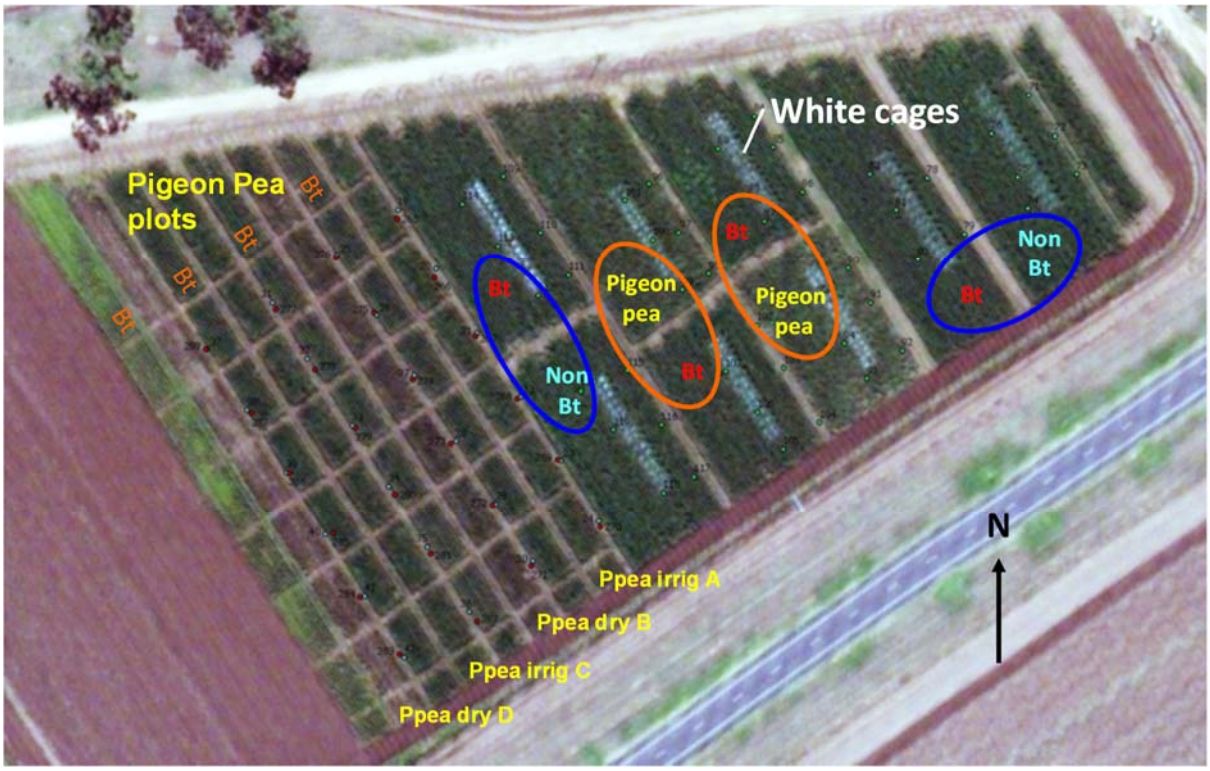

Fig. 1.2 Satellite image of the pairs (circled) of sampling plots at ACRI for the 2014/15 season. The pigeon pea plots are discussed in Section 6.

Helicoverpa identified in the field had their species identity checked using the mitochondrial genes CO1 (cytochrome c oxidase 1) and Cytb (cytochrome b) as molecular markers.

While collecting moths we focused on *Helicoverpa* spp. However, twice a season we also collected other moths caught in the white cages. Of these, only a proportion could be identified to species in the laboratory. Our aim is to more formally identify and analyse these species at a later date.

RESULTS

In the first season (2012/13) despite putting out 1420 cages, we collected only 36 *Helicoverpa* (Fig. 1.3). Of these, one was *Helicoverpa assulta* which emerged from Bt cotton (Fig. 1.3). The majority were *H.armigera* (n=26) with only 9 *H. punctigera*. Overall, out of 36 *Helicoverpa*, 6 emerged from Bt cotton (16.7%) which was much higher than expected.

In the second season (2013/14) we collected large numbers of *H. punctigera* moths (n=332) but similar numbers of *H.armigera* moths (n=30, Fig. 1.4). The majority of *H.punctigera* immerged out of the pigeon pea in one week (5^{th} – 12^{th} of February). The peak immergence in the non-Bt cotton at ACRI was a week later. Out of 362 moths, 25 emerged from Bt cotton. There was quite a bit of variability between crops and location in the proportion of moths emerging from Bt cotton, with the commercial farm producing proportionally more Bt moths (Fig. 1.4).

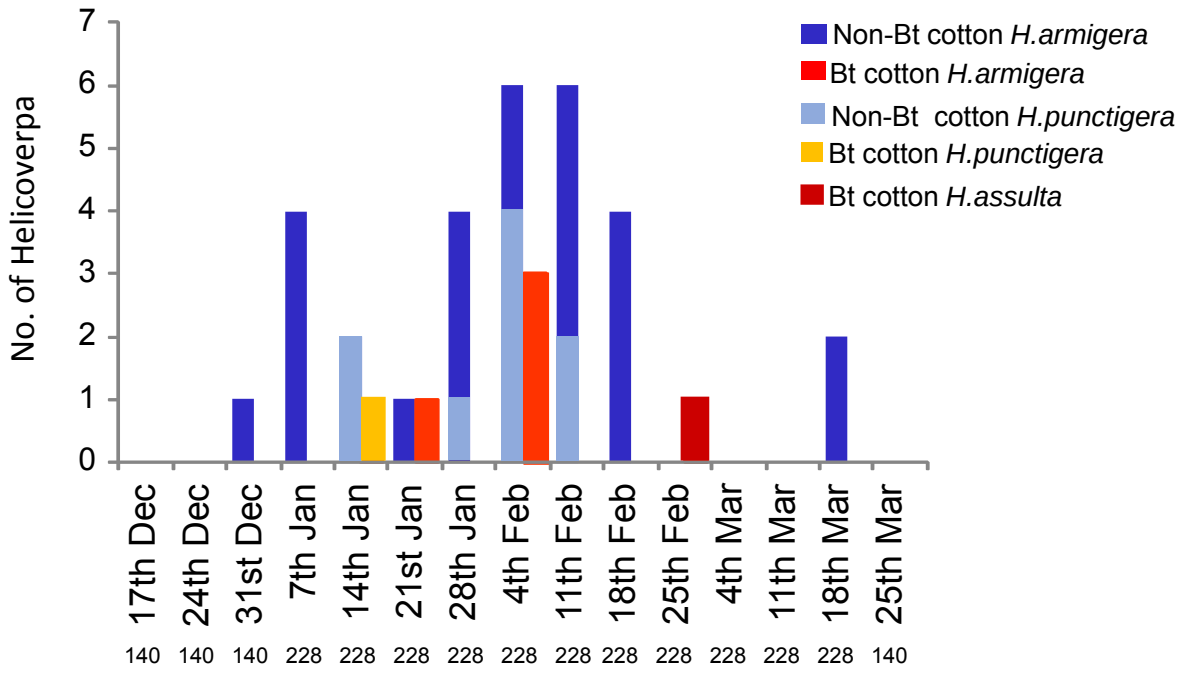

Fig. 1.3 Moths emerging from Bt cotton and non-Bt cotton at ACRI and the commercial farm. The numbers under the dates are the number of cages open.

In the third season (2014/15) we collected more *H.armigera* (n=50) than *H.punctigera* (n=24) in the Namoi. Out of the 74 moths, 8 emerged from Bt cotton (Fig. 1.5). Of the 240 cages we put out at Emerald, we caught 17 moths. Surprisingly, a very high number of those, 11 (65%) emerged from Bt cotton.

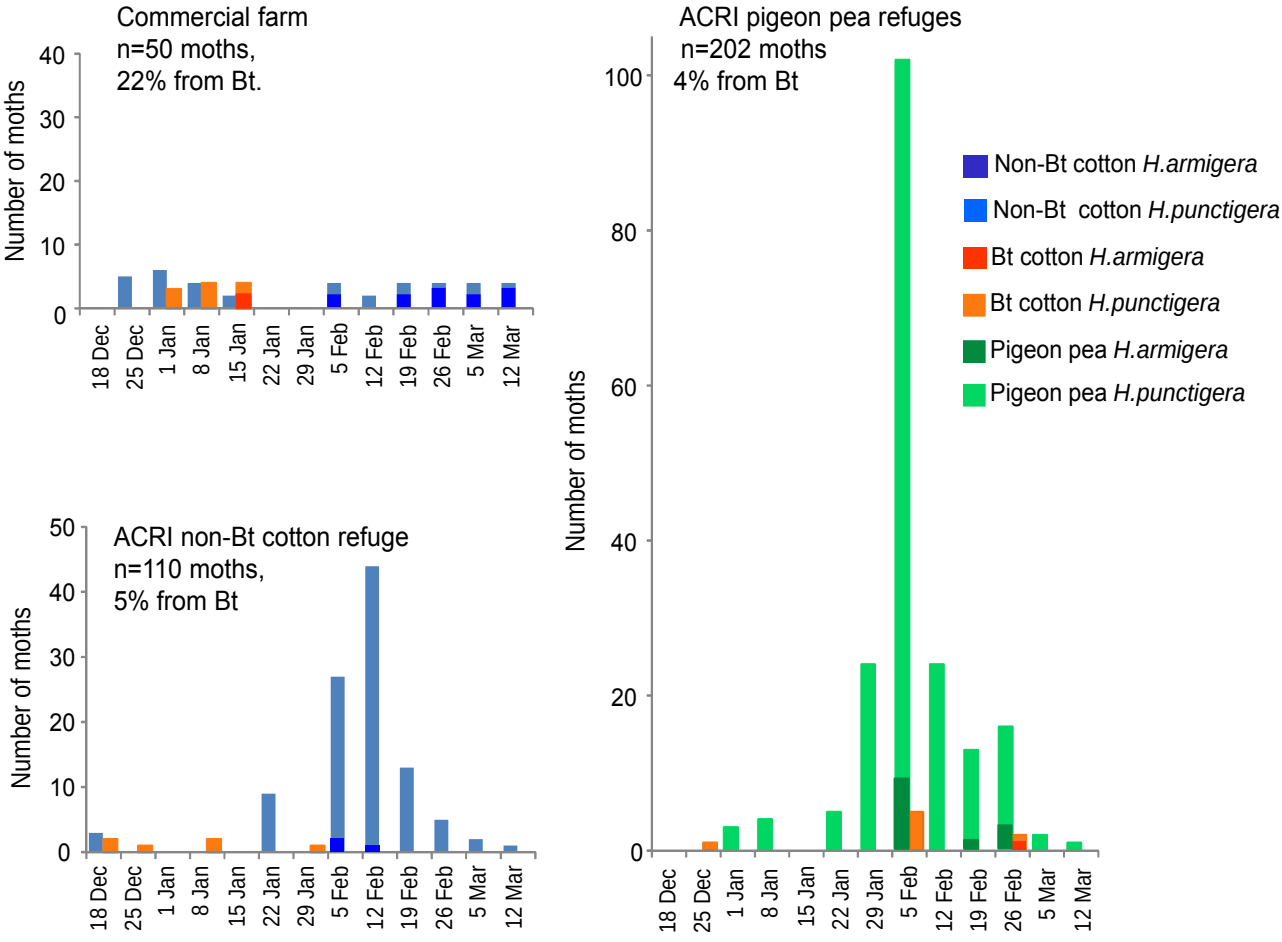

Fig. 1.4. Moths caught in the 2013/14 season in non-Bt cotton, pigeon pea, and their associated Bt cotton.

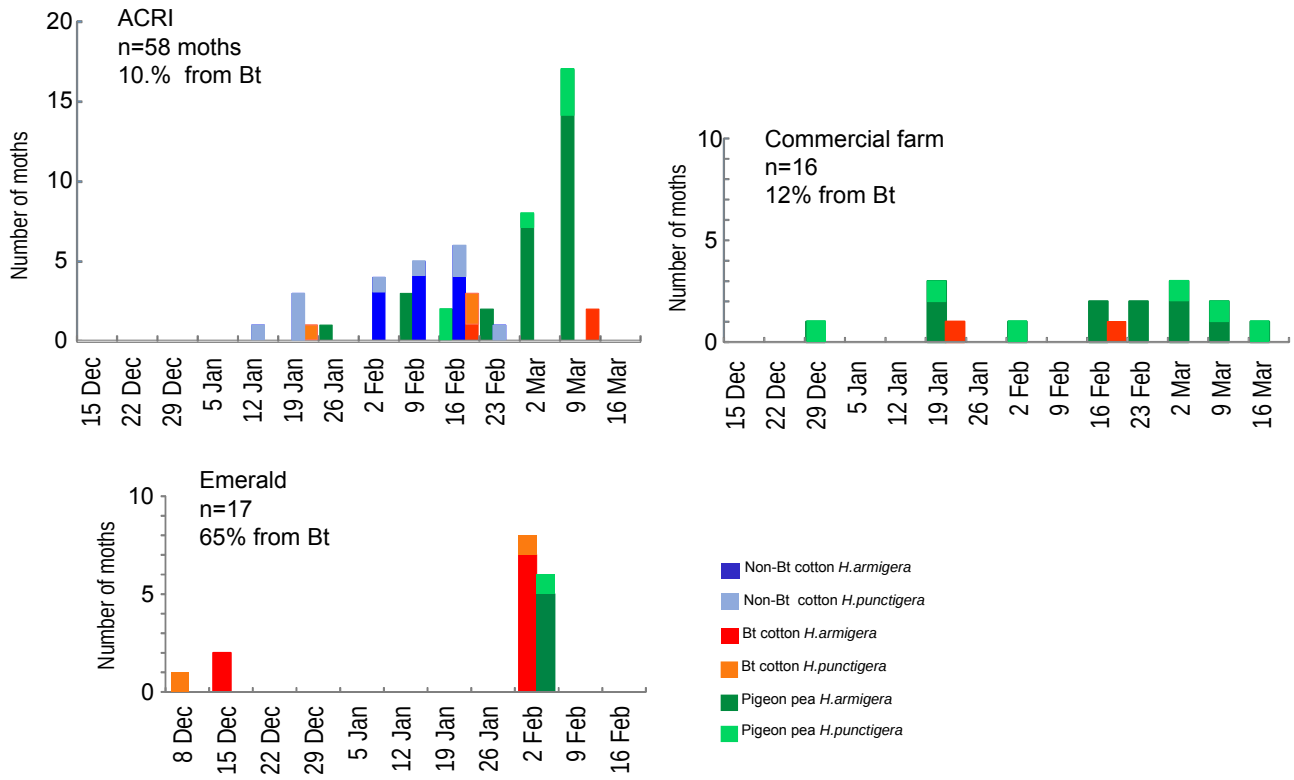

Fig. 1.5. Moths caught during the 2014/15 season in non-Bt cotton, Pigeon pea, and their associated cotton. A very large proportion of the moths in Emerald emerged from Bt cotton.

These three seasons indicate that there is no pattern to when *H.punctigera* or *H.armigera* are more likely to emerge out of cotton crops, and that moths are just as likely to emerge from Bt cotton at the beginning of the season as they are at the end of the season. There is no difference in the likelihood that a *H. punctiger*a or *H. armigera* will emerge out of Bt cotton in any year (Fishers exact test/Chi square analysis. All highly non-significant.).

One of the assumptions of the refuge model is that refuges produce enough moths to dilute the genes of any moths emerging from Bt cotton. All refuges sampled in this study were paired with a Bt crop. Cotton refuges make up 10% of their associated refuge crop, while Pigeon pea refuges make up 5% of their associated Bt cotton crop. Therefore area-wise, the samples from Bt cotton paired with cotton refuges should be multiplied by 9 to match the area represented by these plots, and samples from Bt cotton paired with pigeon pea should be multiplied by 19. If the emergences reported here from Bt crops and their refuges are representative of those in general, then we could multiply out the moth numbers from the Bt crops to estimate the relative proportions of the moths emerging from Bt crops and refuges in the field. This gives an indication of the proportion of moths in the refuge /Bt complex, from Bt cotton. This calculation indicates that about 50% of the moths from the Bt cotton /refuge complex during these seasons in the Namoi valley may have emerged from Bt cotton (Fig. 1.6). The percentage represented by the Emerald samples is a staggering 95%, but this sample is small and may be distorted.

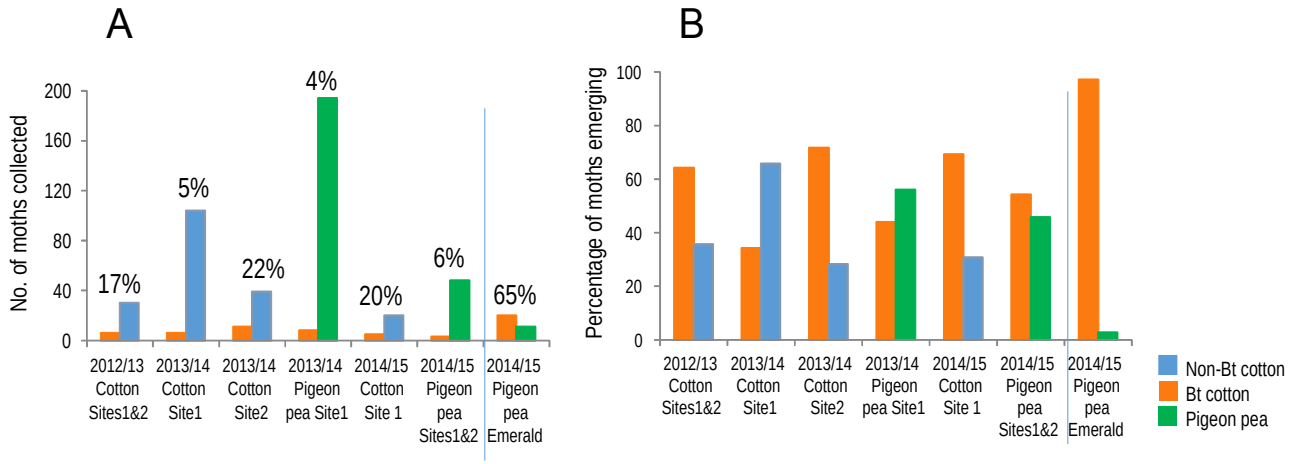

Fig.1.6. A. The actual number of moths emerging out of Bt cotton and its associated refuge over the three seasons, and **B.** the estimated proportion of moths in the refuge/Bt cotton complex that these numbers represent.

Testing Hardy Weinberg assumptions

We calculated the number of moths per metre per species emerging out of each crop over the season, and calculated the ratio of moths emerging from refuges to Bt cotton assuming 5% survival of heterozygotes (Table 1.2). The number of moths emerging per metre was as low as 0.008 per m in Bt cotton (2014/15 season) and high as 1 per m from pigeon pea in the 2013/14 season. (Table 1.2 A). On a straight metre to metre comparison between Bt cotton and its refuges, the lowest ratio was 1:4, while the highest was 1:34 (Table 1.2 B). Following the Hardy Weinberg equation, even if only Cry2Ab was working in *H.armigera* we should expect a ratio of 1: 298 (Table 1.2 C).

A

moths/m	*armigera*			*punctigera*		
	Bt	Non-Bt	pigeonpea	Bt	Non-Bt	pigeonpea
2012/13	0.006	0.023		0.001	0.013	
2013/14	0.004	0.025	0.072	0.030	0.229	1.011
2014/15	0.008	0.052	0.081	0.005	0.043	0.029

B

H.armigera moths Ratio : Bt cotton to refuges		*H.punctigera* moths Ratio : Bt cotton to refuges	
1 : 4	Non-Bt cotton /2012/13	1 : 9	Non-Bt cotton /2012/13
1 : 6.2	Non-Bt cotton 2013/14	1 : 7.7	Non-Bt cotton 2013/14
1 : 6.6	Non-Bt cotton 2014/15	1 : 9	Non-Bt cotton 2014/15
1 : 17.8	Pigeon pea 2013/14	1 : 34	Pigeon pea 2013/`14
1 : 10.1	Pigeonpea 2014/15	1 : 6	Pigeonpea 2014/15

C

F1 screens	Ratio
H.armigera Cry2Ab only:	1 : 298
H.punctigera Cry1Ac only:	1 : 3246
H.Punctigera Cry2Ab only:	1 : 424
H.armigera Cry1Ac &Cry2Ab:	1 : 967,104 (estimated)
H.punctigera Cry1Ac & Cry2Ab:	1 : 1,375,296

Table 1.2. A Number of *H.armigera* and *H.punctigera* moths found per metre in Bt cotton, non-Bt cotton and pigeon pea, calculated per season (Namoi only). **B.** Ratio of *Helicoverpa* moths emerging from Bt cotton and its refuges per season in the Namoi (calculated from the moths per metre data). **C.** Estimated ratios of moths emerging from Bt cotton based on current resistant management frequencies (see Appendix B).

Thus the numbers of *Helicoverpa* moths we found in Bt cotton are much higher than expected on current resistance estimates, even given the survival of 5% of heterozygotes.

Other moths emerging from Bt cotton and refuges

Over the three seasons we sampled 1257 other moths. In the first season we collected 659 moths, and have preliminarily identified and analyzed four species from this collection (*Earias hueyeliara*: rough bollworm; *Mythimna loreyimima*: sugarcane army worm; *Endotricha puncticotalis*, and *Athetis tenuis*).

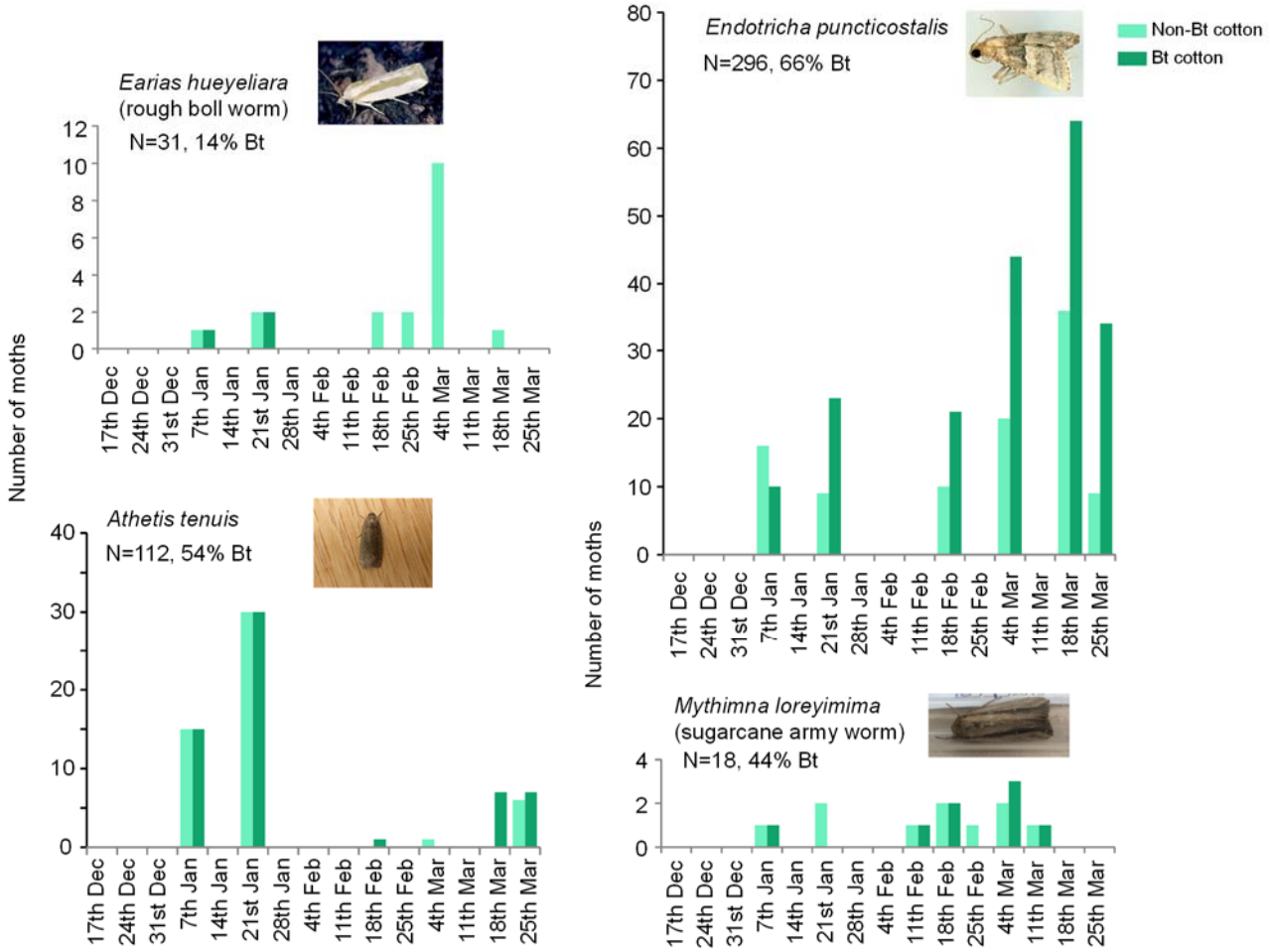

Fig. 1.7. Four of the other moth species caught during the 2012/13 season.

We found that the rough boll worm was controlled to a similar extent as *Helicoverpa*, although in the 2012/13 season, their proportion in Bt cotton was slightly higher at 14%. The sugarcane army worm, which is less susceptible to Bt toxin, had similar numbers in the Bt (44%) as the non-Bt cotton; while other moths, such as *Endotricha puncticostalis*, were in significantly higher proportions in Bt cotton (66%). (Fig. 1.6).

DISCUSSION

Our results show that the numbers of moths emerging from Bt cotton is much higher than expected by the frequencies of resistant moths found in the field, suggesting large numbers of susceptible moths are able to emerge from Bt cotton. The actual proportion of known resistant alleles to susceptible alleles is analyzed in Section 2. How a large number of susceptible individuals are surviving on Bt cotton now becomes key. Two possible explanations are physiological stress causing the plant to temporary reduce toxin production, the larvae seeking out parts of the plant that express less toxin, or some combination of these factors.

If Helicoverpa moths are building up resistance during the season, we would expect more moths to be emerging out of Bt cotton from latter generations at the end of the season (March). This was not the case. With very early emergences, moths could have developed in the previous season's crop and were slow to emerge. We suspect that this may explain some

of the early season emergences in Emerald (P.Grundy pers. comm.). Nevertheless, our results show that conditions suitable for moths to emerge from Bt cotton could occur at any time in the season.

It is possible that the amount of non-Bt plant material in Bt crops is higher than expected. Weeds could be alternative hosts within the crop, but this does not account for the high proportions of *Helicoverpa* emerging from the commercial cotton crops which were very clean.

Another factor that could influence survival is a heterozygous advantage, where RS individuals are better able to survive on Bt toxin than SS individuals. However, the most well-known resistance gene to Cry2Ab toxins (derived from the SP15 colony) in *H.armigera* in Australia (HaR01) is known to be very recessive, as heterozygotes of *Helicoverpa armigera* (Mahon et al 2008) and *Helicoverpa punctigera* (carrying the resistance gene HpR01; Downes et al 2010) are killed as easily as fully recessive individuals. Nevertheless HaR01 resistance genes have been found in only 50% of the resistant field *Helicoverpa armigera* published so far (Tay et al In Press) and the actual proportion of this gene in resistant individuals could be even less (Walsh pers. obs.). Therefore there are other resistance genes in the population that may not be as recessive.

In addition, we know that *Helicoverpa* under laboratory conditions can develop tolerance to toxins that can be passed on to subsequent generations. It is possible that an increase in tolerance, coupled with access to parts of the plant that express less toxin, could enable more individuals to survive on Bt toxin. This is further investigated in Sections 2 and 5 of this report.

The high numbers of *Helicoverpa* emerging from Bt cotton at Emerald in the 2014/15 season is a concern. However, the sample size is small, and the large numbers were only collected on one sampling date, which could have indicated a drop in the efficacy of the crop at this stage for environmental reasons. Nevertheless these results suggest that *Helicoverpa* numbers in Bt cotton in Emerald need close monitoring.

Other moths

Bt cotton has drastically reduced numbers of *Helicoverpa* in cotton which in turn means there is less competition among lepidopteran herbivores for cotton. Moths whose larvae can tolerate Bt cotton could increase in numbers and exploit this opportunity. In particular, moths such as the army worms, which are not as strongly affected by the Bt toxin need to be monitored to check that their numbers do not increase. Likewise there may be other moths which were suppressed by *Helicoverpa* that may become more prominent in Bt cotton.

In this project we presented a preliminary investigation of these animals. While rough boll worm numbers were slightly higher in Bt cotton, they seem to be reasonably controlled. Sugar cane army worms were not well controlled by Bt cotton, but in the 2012/13 season, their numbers were not high in Bt cotton. One moth more common in Bt cotton than non-Bt cotton is *Endotricha puncticostalis*, a species of snout moths. It lives in the detritus at the base of food plants, and is reported as a minor pest of peanuts. At this stage it is unclear whether it would increase in numbers to the extent that it could become a pest in cotton.

SECTION 2.

Resistance and tolerance in field moth emergences and *Helicoverpa* larval survivors from Bt cotton, non-Bt cotton and refuges.

Contributors: Mary Whitehouse, Mahbub Rahman, Tom Walsh, Farzana Rahman, Leense Mathew, Bill James, Melissa Piper, Abbey Johnson

INTRODUCTION

Bt cotton has been highly successful in controlling *Helicoverpa*. However, *Helicoverpa armigera* in particular has developed resistance to most insecticides used in its control. Consequently, a Resistance Management Plan (RMP) was put in place to delay the development of resistance to Bt toxins by both *Helicoverpa armigera* and *H. punctigera*. A key part of the RMP is the use of refuges to produce moths that have not been exposed to Bt toxins. The rationale is that these moths will mate with those from Bt toxin, thereby diluting any resistance genes emerging from Bt cotton. This strategy works because the main resistance gene to Cry2Ab in *H.armigera* and *H.punctigera* (HaR01 and HpR01 respectively) is highly recessive, with heterozygotes showing no increased survivorship (in comparison to fully susceptible individuals) against Cry2Ab (*H. armigera*: Mahon et al 2007; *H. punctigera*: Downes et al 2010).

Given this high level of efficacy, we would expect that the only moths surviving on Bt cotton are those that are RR resistant, or those that have located aberrant plants that have no toxin. Therefore, if the same area is sampled in Bt and non-Bt cotton (assuming a similar plant density and attractiveness to egglays) you would expect to find fewer moths in the Bt cotton, but the same proportion of RS to SS moths, and the same absolute number of RR moths in both crops (Fig 2.1).

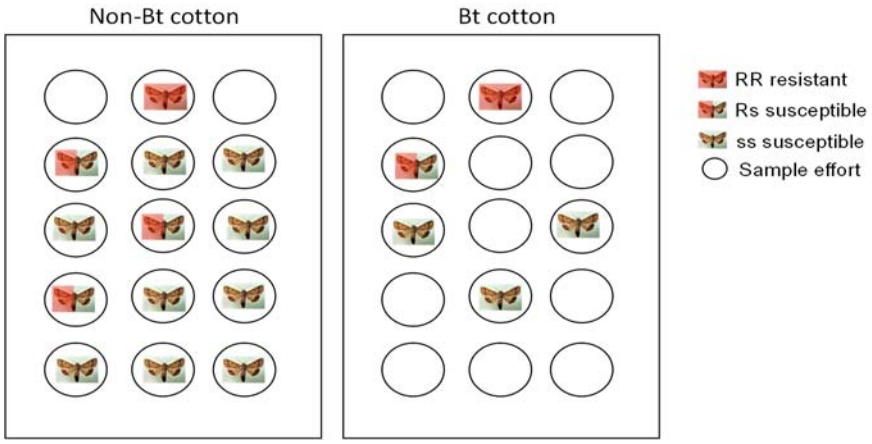

Fig. 2.1. Diagrammatic representation of refuge assumptions with a completely recessive resistant gene. Given the same sampling effort, Bt cotton will yield less moths, but the proportion of RS to SS (1:3 in this case) will match that in non-Bt cotton, and the absolute number of RR moths will be the same in both crops.

Because the frequency of Cry1Ac and Cry2Ab are very low, it would be very difficult to capture frequency information about RR resistant genes, but it may be possible to capture information on the relative frequency of RS genes, and whether there is evidence of other forms of resistance to Bt toxin present in these moths which may be less recessive. Therefore an aim of this study is to test if the frequency of resistance genes is higher in moths emerging from Bt crops than moths emerging from non-Bt refuges.

Increased survival on Bt cotton may not be limited to genetic resistance, but could be enhanced by epigenetic changes. An epigenetic change is one where changes to physical aspects of the organism are caused by external or environmental factors that affect which

genes are turned on or off, and which could be passed on to future generations. In respect to countering toxins, epigenetic effects could turn on genes that control the production of esterases or proteases. These enzymes can interact with certain types of toxins, and reduce the effectiveness of Bt.

Previous studies demonstrated that pests such as the diamond backed moth are capable of developing tolerance to chemical insecticides and pass this onto their offspring (Rahman et al. 2010). In *Helicoverpa*, previous studies (Rahman et al. 2011; Ma et al. 2005, 2007, 2012) showed that under laboratory conditions, larvae fed low doses of Bt toxin over many generations were able to build up tolerance to the toxin. If larvae were exposed to low doses of toxin over a number of generations under field conditions, then this could reduce the efficacy of Bt cotton crops.

Therefore the second aim of this section is to test if the offspring of moths emerging from Bt cotton are more tolerant of Bt toxins than the offspring of moths emerging from non-Bt refuges.

If all resistant alleles are recessive, then populations of larval "survivors" on Bt cotton crops should have the same proportion of heterozygous resistant individuals as *Helicoverpa* in non-Bt crops. However, these larvae have been exposed to some level of Bt toxin. If this can be passed onto their offspring, then their offspring should have higher tolerance levels than those that have not been exposed to Bt toxins. Therefore we also tested if larval "survivors" on commercial Bt cotton crops were more likely to carry resistant genes; and if their offspring were more tolerant of Bt toxins.

METHODS

All moths used in this study were those collected using the white cages described in Section 1. All moths collected alive were either paired with a field moth from the same plot, or with a susceptible virgin moth isolated from the laboratory colony. In the first season when the moths died they were kept at -80ºC for genetic analysis. In the second and third season they were preserved in alcohol for molecular analysis. If they were found dead in the cage they were immediately put in -80ºC (first season) or in alcohol (second and third season).

To obtain larvae for this study, consultants or growers sent in Bt survivors to the Resistant Monitoring Team (headed by S. Downes) who passed the larvae onto us. Alternatively we were told of larval survivors in Bt cotton, which we then collected by visually searching the crop and recording the plant structures on which the larvae were found.

F2 screening

All alive moths collected from the field were taken back to the laboratory to form families.

In the 2012/13 and 2013/14 season, moths were paired and mated with other field moths from the same crop and farm; if none were available, they were mated with a susceptible laboratory moth. In the 2014/15 season, all field moths were mated with susceptible laboratory moths.

Moth culturing followed the standard protocol followed by the Resistance Monitoring Program, described elsewhere (Downes et al 2012). Pairs were mated, their offspring raised and mated together, and their eggs collected for F2 screening of the neonates, or sent to Adelaide for tolerance testing of the neonates.

F2 screening involved taking 45 well trays filled with *Helicoverpa* diet, and adding a layer of either Cry1Ac or Cry2Ab toxin over the top of the diet at the discriminating dose for the *Helicoverpa* species tested. Ninety neonates (one per well) were then placed in the wells, and left for 7 days. If any neonates reached 3rd instar in seven days, it indicated that the family tested could contain a resistant gene.

Tolerance testing.

Families were developed from pairs of surviving larvae and moths collected from the field (mentioned above) by culturing them in the laboratory in the absence of toxins. Over 1000 eggs from the F2 generation of each family were sent to Waite Campus, The University of Adelaide (Dr Mahbub Rahman's Lab) for tolerance testing on full-dose response toxin bioassays which required about 1000 egg-hatched neonates per family. Bioassays were performed using an artificial diet overlaid with a crude bacterial suspension containing Cry1Ac or Cry2Ab. In each bioassay, fresh artificial diet was poured into 45-well plastic trays (~2 ml per well) and left to solidify in a fume hood for about 30 min. Because the exact concentration of toxin in each preparation was not known, a preliminary assay for susceptible *Helicoverpa armigera* and *punctigera* neonates was conducted using a broad range of Cry1Ac or Cry2Ab suspension concentrations to determine the appropriate concentration for the formal assay. Full bioassays were then conducted with 10 concentrations (plus a Milli-Q water as control) and at least 45 larvae for each concentration. Toxin-containing stock solutions were diluted in Milli-Q water to specific concentrations, and desired (75µl for Cry1Ac and 100 µl for Cry2Ab) aliquots were spread evenly on top of the artificial diet in each well. Trays were left to dry in a fume hood for about 30-60 min. Neonate larvae were placed individually in each well and heat sealed with Mylar (heat-sealable polyester film). A total of 20 to 25 fine holes were then punched into the film to allow air exchange. Trays were placed in a ventilated room under standard culture conditions at 25±1°C (14/10h, L/D photoperiod) and the efficacy (larval mortality) of the treatments was assessed on day 10. To estimate the relative tolerance against a susceptible strain, full-dose response toxin bioassays were performed for susceptible *Helicoverpa armigera* and *punctigera* neonates for Cry1Ac and Cry2Ab. Mortality data were analyzed (POLO-PC software; LeOra Software, Berkeley, CA) to estimate the lethal dose concentrations (LDs). For each probit analysis, the mortality was corrected using Abbott's formula. Differences in susceptibility were considered significant when the 95% confidence intervals did not overlap at LD_{50} values. The resistance ratio (RR) was expressed as the ratio of the LD_{50} value of the field population to that of the Waite susceptible control.

Testing for genetic resistance

The resistance allele present in the *H. armigera* lab colony (SP15) has been identified as a mutation in an ABC transporter gene (Tay et al. In Press). The Cry2Ab resistant *H.punctigera,* laboratory colony has been found to have a mutation in the same gene (Tay et al. In Press). These alleles in *H. armigera* have been named HaR01 and in *H.punctigera* are HpR01. While these are the most common genes encountered so far, other resistant genes have been identified, and HaR01 probably represents about 20% of the Australian resistant *H. armigera* population (S. Downes pers. com.). Primers have been developed to sequence the particular part of the gene containing HaR01 and HpR01. The majority of the moths and larvae (that reached maturity) were tested for these known Cry2Ab resistance alleles.

Due to the low numbers collected in the 2012/13 season and because CSIRO has sequenced the *H. armigera* and *H. punctigera* genomes, it was possible to sequence the whole genome of these moths and then examine them for the HaR01 and HpR01 allele. In the 2013/14 and 2014/15 season, many more moths were collected so the specific primers for both species were used.

Initially sequencing was done on individual moths using Sanger sequencing, however due to the high number of heterozygous individuals this was not very successful. An alternative approach was developed using next generation sequencing where pooled amplicon samples were sequenced to look for the presence of the R01 allele in both species. Results were classified as: no allele; < 1% chance of the allele; >1% chance of the allele. Samples in any groups which indicated a >1% chance were retested individually.

RESULTS

In total, 472 Moths were collected over the three seasons from the three different crop types (Table 2.1). Of these, about half were collected alive. These were then paired (with another from the same crop or a member of the susceptible colony) to develop families for F2 screening and tolerance testing.

Season	Species	Total	Crop	Total collected	Total alive	% alive
2012/13	*armigera*	26	Bt cotton	4	2	
			non-Bt cotton	22	10	
			pigeon pea	-	-	
	punctigera	9	Bt cotton	1	-	
			non-Bt cotton	8	4	
			Pigeon pea	-	-	
	assulta	1	Bt cotton	1	-	
TOTALS				36	16	44%
2013/14	*armigera*	30	Bt cotton	3	3	
			non-Bt cotton	14	4	
			pigeon pea	13	8	
	punctigera	332	Bt cotton	22	7	
			non-Bt cotton	132	84	
			pigeon pea	178	111	
TOTALS				362	217	60%
2014/15	*armigera*	50	Bt cotton	5	2	
			non-Bt cotton	11	6	
			pigeon pea	34	12	
	punctigera	24	Bt cotton	3	1	
			non-Bt cotton	9	5	
			pigeon pea	12	4	
TOTALS				74	30	41%

Table 2.1. The total number of moths collected from the white cages and the percentage of moths captured alive.

In total 410 larvae, who ranged in size from 3rd to 5th instar, were collected from 21 outbreak events in the Lower Namoi, St George, the Darling Downs and Emerald. Of these, 192 (47%) reached maturity. In all three seasons most of the larvae (82%) that survived to maturity were *H. punctigera* (Table 2.2).

Season	Total larvae	No. of farms	Species	No. reaching maturity
2012/13	23	1	armigera	-
			punctigera	21
2013/14	277	17	armigera	27
			punctigera	87
2014/15	110	4	armigera	8
			punctigera	49

Table 2.2 The number of *Helicoverpa* larvae collected from Bt cotton surviving to maturity in the laboratory.

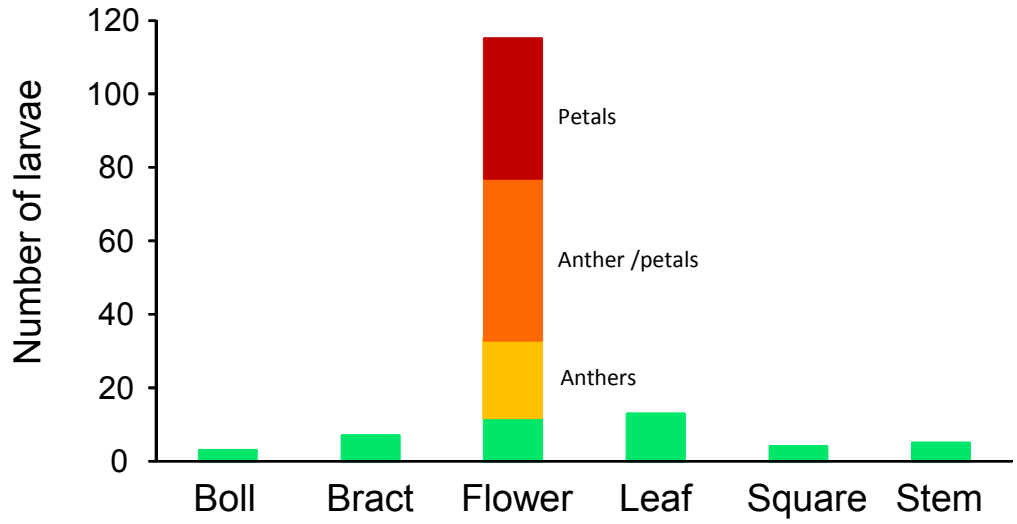

Fig. 2.2 The number of surviving larvae found on different Bt cotton structures in the field.

Most of the larvae found were located on the flowers (Fig. 2.2) in particular, there were signs that they had been consuming the anthers and pollen, and many petals also contained a small hole.

Genetic resistance to Cry2Ab (R01 gene)

Of the 472 moths caught in cages, 30 from 201213 season, 329 from the 201314 season, and all 74 from the 2014/15 season were tested for the R01 allele, which gives resistance to Cry2Ab toxin. None of the moths tested in the 2012/13 or 2014/15 season yielded positive results. In the 2013/14 season, results from one *H. punctigera* moth (M1516) indicated that it was likely to be carrying the resistance R01 gene. It had emerged from pigeon pea.

The argument is that if you compare the same amount of area in Bt and its refuges (although all moth numbers will be lower in the Bt cotton) the proportion of individuals carrying a resistance gene should be the same (if there is no advantage of carrying a resistance gene while being susceptible: i.e, RS). As we only found 1 moth that could be carrying the resistance gene, our numbers are too small to test this argument.

Of the 192 larvae survivors (157 *punctigera*, 35 *armigera*) collected from Bt cotton crops that reached maturity, we tested 176 for the Ha and Hp R01 allele. None were positive for either Ha or HpR01.

F2 Screens

Of the 263 moths that were collected alive from cages, and the 192 larval survivors of Bt cotton that reached maturity, we created 91 families (from 166 wild moths and larvae) that produced sufficient F2 progeny to undertake F2 resistant screen tests.

Of the 28 larval families (7 *H. armigera*, and 21 *H. punctigera*) 2 *H. armigera* and 2 *H. punctigera* tested positive for Cry2Ab (allele frequency for *H. armigera*: 0.09, 22 alleles, and for *H. punctigera*: 0.03, 66 alleles).

Of the 63 moth families (93 moths) tested with F2 screens, one *H. armigera* family tested positive for Cry1Ac (allele frequency= 0.006, 170 alleles). The field moth for this family originated from pigeon pea. In addition six families (2 *H. punctigera*, 4 *H. armigera*) tested positive for Cry2Ab (allele frequency for both *H. armigera*: 0.15, 22 alleles, and *H. punctigera*: 0.011, 186 alleles). The proportion of positive families from Bt and non-Bt crops for each species was not significantly different (Table 2.3) indicating that there was no

significant difference in the ratio of Heterozygous moths on Bt cotton and non-Bt refuges, for either species, using the F2 screening tests.

Species	Crop origin	F2 +ve	F2 -ve
H armigera	Bt	1	0
	non-Bt crop	3	7
H.puctigera	Bt	0	8
	non-Bt crop	2	83

Table 2.3 number of moths from cages in the F2 screens that tested positive for Cry2Ab resistance. The proportion of positives from Bt and non-Bt crops in either species was not significantly different.

Comparing F2 Screen results with Molecular Tests and Tolerance Bioassays

We tested if the results of the F2 screen tests were reflected in the molecular tests for the R01 resistance allele; and the F2 screenings for tolerance. The genetic tests provided no evidence that any of these families carried the R01 Cry2Ab resistant gene (Table 2.4 A).

Bioassays for tolerance tests could indicate the presence of a resistance gene, if the ratio of family to control survivors in the LD50 test is relatively low, but the ratio of survivors in the LD90 tests is high. A low LD50 and High LD90 indicates that individuals that survive the LD50 dose will also survive the LD90 dose. That is, there is no reduction in survivors, as you would expect with a normal distribution of tolerant individuals. In four of the five families with F2 survivors tested for tolerance, the LD90 ratios were low, and in some cases the families did worse than the control. The exception is the M1197/ M1198 family, which was tested for tolerance in a group with four other families. These combined families in the Cry2Ab bioassays expressed a low ratio for LD50 (1.6) but a high ratio to the susceptible colony in LD90 (18.2) suggesting that the individuals that survived the LD50 test would also survive the LD90 test. This supports the F2 screen test, indicating that there could a resistant allele in this family, even if it is not R01. This lower, more conservative number of families carrying a resistance gene for Cry2Ab again supports no difference in the number of heterozygous individuals in Bt and non-Bt cotton, although the samples are small.

Moth Family	Season	Species	F2 screen survival	Crop	Resistance gene to Cry2Ab -	Tolerance LD50 Cry2Ab	Tolerance LD90 Cry2Ab	Tolerance LD50 Cry1Ac	Tolerance LD90 Cry1Ac
M1197 /M1198	201314	punctigera	Cry2Ab	Pigeon pea	<1%	1.6	**18.2***	**12***	**10.2***
m1665	201415	punctigera	Cry2Ab	Pigeon pea	<1%	-	-	2.5	2
m1624	201415	armigera	Cry2Ab	Bt	none	-	-	-	-
m1651	201415	armigera	Cry2Ab	Pigeon pea	none	2.22	1.9	1.88	0.62
m1675	201415	armigera	Cry2Ab	Pigeon pea	none	0.56	0.26	**4.64***	**8.97***
M1680	201415	armigera	Cry2Ab	non-Bt	none	0.81	0.8	0.68	2.55
M1701	201415	armigera	Cry1Ac	Pigeon pea	none	**3.8***	1.7	-	-
Larval family	Season	Species	F2 screen survival	instar collected from crop	Resistance gene to Cry2Ab -	Tolerance LD50 Cry2Ab	Tolerance LD90 Cry2Ab	Tolerance LD50 Cry1Ac	Tolerance LD90 Cry1Ac
L0303	201415	armigera	Cry2Ab	5	none	1.43	0.88	**7.38***	**14.26***
L0355	201415	punctigera	Cry2Ab	3	<1%	-	-	-	-
L0364	201415	punctigera	Cry2Ab	4	<1%	-	-	0.53	0.52
L0383	201415	punctigera	Cry2Ab	5	<1%	-	-	-	-

Table 2.4 Table of families with F2 survivors and the results of their molecular tests (R01) and tolerance tests (LD50 and LD90 for both Cry1Ac and Cry2Ab).In the tolerance tests, the numbers are the ratio of survivors to the control susceptible colony. Asterisks indicate tolerance levels significantly higher than the controls.

Tolerance tests

The number of *H. punctigera* families that successfully undertook the bioassay for tolerance to Cry2Ab toxins were 36 and for Cry1Ac toxins were 27. The bioassays revealed that *H. punctigera* from the field had higher levels of tolerance to Cry1Ac than Cry2Ab, as 19 out of 27 bioassays in the Cry1Ac tests were significantly more tolerant than the control, while only 8 out of 25 bioassays for Cry 2Ab toxin were likewise more tolerant (Fig. 2.3, Chi sq test stat= 7.7, df=1, P=0.006). In the *H. armigera* bioassays there was no difference in levels of tolerance to Cry1Ac and Cry2Ab (Fig. 2.4).

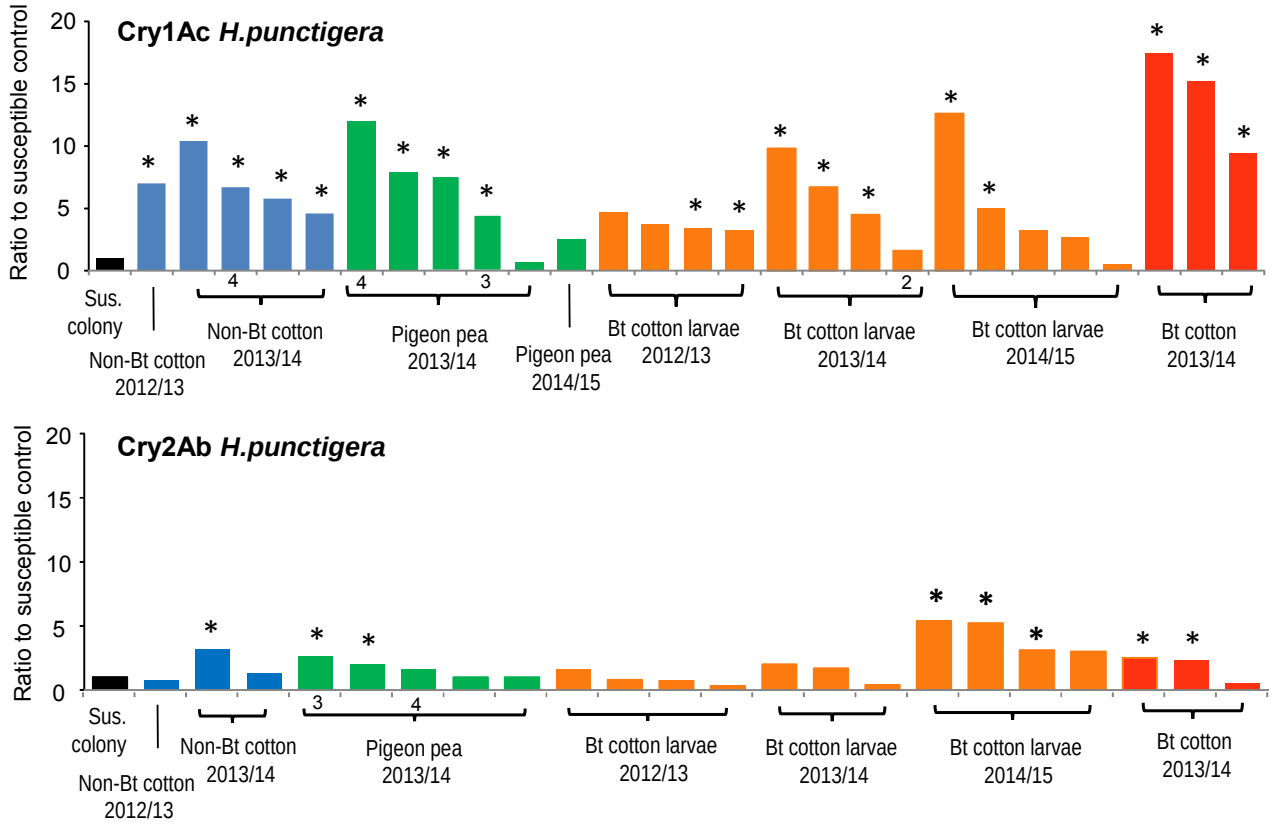

Fig. 2.3 The LD50 (expressed as a ratio between the family and the susceptible control) of *H.punctigera* moth and larvae families tested on Cry1Ac and Cry2Ab toxin. The numbers under the histogram indicate when the bioassay involved multiple families. The asterisk above the histogram indicates tests where the family's tolerance to the toxin was significantly higher than that of the control.

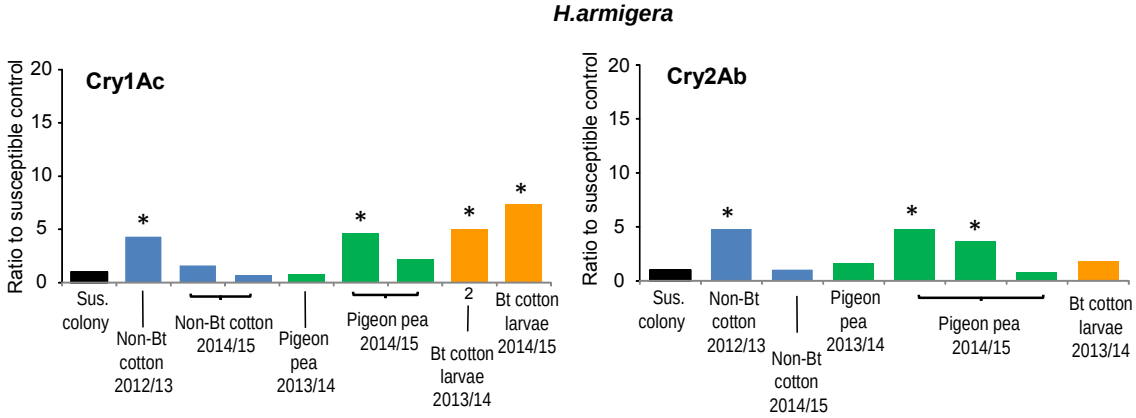

Fig. 2.4 The LD50 (expressed as a ratio between the family and the susceptible control) of *H.armigera* moth and larvae families tested on Cry1Ac and Cry2Ab toxin. The numbers under the histogram indicate when the bioassay involved multiple families. The asterisk above the histogram indicates tests where the family's tolerance to the toxin was significantly higher than that of the control.

While crop type did not seem to affect the amount of tolerance in Cry2Ab bioassays, there appears to be some evidence of tolerance to Cry1Ac toxins by *H. punctigera* families (Fig. 2.3). Further analysis of the 2014 season results using a generalized linear model (undertaken by Mr. S. Harden, NSW DPI) indicated that there was an effect of crop type on the survivorship of offspring (Fig. 2.5) with those from Bt crops surviving the best ($P<0.0001$, df=3,589; change in deviance by adding crop = 182).

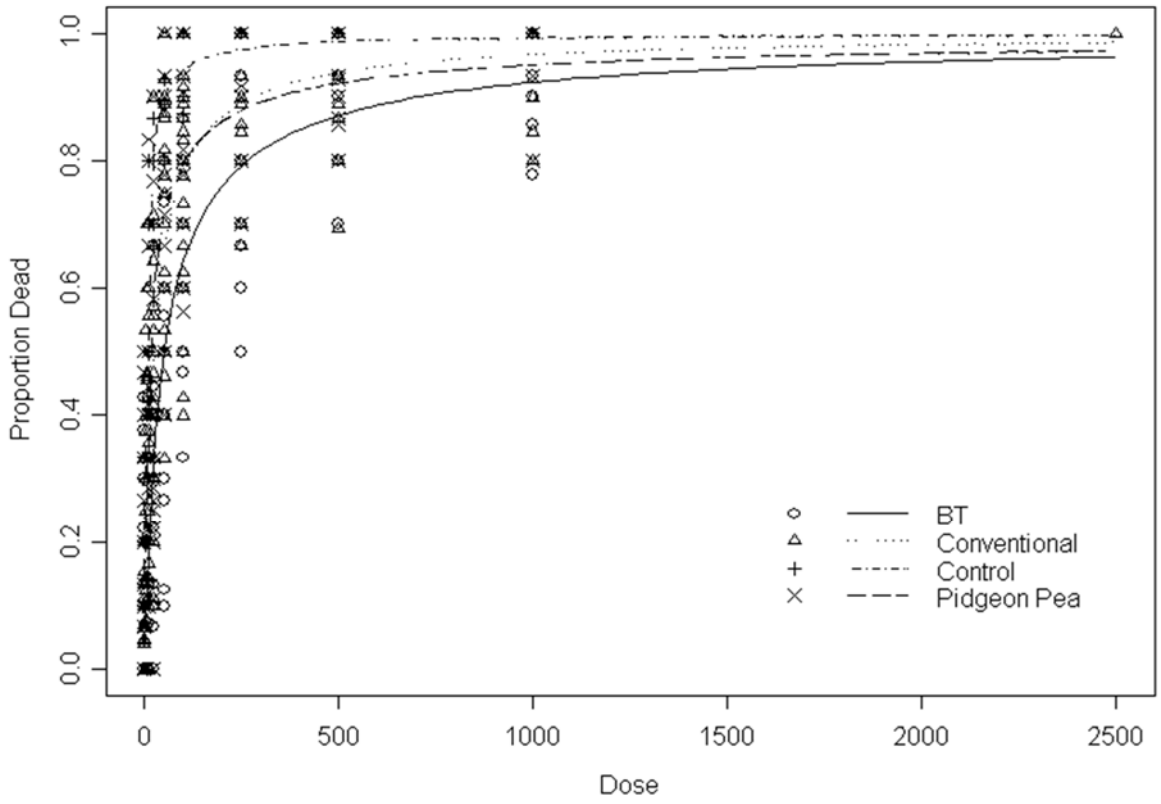

Fig. 2.5 Generalized linear model showing the effect of crop type on the survival of neonates in the 2013/14 tolerance tests for *H. punctigera*. Control larvae had the lowest survival while the Bt larvae had the highest.

One of the *H.punctigera* larvae survivor families (L0395) from a Bt crop in the 2014/15 season had a ratio reading of 3.3 at LD50, but a ratio reading of 32.5 at LD90 for Cry1Ac. This again indicates animals surviving the lower discriminating doses also surviving the higher doses, suggesting that this family may have a resistance gene for Cry1Ac. Cross checking with the F2 screens revealed some survivorship on the discriminating dose, but only to 2nd instar which is why they were not scored as a positive result. Nevertheless this level of survivorship may indicate some level of tolerance to the toxin. These results suggest that further analysis of the genetic component of this family in respect to Cry1Ac resistance could be fruitful.

DISCUSSION

Analysis of the Cry2Ab resistance gene in *H.armigera* and *H.punctigera* indicates that it is highly recessive (*H. armigera*: Mahon et al 2007; *H. punctigera*: Downes et al 2010). Therefore, as explained in the introduction, the proportion of heterozygous moths carrying the resistance gene should be the same in Bt and non-Bt crops (all things being equal). The F2 screening tests identified only a few potentially resistant families, and of these there was no difference in the proportion of resistant genes in families from Bt and non- Bt crops.

However, the genetic screening tests of *H.armigera* did not identify the R01 resistance allele in any of these families. While a higher proportion of RS individuals in Bt cotton compared to non-Bt crops could occur if other genes coding for resistance were not completely recessive, we only tested for R01 which is completely recessive

While *H. armigera* may be more likely to develop resistance to Cry2Ab, *H. punctigera* may be more likely to develop tolerance to Cry1Ac. The tolerance bioassays for Cry1Ac toxin indicated that more *H. punctigera* families had significantly higher tolerance ratios (to the control) than *H. armigera* families but that the relatively low LD90s indicated that the tolerance was not high enough to be scored as positive for resistance. In addition, there was a crop effect on tolerance in *H. punctigera*, with families from Bt cotton expressing the highest level of tolerance. Thus exposure to Bt toxin in the field may increase the amount of tolerance to Cry1Ac in subsequent generations, at least in *H. punctigera*.

Interestingly, the larvae collected from Bt crops did not have the same level of tolerance to Cry1Ac toxins as the moths. This may be because larvae were collected as 3rd to 4th instars. The Summer Scholarship work (Appendix A) demonstrated that 3rd instar and above was a critical period for the development of tolerance. If larvae are removed before or during this critical period, they would not have had the chance to develop tolerance, which would explain why their grandchildren are not more tolerant.

Larval survivors

Across all three seasons more *H. punctigera* than *H. armigera* larvae (that became moths) were found on Bt cotton despite the fact that for two seasons *H. punctigera* were not very common. There is a huge amount of variation in the relative number of *H. armigera* to *H. punctigera* emerging out of any crop, so this finding could represent the normal variation of the system. Also, there was no evidence from the moths caught in Bt and non-Bt crops that *H. punctigera* were more common in Bt cotton than *H. armigera*. Nevertheless, any differences between these two species may result in differences in how they respond to Bt cotton, which could be important in their management.

Most of the larvae found in Bt crops were on the flowers. Flowers are known to produce less toxin than other parts of the plant, indicating that the larvae may have been seeking out lower levels of toxin, as has been seen in laboratory experiments (see Appendix A). However while pollen is known to contain lower levels of toxin, our work suggests that petals do not have lower levels of Cry2Ab toxin (Section 5). In this study, most of the petals had a small hole in them rather than being completely consumed. It is possible that the larvae were sampling the petals rather than feeding on them.

SECTION 3.

The relatedness of Helicoverpa moths emerging from different crops in two locations.

Contributors: Mary Whitehouse, Wee Tek Tay, Bill James, Melissa Piper

INTRODUCTION

The most destructive pests in Australian cotton are the larvae of *Helicoverpa* moths. Bt cotton was developed to control these pests, but *Helicoverpa*, particularly *H. armigera*, are notorious for developing resistance to any chemical insecticide used in their control. To counter this threat, Monsanto, in consultation with the Bt cotton industry, developed the Resistance Management Plan, which aims to ensure that any resistance genes in the population do not increase or become concentrated. As part of this plan, the role of refuges is to dilute resistance genes, but in order for refuges to function well there needs to be complete mixing of moths emerging from Bt cotton and its refuges. Thus populations ideally should be continually mixed, both between Bt and non-Bt originating populations and within regions. The thorough mixing of the population within a region area would reduce the likelihood of resistance genes concentrating in localized pockets, which could result in localized populations of resistant *Helicoverpa*.

In the Australian cotton growing region there are two main types of *Helicoverpa* that attack cotton *Helicoverpa armigera* and *punctigera*. These moths are thought to have quite different distribution patterns. *Helicoverpa armigera* is thought to recruit locally, so that individuals from one season are related to those from the previous season, whereas *Helicoverpa punctigera* are thought to migrate in from central Australia, and so are unlikely to be closely related between seasons. Because of these differences, *Helicoverpa armigera* is possibly more likely to develop localized populations where resistant genes can develop, while *Helicoverpa punctigera* should be less likely to develop resistance as more genes are continually entering the system.

The aim of this section is look at relatedness and therefore mixing of both *H. armigera* and *H. punctigera*, both between seasons, between Bt and its refuges, and between two locations within the Lower Namoi. This section helps to address Milestone 2, exploring the degree to which Helicoverpa moths mix within regions and between crops.

METHODS

Emerging moths were collected in the Namoi Valley using large white cages (described in Section 1) at two locations ACRI (-30.31, 149.77) and a commercial farm (-29.94, 149.38) which were 36 km apart. All moths during the 2012/13 season and the first few moths collected from the 2013/14 season were used in the analysis.

The genetic analysis was undertaken by Dr. Tay in Canberra. Relatedness estimates between *H. armigera* populations and between *H. punctigera* populations from separate field sites (ACRI and the commercial farm) were inferred by taking the population-wide frequencies of the alleles to calculate the probability of shared alleles at the five nuclear loci (RpL3, RpS2, RpS6, RpS12, and RpL29). The Rp nuclear loci have had the development of their EPIC-PCR markers described elsewhere (Tay et al. 2008, Behere et al. 2013). Each of the loci were labelled with fluorescent dye (NED, 6-FAM, VIC) as described in Behere et al. (2013) and all *H. armigera* and *H. punctigera* were PCR-amplified individually. PCR amplicons from all five loci were pooled per individual prior to the electrophoresis run (at the ANU ACRF-BRF facility).

Alleles were manually scored using the molecular biology software Geneious R8 (Biomatters Ltd. Auckland, New Zealand) by setting allele-specific bins with a one-base pair repeat size range. A one-base pair repeat unit was the preferred setting because EPIC-PCR markers may detect single base pair insertion/deletion (INDEL) mutations that may cause intron length variations (Tay et al. 2008). As such, these markers differ from microsatellite DNA markers where alleles of a locus typically differed at predictable repeat unit variations (e.g., Tay and Crozier 2001; Tay et al. 2010). Symmetrical relatedness estimates between *H. armigera/H. punctigera* were calculated using the software Relatedness v5.0.4 (Queller and Goodnight 1989). The population genetic program Genepop <http://genepop.curtin.edu.au> (Raymon and Rousset 1995; Rousset 2008) was used to infer population differentiation between ACRI and the commercial farm populations of *H. armigera* and *H. punctigera* based on allele frequencies from the EPCI-PCR markers. The Hardy-Weinberg exact test was used to ascertain the robustness of the EPIC-PCR markers with respect to effects from possible null alleles, inbreeding, and/or genotyping errors such as relating to allele scoring errors.

RESULTS

			201213	201314
Acri	armigera	Bt cotton	1	0
		cotton ref	7	0
		pp	0	0
	punctigera	Bt cotton	2	0
		cotton ref	4	1
		pp	0	1
Warrianna	armigera	Bt cotton	3	1
		cotton ref	9	0
		pp	0	0
	punctigera	Bt cotton	1	3
		cotton ref	10	5
		pp	0	0

Table 3.1. Moths used in this analysis, showing where they were caught, the species, and the crop.

Due to time constraints, only the moths collected from the first season and the very beginning of the second season were used in this analysis. The sample consisted of 48 moths (Table 3.1): 21 *H. armigera*, and 27 *H. punctigera.* Of these, 11 emerged from Bt cotton, 36 from conventional cotton and one from pigeon pea.

A total of 21 *H. armigera* (ACRI, n=8; Commercial farm, n=13) and 27 *H. punctigera* (ACRI, n= 8; Commercial farm, n= 19) were genotyped using the five EPIC-PCR markers. These included one *H. armigera* from the cropping season of 2013/14, and five *H. punctigera* from the cropping season of 1012/13. Due to the limited number of individuals from each species that were sampled each season from the Bt-host sites, population genetic parametres were estimated between sampling sites per moth species per year. The presence of a heterozygous deficit (i.e. less variation than expected) in each marker was tested using the Hardy-Weinberg exact test. The test showed that RpS6 and RpL29 had no significant deficit, while RpL3, RpS2, and RpL12 were more homozygous than expected by chance; particularly RpL12, which was highly significant for *H. armigera* (Table 3.2).

In *H punctigera* two of the five markers (RpL3, RpS2) were more homozygous than expected by chance. When *H. armigera* and *H. punctigera* from each site were tested separately, only *H. armigera* at ACRI was as heterozygous as expected (*H. punctigera* were below the p-value of 0.05 when the S.E. estimates were taken into consideration). When combined across all populations and all loci, both *H. armigera* and *H. punctigera* had a heterozygous deficit

(Table 3.2). These results indicate that this *H. armigera* population has not reached a Hardy-Weinberg equilibrium, and contains more homozygous individuals than expected by chance. This finding is consistent with those of other studies of *H. armigera* populations, (e.g., Endersby et al. 2007; Behere et al. 2013) regardless of the marker system applied (e.g., microsatellite DNA; EPIC-DNA).

	H. armigera		*H. punctigera*	
	P-value	S.E.	P-value	S.E.
RpL3	0.0199	0.0017	0.0280	0.0026
RpS2	0.0110	0.0018	0.0340	0.0043
RpS6	0.6786	0.0367	1.0000	0.0000
RpL12	0.0000	0.0000	0.2201	0.0020
RpL29	0.2659	0.0235	0.5963	0.0209
ACRI	0.1467	0.0175	0.0528	0.0062
Warriana	0.0026	0.0010	0.0519	0.0097
All loci & all pop	0.0011	0.0004	0.0162	0.0036

Table 3.2: Global Hardy-Weinberg tests involving five loci and two populations each for *H. armigera* and *H. punctigera* at the ACRI and Warriana field sites. Significant P-values indicate support for the alternative hypothesis of heterozygote deficit that may be due to the presence of null alleles, allele drop-outs, genotype scoring errors and/or inbreeding.

Population differentiation and Relatedness estimates

Genotypic differentiation (exact G test) indicated that *H. armigera* populations were not significantly different, suggesting population connectedness at the spatial scale tested (P-value: 0.7073). Similarly for *H. punctigera* the P-value was also non-significant (P-value: 0.6218). Thus there is significant gene flow for both species between ACRI and the commercial farm.

Population-wide (i.e., combining ACRI and the commercial farm as a panmictic population) relatedness estimates for both *H. armigera* and *H. punctigera* were significantly greater than zero. As zero indicates that individuals are unrelated, the results suggest that the individuals tested from both species at both sites were related. Relatedness for *H. armigera* remained above zero even when estimated using only four of the EPIC-markers (i.e., by excluding the RpL12 marker which showed less heterozygoticity than expected, see above; and Table 3.3). The same positive relatedness estimates were observed for *H. punctigera* again suggesting individuals were possibly related. Thus the point-estimate of relatedness values at the population wide scale, as well as within individual sampling sites, were all at the sib-ship level (0.5 = average relatedness between full sibs) indicating that the population is closely related, possibly with highly inbred parents.

	5-loci	S.E.	95% CI	4-loci	S.E.	95% CI
H. armigera (All)	0.505	0.158	0.439	0.347	0.066	0.208
ACRI	(N.D)	-	-	0.380	0.098	0.310
Warriana	(N.D)	-	-	0.315	0.046	0.145
H. punctigera (All)	0.494	0.149	0.413	(N.D)	-	-
ACRI	0.523	0.217	0.692	(N.D)	-	-
Warriana	0.527	0.150	0.479	(N.D)	-	-

Table 3.3: Relatedness estimates of *H. armigera* and *H. punctigera* at population wide (i.e., 'All') and at the ACRI or the commercial farm sites (i.e., intra-population estimates). Number of loci used in *H. armigera* analyses were all five loci, or four loci (excluding RpL12). Standard errors (S.E.) and 95% confidence intervals (95% CI) are indicated, and are obtained by jackknife over loci. For estimating relatedness in *H. punctigera* all five loci were used. (N.D): not determined.

DISCUSSION

The analysis presented here indicates that both *H. armigera* and *H. punctigera* populations in this region had not reached a Hardy-Weinberg equilibrium, as there were larger numbers of homozygous individuals than expected by chance. The overall trend of the relatedness estimates suggest that individuals were potentially more related than full-sibs, with a potential scenario of highly inbred parents. This has ramifications for the spread of resistance genes. If the *Helicoverpa* are mating with close relatives, this could result in concentration of resistance genes.

However the cause of the high degree of relatedness is unclear. The results also indicate that there was no spatial segregation over 36 km, as the populations for both species were not segregated between the two sites. If there is a lot of mixing within this region, then the high numbers of homozygous individuals could be caused by a limited number of individuals colonizing the region (i.e. a Founders Effect).

What is interesting is that the genetic pattern seems to be the same for both species of *Helicoverpa*, even though the dispersal patterns of these animals is supposed to be quite different. The *H.punctigera* population should be descended from a recent wave of immigration from the inland. Theoretically, individuals in one season should be closely related, while there should be a distinct difference between years. Alternatively *H. armigera* should have been established in the region for some time, reducing the likelihood of differences between seasons (Endersby et al. 2007; Behere et al. 2013) and possibly a more closely related population. Thus between seasons, *H. punctigera* are not expected to be closely related, while *A. armigera* are expected to be closely related.

However, there are caveats on forming strong conclusions from these results. Firstly there are potential problems with the molecular markers used (e.g., see Behere et al. 2013) as well as the small sample size, which could impact on relatedness estimates. For example, although ACRI *H. punctigera* individuals have an average point-estimate relatedness of 0.523, the variance is very large with a 95% confidence interval (CI) of 0.692, suggesting there is a chance that the individuals could be un-related. Thus firm conclusions on relatedness would be premature given the large 95% CI; without utilising more robust DNA markers; and without analysing the whole moth data set (i.e., increasing DNA markers and/or applying

whole genome SNP markers, and significantly increasing population sizes to include analyses of more parental generations).

To overcome some of these challenges it would be ideal to analyse the complete moth data set of 472 individuals that was collected in this study. This would enable a more robust analysis of whether moths from the same location but different seasons are closely related, and whether moths emerging from the three crop types (Bt cotton, non-Bt cotton and pigeon pea) are also closely related.

Nevertheless, at this stage findings suggest that in the Namoi both the *H. armigera* and *H. punctigera* populations (at least within the 36 km tested) may be more related than expected by chance and possibly inbred (as has been found in other studies). Underlying factors leading to these genetic patterns in both species will require further study.

SECTION 4.

Characterization of tolerant colonies.

Contributors: Mahbub Rahman, Farzana Rahman, Mary Whitehouse, Leense Mathew, Tom Walsh

INTRODUCTION

Although the frequency of resistance alleles to Bt toxins in Australian field populations of *H. armigera* in Bollgard II cotton has not increased significantly, surviving larvae are found occasionally - particularly in late season or on low toxin-expressing plant tissues. Bt-resistance is thus a continuing threat. Our understanding of the diverse biological pathways leading to insect resistance against Bt-toxins outside mutations in major Bt-receptor genes (type I resistance) is still limited. Moreover, little is known about Cry1Ac resistance in Australia (Akhurst et al 2003, Teese et al 2013) which is still very rare (<0.001 allele frequency in both species; S.Downes, resistance report for 2014/15 season). Alternatively, Cry2Ab resistance (referred to here as "type 1 resistance") is more common in both species (see Section 2), the majority of this resistance in both *H.armigera* and *H.punctigera* has been caused by the same modification (deletion of 100 base pairs) to the same gene (Tay et al. In Press.). This form of resistance (R01) is highly recessive, with RS larvae as equally susceptible as SS larvae (*H. armigera*: Mahon et al 2007; *H. punctigera*: Downes et al 2010)

In addition to genetic resistance based on target site mutations (which produces individuals resistant to high toxin concentrations) laboratory work has shown that exposure of insect larvae to low - medium levels of Bt crystal toxins causes the induction of immune and metabolic responses, resulting low-level resistance (which we refer to here as inducible tolerance) in insect populations that can be transmitted to offspring by epigenetic inheritance mechanisms (caused by gene and protein regulatory mechanisms) (Rahman et al. 2004, 2011, Ma et al. 2005, 2007, 2012). To determine the cause of *H. armigera* survival in Bolgard II cotton, we proposed to test survivors to identify the relative contribution of inducible tolerance mechanisms to their overall resistance. The task was to test the genetic and epigenetic potential of inducible tolerance to Bt-toxins in *H. armigera* populations. This involves establishing many laboratory Cry1Ac and Cry2Ab tolerant *H. armigera* colonies concurrently by exposing them to sub-lethal doses of either toxin separately, or both together, by incorporating the toxins in the artificial diet.

The key questions in this section were: Can inducible tolerance develop in field and laboratory populations of *H. armigera* if they are exposed concurrently to Cry1Ac and Cry2Ab toxins? If so, by what magnitude can the tolerance be increased under continuous selection pressure over many generations? Can laboratory tolerant *H. armigera* survive on glasshouse grown and field bollgard II cotton (flowers & leaves) as well as single toxin expressing plant (Cry1Ac & Cry2Ab)? If so, what is the relative contribution of type 1 resistance and inducible tolerance to their ability to survive on Bt cotton?

Resistance traits caused by recessive mutations and inducible mechanisms may coexist in field insect populations under continuous selection pressure. Given that many type I resistance mechanisms are incompletely recessive, the main question was whether additive effects of the two mechanisms in heterozygotic pest insects can overcome toxin levels in transgenic crops, or allow them to survive when toxin expression declines in crops. The outcomes from this section have direct impacts on the management of Bt resistance in cotton cropping systems.

MATERIALS AND METHODS

a. *Bacillus thuringiensis (Bt) crystal toxins*

The toxins used were *B. thuringiensis* crystal toxins Cry1Ac and Cry2Ab. Cry1Ac was a bulk, crude, bacterial suspension of *B.thuringiensis* strain HD73 containing Cry1Ac toxin (kindly supplied by Dr. John L. Reichelt, Bacterial Fermentation Pty. Ltd., Arundel, Queensland, Australia); and Cry2Ab synthesised from a coding sequence gi|40311|emb|X55416.1| *B. thuringiensis ssp. kurstaki* HD-1 plasmid gene (for the crystal protein Cry2Ab 1902 bp) cloned into an *E. coli* expression vector pQE30 (obtained from Dr Tom Walsh, CSIRO Canberra). Initially, bioassays were performed with egg-hatch neonate larvae and confirmed the relative toxicity of the Cry1Ac and Cry2Ab-containing suspension at different dilutions compared to the negative control preparation (MQ). Aliquots of this bulk preparation were used throughout the project.

b. Insect populations

An established Bt toxin-susceptible *H. armigera* laboratory strain (obtained during 2011 from Dr Sharon Downs, CSIRO Narrabri) was maintained at 25±0.5°C (14/10h, L/D photoperiod) on artificial diet (modified from Teakle and Jensen, 1985) in constant temperature room, Waite Campus, University of Adelaide, South Australia, without exposure to any insecticides. Freshly prepared artificial diet (2ml) was dispensed in 45-well plastic trays (45×12) and left to dry in a fume hood for about 15~20 minute. Neonate larvae were placed individually in each well and heat sealed with Mylar (heat-sealable polyester film). A total of 20 to 25 fine holes were then punched into the film to allow air exchange. Trays were placed in constant temperature room under standard culture conditions (see above). After seven days about 432 (36×12) late 3rd to early 4th instar larvae were transferred individually to comparatively larger 36-well plastic trays containing freshly prepared artificial diet for growing larvae, heat sealed with 11x6" 3MIL/10MP DBL-PNCHD/PERF lids (Oliver-Tolas Healthcare Packaging, Grand Rapids, MI 49504 USA) and placed in constant temperature room. About 300-350 pupae were surface sterilized with 0.1% bleach for 2~3 minutes and rinsed well under cold running water. Once dry, the pupae were set in rectangular (50×30cm) cage for emerging adults. Sterile 0.02% honey solution containing ascorbic acid (2g/L) was supplied in honey pots for the adults. Once mated, about 40~50 moths were then moved to an egg-laying bucket (2.5L, surface painted with black paint) covered with nappy liner for egg laying. Eggs were collected from the nappy liner on a daily basis and sterilized in 0.1% bleach solution before being set for larval emergence. The population was maintained on a weekly basis for a constant supply of egg and/or neonate larvae for experimental purposes. Sub-populations of this susceptible strain was used for laboratory selection with Cry1Ac and Cry2Ab both individually and combined incorporated with artificial diet.

C. Toxin bioassays

Bioassays were performed using an artificial diet overlaid with a crude bacterial suspension containing Cry1Ac or Cry2Ab. In each bioassay, fresh artificial diet was poured into 45-well plastic trays (~2 ml per well) and left to solidify in a fume hood for about 30 min. Because the exact concentration of toxin in each preparation was not known, a preliminary assay was conducted using a broad range of Cry1Ac or Cry2Ab suspension concentrations to determine the appropriate concentration for the formal assay. Full bioassays were then conducted with 10 concentrations (plus a Milli-Q water control) and at least 45 larvae for each concentration. Toxin-containing stock solutions were diluted in Milli-Q water to specific concentrations, and desired (75µl for Cry1Ac and 100 µl for Cry2Ab) aliquots were spread evenly on top of the artificial diet in each well. Trays were left to dry in a fume hood for about 30-60 min. One neonate larva was placed in each well, and the tray sealed as described above. A total of 20 to

25 fine holes were then punched into the film to allow air exchange. Trays were placed in a ventilated room under standard culture conditions (see above) and the efficacy (larval mortality) of the treatments was assessed on day 10.

D. Laboratory selection of susceptible strain to Cry1Ac and/or Cry2Ab toxins

About 450 neonate larvae were individually selected on artificial diet incorporated with sub-lethal concentrations of Cry1Ac and/or Cry2Ab crystal preparation (100µg/ml, note that the concentration refers to total protein within the crude preparation) in 45-well plastic trays (45×10) to establish whether continuous exposure to Bt crystal toxins increases tolerance to the corresponding toxin. Given the high numbers of individual larvae required for bioassays and selected lines, careful operational and logistic preparations went into the implementation of the selection process, including the physical separation of each insect culture in different rooms to prevent any larval escapees from contaminating any of the other strains. After trial and error, we established Cry1Ac (C1 strain), Cry2Ab (C2 strain), and Cry1Ac and Cry2Ab combined (C1C2 strain) tolerant strains under continuous selections. The toxin concentration was maintained at the same level for at least ten generations except for the neonate using in bioassays. To estimate relative tolerance against susceptible strain, full-dose response toxin bioassays (mentioned above) were performed for both selected and unselected control (susceptible strain) for Cry1Ac and Cry2Ab. The tolerant strains were compared with the susceptible strain to estimate the relative tolerance and/or resistance (RR) levels in tolerant strains against respective toxin.

E. Loss of tolerance

If tolerance in the field is due to differential regulation of immune and/or metabolic activities (Rahman et al 2011, Ma et al 2012, and also Mahbub Rahman, CRDC Project UA1201) then it would be transient and in the absence of the toxin and elicitors, the tolerance would disappear within a few generations.

After the C2 and C1C2 colonies had been exposed for 20 and 15 generations respectively, toxin was withdrawn, and bioassays were conducted after 2, 5 and 8 generations of no exposure.

F. Reciprocal genetic crosses

To establish whether the acquisition of tolerance under continuous incremental selection pressure is caused by differential immune and metabolic responses with an epigenetic transmission, or by resistance alleles that pre-existed in the field populations in low frequencies and which were increased in selected populations, we performed reciprocal genetic crosses between susceptible and tolerant strains, and analysed tolerance levels in the offspring.

About 150 pupae each from susceptible (S♀xS♂), C1, C2 and C1C2 tolerant strains (T♀xT♂) were sexed. For T♀xS♂, the tolerant female pupae (T♀) were crossed with susceptible male pupae (S♂), and for S♀xT♂, susceptible female pupae (S♀) were placed with tolerant male pupae (T♂). Full-dose response toxin bioassays were performed following the methodology above with F_1 and F_2 neonate offspring from S♀xS♂, S♀xT♂, T♀xS♂, and T♀xT♂ genetic crosses.

G.Bt cotton flower and leaf bioassays

When the C1, C2, and C1C2 colonies reached their 13th, 13th, and 6th generation respectively, we undertook laboratory bioassays to see if these animals could survive on Bollgard II plant

materials. Using glasshouse materials, we tested the colonies on Bollgard fruit (flowers and bolls) and leaves at CSIRO Narrabri. At generation 16-17 (Year 2013-14) and at generation 25-28 (Year 2014-15), neonates were individually exposed to glasshouse grown cotton leaves (conventional & Bollgard II) embedded on settling agar on 32-well plastic trays as well as on growing cotton plants at glasshouse at Waite Campus, Adelaide University. Larval mortality & larval development (instar) were assessed routinely, and surviving larva moved to fresh leaves to prevent fungal contamination. There were at least 192 neonates in each bioassay and a total of four bioassays for each insect strain. Note: C1: Cry1Ac selected strain; C2: Cry2Ab selected strain; C1C2: Cry1Ac&Cry2Ab selected strain; Sus: susceptible strain; Conv: non-Bt cotton leaf; BGII: Bollgard leaf; GHBGII: bioassay performed in glasshouse on growing Bollgard II cotton plants (n:120 neonates).

G. Statistical analysis

Mortality data were analyzed (POLO-PC software; LeOra Software, Berkeley, CA) to estimate the lethal dose concentrations (LCs). For each probit analysis, the mortality was corrected using Abbott's formula. Differences in susceptibility were considered significant when the 95% confidence intervals did not overlap at LC_{50} values. The resistance ratio (RR) was expressed as the ratio of the LC_{50} value of the relevant sample to that of the Waite susceptible insects.

RESULTS

We exposed susceptible *Helicoverpa armigera* populations to low levels of Cry1Ac and Cry2Ab toxins, either alone (these colonies are currently at 32 generations) or in combination (this colony is currently at 26 generations) by incorporating the toxin into the artificial diet. Given that *H. armigera* can suffer from inbreeding depression, we always had 300+individuals in each strain to maintain genetic diversity in our selected insects. In none of the strains maintained for the past three years we didn't detect any reduction in reproductive success due to inbreeding depression.

Tolerance after continuous exposure to Cry1Ac and Cry2Ab concurrently

When susceptible neonates were selected with Cry1Ac alone (which we called C1 strain), or in combination with Cry2Ab (which we called C1C2 strain) the level of acquired tolerance to Cry1Ac increased significantly within ten generations of selection (Table 4.1, resistance ratio (RR) measured as T♀×T♂/ S♀×S♂ for LD_{50}, and LD_{90} values; in each comparison the resistance level in the selected strains was significantly higher than that in the control).

The bioassay results indicated that in both the C1 and C1C2 strains, tolerance developed in a similar manner, but as the selection progressed, the C1 strain gained higher levels of tolerance. The level of tolerance for C1 and C1C2 at generation 20 increased to 46 (C1) and 26.5 (C1C2) fold (RR) at $LD_{50;}$ and 83 (C1) and 21.7 (C1C2) fold (RR) at LD_{90}. At Generation 31 C1 level of tolerance was 115 fold (RR) at LD_{50} and 173 fold (RR) at LD_{90}. Because the C1C2 population was initiated latter than C1, it is not possible to compare C1 and C1C2 at generation 31. Transmission of tolerance could be due to an epigenetic mechanism showing a strong maternal effect at earlier stages of selection similar to that of Rahman et al, 2011 (also reported in CRDC UA1201), and as the selection progresses other mechanisms may be more likely to contribute to overall tolerance and/or resistance.

Selection	Generation	C1 (Cry1Ac) Strain		C1C2 (Cry1Ac+Cry2Ab) Strain	
		RR at LD_{50} Value	RR at LD_{90} Value	RR at LD_{50} Value	RR at LD_{90} Value
50µg/ml	G5	-	-	13.9	24.2
	G8	-	-	15.4	31.8
	G10	9.3	33.3	-	-
100µg/ml	G13	-	-	17.9	41.4
	G15	20	62.2	-	-
	G20	46	83	26.5	21.7
250µg/ml	G25a	51	38.2	-	-
	G25b	39	34.4	-	-
	G26a	44	45	-	-
	G26b	45	37	-	-
	G29	43	76	-	-
	G31a	115	165	-	-
	G31b	104	173	-	-
SELECTION OFF					
C2 (Cry1Ac) Strain			C1C2 (Cry1Ac+Cry2Ab) Strain		
Generation	RR at LD_{50} Value	RR at LD_{90} Value	Generation	RR at LD_{50} Value	RR at LD_{90} Value
G20	46	83	G13	17.9	41.4
G20	SELECTION OFF		G15	SELECTION OFF	
USG2	46.2	79	USG2	21	22.7
USG5	40	45.3	-	-	-
USG8	12.1	43.3	USG8	13.1	13

Table 4.1. Tolerance and reverse tolerance to Cry1Ac in laboratory *H.armigera* population selected with Cry1Ac alone, and in combination with Cry2Ab on artificial diet. RR: Resistance Ratio compared to susceptible control. In all cases shown above, the selected strain was significantly more tolerant that the control.

When susceptible neonates were selected with Cry2Ab alone (which we called C2 strain), or in combination with Cry1Ac (as mentioned above, C1C2 strain) the level of acquired tolerance to Cry2Ab increased steadily in a similar manner for both populations in the first ten rounds of selection. Again the level of tolerance attained was significantly higher than that of the control (Table 4.2, resistance ratio (RR) measured as T♀×T♂/ S♀×S♂ for LD_{50}, and LD_{90} values, Fig. 4.1). The level of tolerance increased to 64.4, 34.4 fold (RR) at LD_{50} and 39.2, 62.5 fold (RR) at LD_{90} for C2 and C1C2 strain respectively at generation 20; and upto 233 fold (RR) at LD_{50} and 772 fold (RR) at LD_{90} for C2 strain at generation 30. Like Cry1Ac, a comparison between C2 and C1C2 population for Cry2Ab tolerance and/or resistance at generation 31 was not possible due to the time constraints of the current project. Bioassays of neonates from reciprocal genetic crosses at generation 12, 20 and 27 for C2 strain revealed a strong maternal effect prior to significant shift of tolerance and/or resistance (Table 4.3, Fig. 4.1).

Selection	Generation	C2 (Cry2Ab) Strain		C1C2 (Cry1Ac+Cry2Ab) Strain	
		RR at LD_{50} Value	RR at LD_{90} Value	RR at LD_{50} Value	RR at LD_{90} Value
50µg/ml	G1	-	-	-	-
	G3	-	-	6.5	4.8
	G5	6.3	10.6	4.5	16.1
	G10	6.8	21	-	-
100µg/ml	G11	-	-	-	-
	G12	11.6	19.6	-	-
	G13	-	-	38	74
	G15a	11.9	52.4	-	-
	G15b	12	45.5	-	-
	G18	-	-	42	109
	G20	64.4	39.2	34.4	62.5
250µg/ml	G21	-	-	-	-
	G27	77.3	527	-	-
	G30	233	772	-	-
SELECTION OFF					
C2 (Cry1Ac) Strain			C1C2 (Cry1Ac+Cry2Ab) Strain		
Generation	RR at LD_{50} Value	RR at LD_{90} Value	Generation	RR at LD_{50} Value	RR at LD_{90} Value
G20	64.4	39.2	G13	38	74
G20	SELECTION OFF		G15	SELECTION OFF	
USG2	26.5	43.3	USG2	32.75	28.1
USG3	28.9	32.8	USG3	21.3	27.4
USG5	22.3	34.5	USG5	5.1	6.28
USG8	18	21	USG7	13.2	6.25

Table 4.2. Tolerance and reverse tolerance to Cry2Ab in laboratory *H.armigera* population selected with Cry2Ab alone, and in combination with Cry1Ac on artificial diet. RR: Resistance Ratio compared to susceptible control.

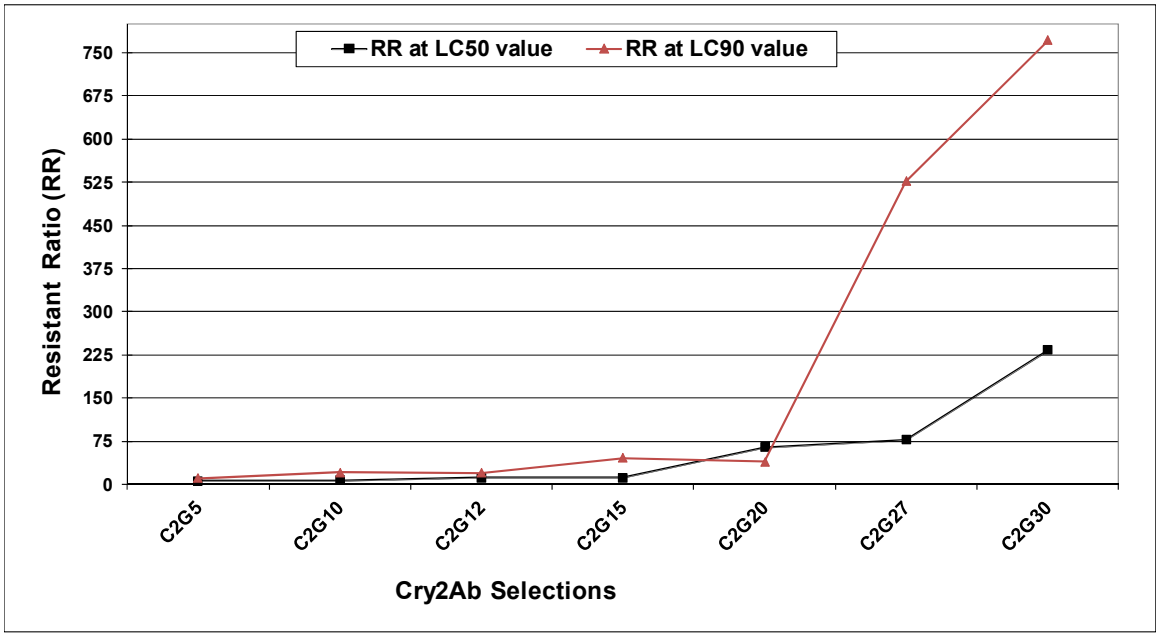

Fig 4.1. Low level selections on susceptible *H.armigera* neonate with Cry2Ab toxin over 30 generations. Susceptible *H.armigera* obtained from CSIRO Narrabri (in 2011) reared on artificial diet incorporated with low Cry2Ab for the last 30 generations. During the course of selections, we performed full-dose response bioassays to estimate acquire tolerance and/or resistance and compared with parental susceptible strain. There are at least 450 neonate for each bioassay.

Insect Strain	LC50	CL	RR	LC90	CL	RR	Slope
Susceptible	150	120.4-188.5	1	817	599-1215	1	1.745+/-0.141
C2G12	1742	1327-2294	11.6	15973	10821-26123	19.6	1.332+/-0.095
(C2G12FXSusM)F1	244	197-301	2.6	1131	845-1650	0.72	1.924+/-0.163
(C2G12FXSusM)F2	304	210-442	3.3	4088	2394-8475	2.61	1.136+/-0.085
(SusFXC2G12M)F1	453	361-566	4.9	1989	1476-2940	1.27	1.994+/-0.169
(SusFXC2G12M)F2	1729	1230-2333	18.8	9044	6230-15355	5.77	1.784+/-0.205
Susceptible	122	70-184	1	1291	803-2717	1	1.254+/-0.154
C2G20	7860	5520-14781	64.4	50604	22757-368258	39.2	1.585+/-0.358
(C2G20FXSusM)F1	5786	4105-15425	47.4	22164	10388-428377	17.2	2.197+/-0.680
(SusFXC2G20M)F1	1848	1219-3188	15.2	14487	6830-64381	11.2	1.433+/-0.211
Susceptible	194	171-219	1	1241	1033-1533	1	1.590+/-0.076
C2G27	14995	4765-354004	77.3	653752	64758-89077765	527	0.782+/-0.169
(C2G27FXSusM)F1	1711	1299-2344	8.8	5744	3912-9957	4.6	1.954+/-0.171
(C2G27FXSusM)F2	3043	2046-5062	15.7	60775	26503-224847	49	0.986+/-0.112
(SusFXC2G27M)F1	1509	906-2640	7.8	6488	3490-21550	5.2	2.023+/-0.175
(SusFXC2G27M)F2	2507	1605-4447	12.9	50109	20304-230682	40.4	0.985+/-0.110
C2G30	45234	19286-334770	233	958913	70353-14541855	772	0.952+/-0.242

Table 4.3. Resistant for Cry2Ab selected *H. armigera* strain and its reciprocal genetic crosses in different stages of selections. There are at least 450 neonate for each bioassay.

Reversion of tolerance when no longer exposed to Bt toxin

To investigate whether the observed acquisition of tolerance is reversible or not, sub-population of C1, C2 and C1C2 strains had been raised on toxin-free diet respectively from generation 20 for C1 and C2, and from generation 15 for C1C2 strain for 8 generations alongside with their parental tolerant strains. Irrespective of strains and toxins, tolerance levels drop gradually to about half to one quarter of that of parental strains (Table 4.1 and 4.2, Fig. 4.2). This suggests that the induction is reversible, probably to prevent the excessive expenditure of internal resources if toxin is no longer present.

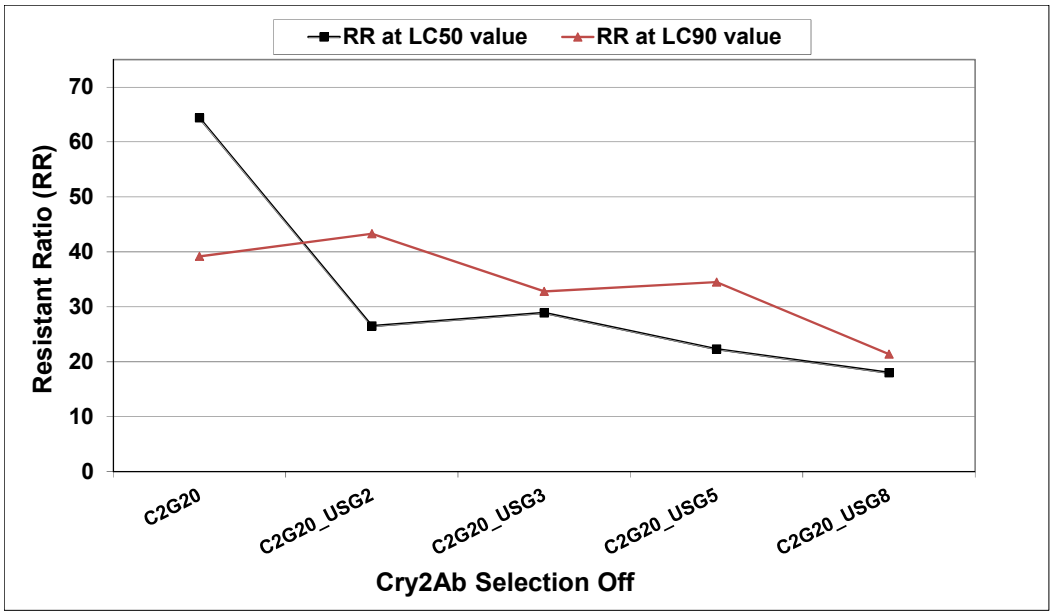

Fig 4.2. Cry2Ab selected strain at generation 20 and its subsequent 8 generations without any selection. Full-dose response bioassays were performed for estimated resistant ratio and compared with parental susceptible strain.

Inducible tolerance for potential to acquire genetic resistance

Since long-term toxin exposure may result in the emergence of recessive target site mutations (Kranthi et al. 2000, Gahan et al. 2005, Liang et al. 2008), we exposed larvae to a Bt toxin preparation at concentrations where lethality was low, which enabled us to focus on inducible metabolic effects that are reversible. Previously, we have identified interactions between mature Bt toxin and lipid particles in the gut lumen of Bt-tolerant insects (Ma et al. 2012) that might affect the growth and the physiology of the toxin exposed insects. Further, we uncovered that significant developmental penalties were associated with inducible tolerance in field-derived laboratory selected Bt tolerant insects, as evidenced by lowered larval weights and increased developmental times (Rahman et al. 2011). However, once the insects were kept at constant toxin levels, the developmental penalties slowly diminished over subsequent generations. This could indicate possible genotypic selection of allelic combinations of multi-gene functions that reduce developmental penalties. This may, in the long term, provide tolerant insects with the adaptive potential to acquire resistance mechanisms that are genetically transmitted and involve target site mutations in important resistance genes.

To test the possibility of genetic resistance in our laboratory Cry1Ac and/or Cry2Ab tolerant *H.armigera* strains, we set up over 60 single pairs that were derived from susceptible GR colony (WS), and C1, C2 and C1C2 strains. The results from the discriminating dose bioassay using the F2 generation of families derived from these *H.armigera* colonies, showed that many larvae survived on Cry2Ab, but fewer survived on Cry1Ac. When we tested the F3 generation of the survivors on Cry2Ab, we again found very high rates of survival on Cry2Ab. Given these high and consistent rates of survival, we suspected that Cry2Ab tolerant strains may have developed genetic resistance to Cry2Ab. Therefore, in collaboration with Dr Tom Walsh (CSIRO Canberra), we investigated larvae and moths from C1, C2 and C1C2 strains together with original parent moths for genetic resistance to Cry1Ac and Cry2Ab. The resistance allele for Cry2Ab present in the *H. armigera* lab colony (SP15) has been identified as a mutation in an ABC transporter gene (Tay et al. In Press). Analysis of the F1 moths from C2 and C1C2 strains showed high numbers carried a resistance gene (HaR01) for Cry2Ab. We have detected Cry2Ab resistance allele (HaR01) in parental moth samples as well.

To date, a total of 8 cadherin alleles genetically-linked to Bt resistance were identified in *H. armigera* (Zhao et al. 2010). We aimed to investigate tolerant larvae for possible mutations in *Ha_BtR* alleles at the genomic DNA level. Although we have not yet analysed the samples

for genetic mutations in *Ha_BtR* alleles at highly tolerant insects (generation 10 onwards), we have previously reported a reduction in diversity in r6 allele between susceptible and tolerant individuals from C1 strains at very early stage of selection (Mahbub Rahman, CRDC UA1201). Sequence results showed close homology with published cadherin sequence with one exception. In the r6 PCR products from the C1, and C1C2 larvae, sequence from certain individuals showed close homology to a mariner transposon. This is the same type of mutation associated with resistance in China where a transposon is inserted into the gene resulting in a premature stop codon. Further sequencing of individuals from these groups identified three individuals that seemed to be carrying this mutation. Some of the sequence was of poor quality suggesting that these individuals may be heterozygotes at this loci. Another possibility could be miss-priming of the r6 primers and as a result we would be sequencing the wrong fragment. However, the 5 prime 60bp of the sequence matched the cadherin gene sequence while the rest was a close match for a mariner transposon which suggests that the sequence is accurate. This type of resistance mutation does not require diversity in the population under selection and can occur at any time. Disruption or enhanced expression of genes by transposons has been associated with the development of resistance in other insect species. Although it is expected that extended incremental selection in the laboratory eventually select for genetic resistance it was a little surprising that it could happen so quickly. If this is truly a Cry1Ac resistant line, then attempts should be made to isolate it from the background and maintain it as a separate line.

Cross-tolerance under incremental selection

Previously, we observed that Bt cry toxins (Cry1Ac and Cry2Ab) have similar glycolipid binding properties (Ma et al. 2012) although bind to different glycoprotein receptors (Tabashnik et al. 2009). We showed that while Cry1Ac and Cry2Ab bind to different glycoprotein receptors on the BBM causing toxicity by different pathways (Tabashnik et al. 2009), their binding to the same glycolipid moiety suggest that sequestration of the toxins resulting in tolerance, may be based on the same mechanism (Ma et al. 2012). Therefore during the course of incremental selection, we assessed cross tolerance to Cry1Ac and Cry2Ab in C1 and C2 strains, and compared with susceptible control. Bioassays results revealed a low but significant cross-tolerance between Cry1Ac and Cry2Ab at the initial phase of the selection with very low concentrations, and that as tolerance increased with incremental increases in selection, the cross-tolerance disappeared (Figs 4.3 & 4.4). However, whether this cross-tolerance will threaten management of *H. armigera* populations in the field will depend on the correlation between developmental penalties and/or fitness costs, mechanism and the relative contribution of the tolerance to overall resistance and extent of cross-resistance under extreme selections.

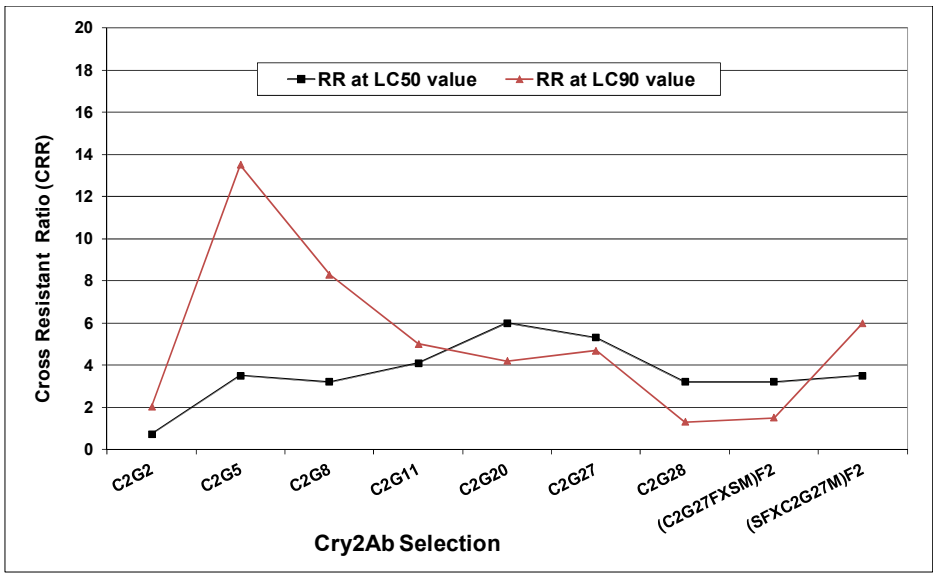

Fig 4.3. Cross-tolerance with Cry1Ac. Cry1Ac bioassays were performed for Cry2Ab neonate and compared with parental susceptible strain.

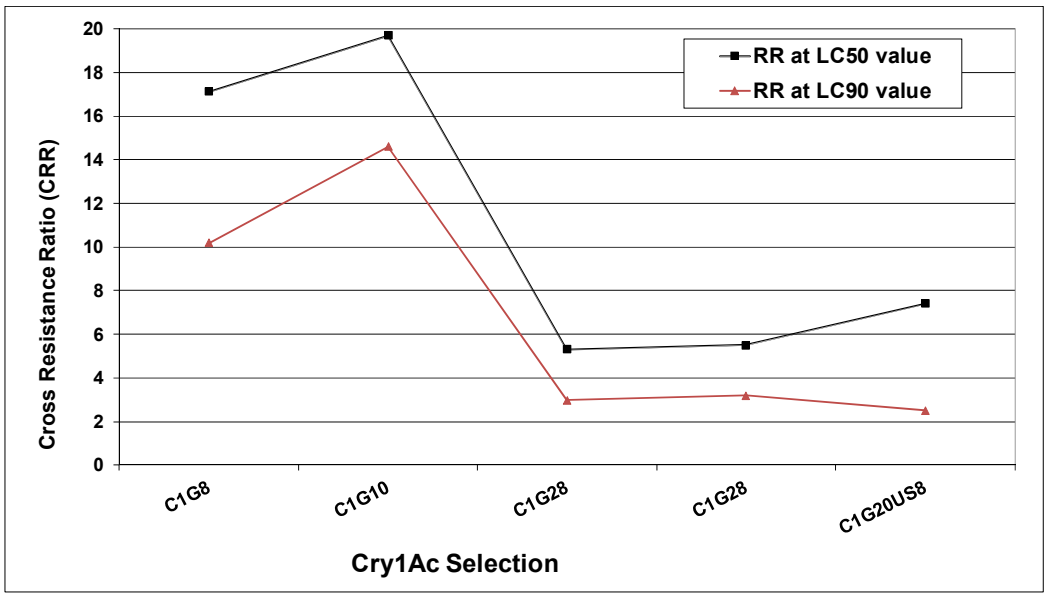

Fig 4.4 Cross-tolerance with Cry2Ab. Cry2Ab bioassays were performed for Cry1Ac neonate and compared with parental susceptible strain.

Survival of tolerant neonate on Bt cotton containing Cry1Ac and Cry2Ab toxins

The aim of this work was to determine whether laboratory tolerant C1, C2 and C1C2 neonates can survive on glasshouse grown plant materials containing Cry1Ac and/or Cry2Ab toxins. Since it took too long to get OGTR permission to grow Bt cotton in Adelaide, we set up several bioassays on glasshouse grown Bollgard fruit (flowers and bolls) and leaves at CSIRO Narrabri when the C1 and C2 colonies reached their 13th generation. None of the larvae in any strains survived on leaves but a very high number of neonates from C2 (but not C1) strain survived on flowers (Fig. 4.5 and 4.6).

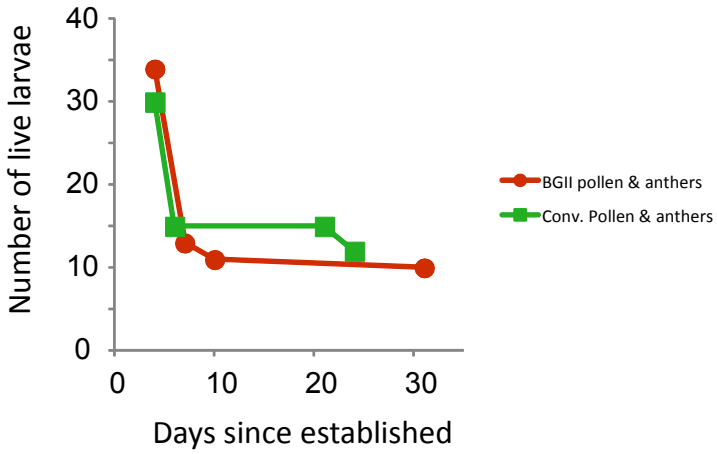

Fig. 4.5 Cry1Ac colony (13th gen) fed on Bollgard II and conventional pollen and anthers. There was no difference in survival.

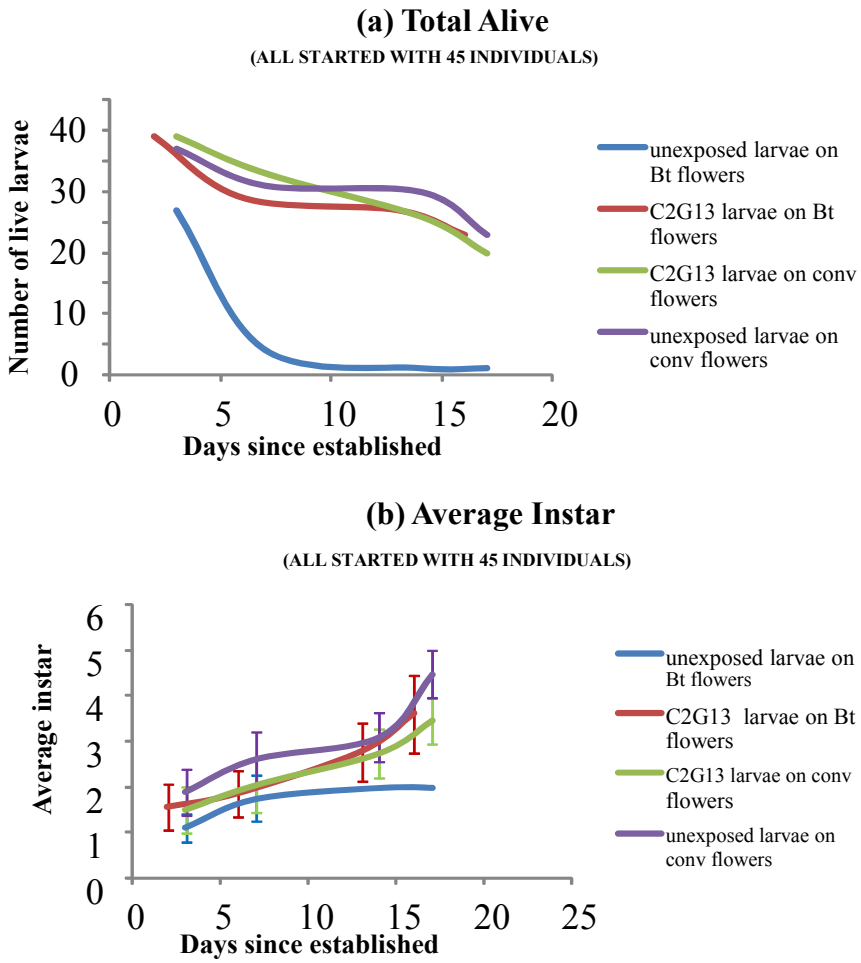

Fig. 4.6. Cry2Ab colony (13th gen) and unexposed larvae on Bollgard II and non-Bt pollen and anthers. (a) There was no difference in survival between C2G13 on Bt or non-Bt pollen and anthers, while larvae that had not been exposed to Cry2Ab died quickly on Bt flowers. (b) The rate of development.

At generation 16-17 (Year 2013-14) and at generation 25-28 (Year 2014-15), C1, C2 and C1C2 neonate were individually exposed to glasshouse grown cotton leaves (conventional & Bollgard II) embedded on settling agar on 32-well plastic trays as well as on growing cotton plants at glasshouse at Waite Campus, Adelaide University. Our 2013-14 bioassay results for neonates that have been selected for three to four additional generations are very consistent with that of Narrabri's earlier bioassay results. At generation 25, we observed very high numbers of C2 and C1C2 neonates surviving on Bollgard II cotton leaves, a significant proportion of the them reached 5th instar, many of them completed their entire life cycle producing viable eggs where susceptible and C1 neonates suffered nearly 100% mortality within seven days of exposer to leaves (Fig. 4.7). Although, initially we thought our glasshouse grown plants were under stress and contained no or significantly less toxin, ELISA analysis of the leaf material indicated that these plants contained normal toxin levels (data not shown) and we had 100% control of susceptible neonates. Similar results were observed in subsequent generations for C2 and C1C2 neonates throughout the season.

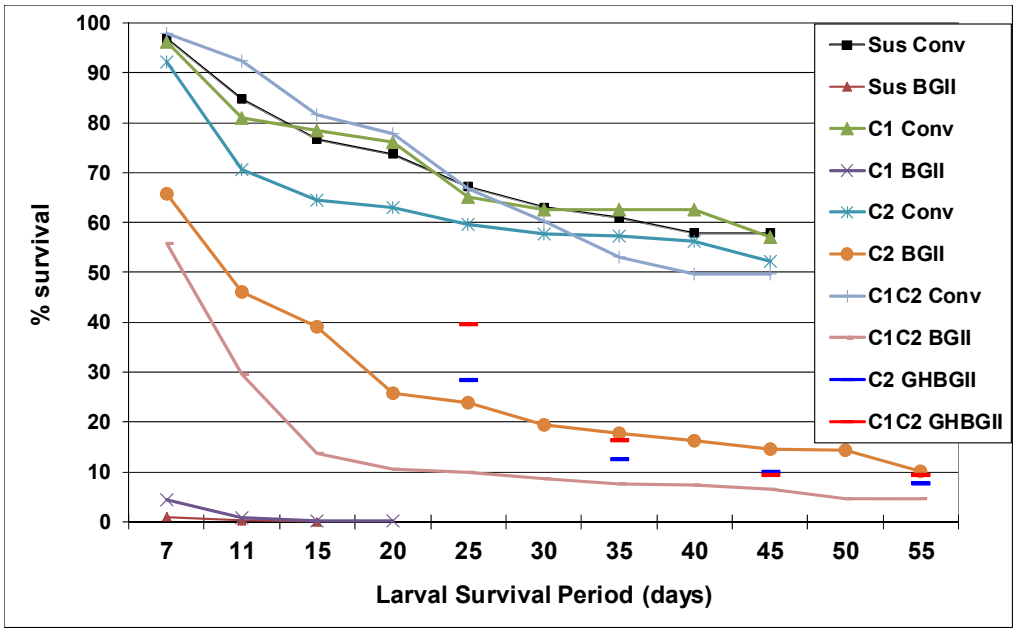

Fig. 4.7. Laboratory selected *H.armigera* neonates (Generation 25: C1C2; 28: C2) surviving on glasshouse grown bollgard II cotton leaves.C1: Cry1Ac selected strain; C2: Cry2Ab selected strain; C1C2: Cry1Ac&Cry2Ab selected strain; Sus: susceptible strain; Conv: conventional cotton leaf; BGII: Bollgard leaf; GHBGII: bioassay performed in glasshouse on whole plant (n:120 neonate).

We back-crossed males and females of our C2 colony with the susceptible colony, and then tested the ability of the offspring (F1 generation) and their offspring (F2 generation) to survive on Bollgard II grown in the glasshouse. We found that the C2 neonates were better able to survive on BGII than their offspring, but that the F2 generation appeared to survive better than the F1 generation (Fig. 4.8). The sex of the C2 moths used to create the back-crosses had no effect on neonate survival.

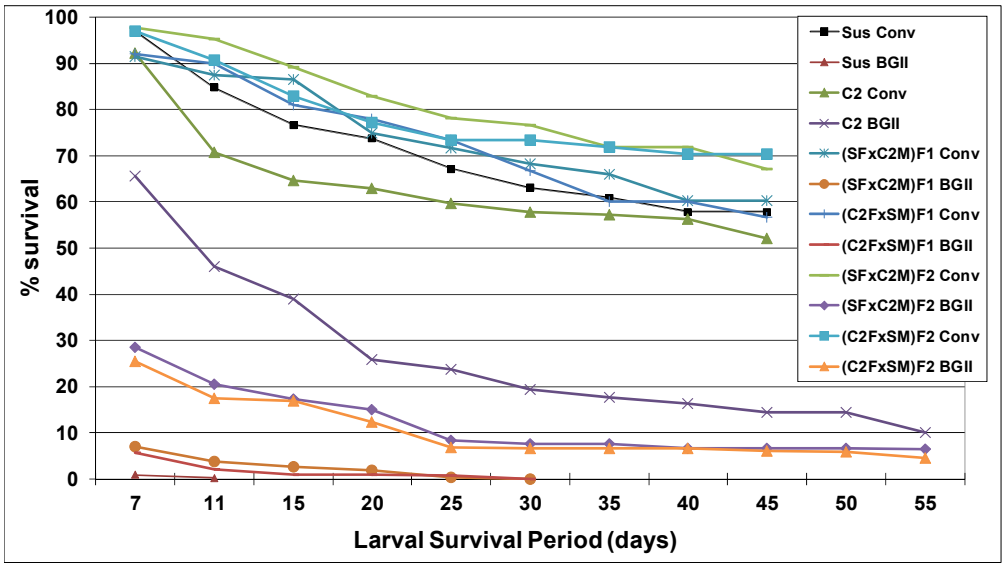

Fig. 4.8. Glasshouse grown Bollgard II leaves bio-assayed for neonates of the Cry2Ab selected strain (generation 27) and its reciprocal genetic crosses (F1 & F2). Susceptible neonates used as control. Note: C2: Cry2Ab selected strain; Sus: susceptible strain; SF, susceptible female; SM, susceptible male; C2F, Cry2Ab selected female; C2M, Cry2Ab selected male, Conv: conventional cotton leaf; BGII: Bollgard leaf; n:120 neonate.

DISCUSSION

Bt-resistance in field populations of cotton bollworm, *Helicoverpa armigera* poses a great threat to transgenic cotton's long-term sustainability. Although the second generation of Bt-cotton Bollgard II® (contains Cry1Ac and Cry2Ab toxins) is expected to provide excellent

season-long control of *Helicoverpa* species, surviving larvae are found on Bt cotton (Section 1). Researchers often comment upon (but generally do not publish) low levels of decreased susceptibility to pesticides that seem to wax and wane irregularly and are difficult to repeat (Ahmad 1999). These effects seem to be separate from target site mutations, providing protection from low to medium pesticide doses. The refuge strategy for transgenic cotton is an integrated set of practices to avoid or delay the evolution of genetic resistance in insect pests. It is based on the assumption that any mutants emerging as heterozygotes in transgenic cotton plantations are less likely to mate with each other if surrounded by susceptible insects, thus avoiding the emergence of homozygous insects, which would become difficult to control even with transgenic plants expressing high levels of the toxin. Since the refuge strategy relies on resistance levels of heterozygotes being well below the levels of toxin expressed by the transgenic crop, it is important to understand whether other resistance mechanisms could interact with recessive type I resistance to contribute to the overall resistance in the field.

Our results show that after low level exposure to Cry2Ab toxins our colonies have the HaR01 Cry2Ab resistance gene. The likelihood of this developing spontaneously as a result of low level exposure in Adelaide is unlikely. The most parsimonious scenario is that the original 2011 susceptible colony had an undetectable level of this gene that increased in concentration with exposure to low level toxins. However, this gene is well known for being completely recessive, with RS individuals providing no reduced susceptibility to Cry2Ab toxins (Mahon et al. 2007). A possible scenario is that the resistant genes were abundant enough that RS individuals were able mate together, producing RR individuals in sufficient numbers to increase. Modelling would need to be done to ascertain what proportion of the population would need to be resistant in order for RR individuals to increase under these conditions. As the initial colony was considered susceptible, we would expect it to contain very low numbers of resistant genes. Another scenario is that low level exposure to the toxin increased the larvae's immune and metabolic responses, thereby enabling more larvae to survive. From this, even the smallest advantage of RS individuals could be magnified to spread through the population. Therefore, the development of tolerance may not only enable more larvae to survive on Bt cotton, but it may also facilitate the development of resistance by enabling a higher proportion of RS individuals to survive.

The development of preventative resistance management strategies is a crucial objective of Program 4 (Crop Protection), given the emphasis on cotton plant resistance against insect pests. The immediate threat to transgenic cotton is the emergence of type I resistance in *H. armigera* populations, which is now managed with the introduction of Bollgard II. Nevertheless, other less known resistance mechanisms (i.e., dominant resistance traits, Gunning et al. 2005; inducible tolerance, Rahman et al. 2004, 2010, Ma et al., 2005) alone or in combination with type I resistance have the potential to create problems particularly when pest populations are continuously exposed to the toxin over many generations. Our observations suggest that incremental increases in tolerance to Bt toxins in *H. armigera* are achievable under laboratory conditions, and the potential exists for emergence of high levels of tolerance and/or resistance in the laboratory and field insects. One way to prevent this from occurring is to interrupt the linage of induced females. i.e., it is possible to prevent high levels of tolerance emerging in the field by ensuring out-crossing selected populations with susceptible females.

A conclusion from this section is the need to enhance strategies that encourage matings between *Helicoverpa* that have been exposed to Bt toxins, and those that have not. Not only does this disrupt the development of type 1 resistance, but it may counter the development of tolerance. This conclusion is consistent with the refuge strategy used in the cotton industry, which encourages the out-crossing of spontaneous mutants with susceptible insects, thereby delaying resistance by maintaining heterozygosity in mutant field populations.

SECTION 5.

Raising susceptible larvae on Bollgard II (Bt) cotton plants

Contributors: Mary Whitehouse, Abbey Johnson, Finnihika Saafi, Thea Liddle, Nikita Holland

INTRODUCTION

From previous sections it is clear that more larvae are surviving on Bt cotton than expected (Sections 1 & 2) and that this is not due to an increase in the number of resistant larvae (Section 2). The survival of susceptible larvae in Bt crops is thought possible by larvae feeding on poorly expressing plants, or parts of the cotton plant that expresses less Bt toxin. In Section 2 we showed that the majority of larvae in Bt cotton were found on flowers, which are thought to produce less toxin. However even to survive on poorly expressing Bt cotton, the larvae need to tolerate some toxin.

In Section 4 we have shown that larvae can develop tolerance by feeding on low levels of toxin, and that this can be passed onto their offspring. In addition families of *H. punctigera* moths emerging from Bt cotton may have higher tolerance to Cry1Ac toxin than those emerging from other crops (Section 2). Our aim with this section is to test if larvae can develop on Bt cotton whose expression of Bt toxin is compromised; and if they can, the second aim is to establish if the offspring of the survivors are better at surviving on Bt cotton than other larvae.

The work of our summer scholarship student (Appendix A) showed that tolerance can be present after only one generation of exposure, and that older larvae of *H. punctigera* were better able to survive the toxin and to pass this tolerance onto their offspring. This situation mimics the situlation when larvae move into a Bt cotton field from another non-Bt field which has become unsuitable. Therefore our third aim is to see of older, susceptible larvae are better able to survive on Bt toxin.

METHODS

Culturing larvae

In order to raise large numbers of larvae on Bt cotton plants we needed to develop some techniques. Neonate larvae were placed in 32 well trays filled with agar. Here they were given *Helicoverpa* eggs to consume (as additional supplementary food) and given twice a week fresh anthers and pollen. When they reached 2nd instar they were moved onto a flower (petals removed) where they could still eat the pollen, but could also access the base of the flower. Once they reached 4-5 instar, they were moved onto bolls where we had pre-cut a hole through the boll's skin to enable the larvae to avoid feeding on the outer surface of the boll and feed in the center. At about 4th instar we often provided both flowers and bolls so larvae could choose their food source (Fig. 5.1).

Experimental designs

1. *Raising* Helicoverpa *larvae on Bt plant material from neonates*

During the 2012/13 season both *H.armigera* and *H.punctigera* neonates were established on both Bt and non-Bt plant material. Those set up on Bt plant material (Sicot 71 BRF) were cultured following the techniques above. Those set up on non-Bt plant material (Sicot 71 RRF) were initially given leaf discs on the algar wells (in 32 well trays) and then transferred to Bt leaves in vials. Their development was recorded.

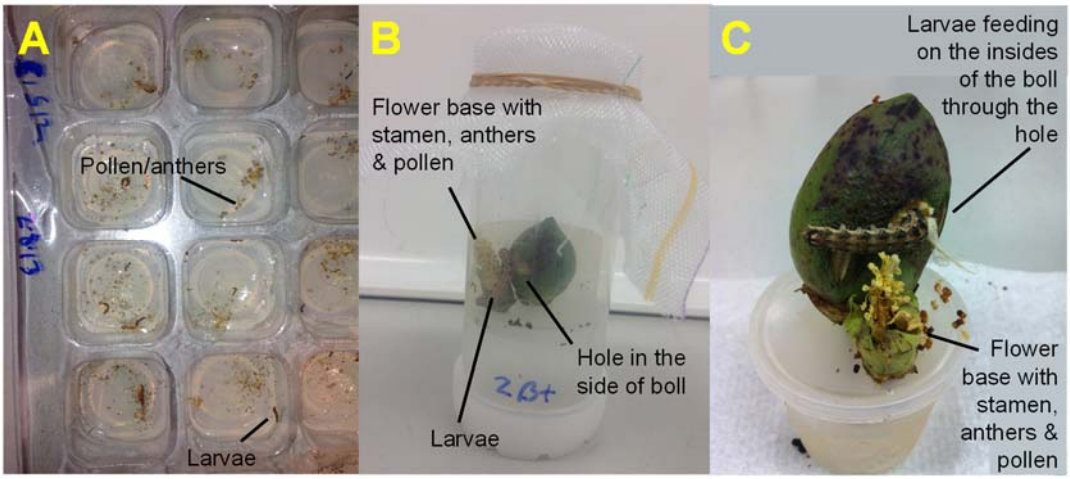

Fig. 5.1 plant food provided to larvae at different stages of development. A: for neonates to 2nd instar; B: from 2nd instar to about 4th instar; C: Bolls from 4th to pupation

2. *Raising* Helicoverpa punctigera *larvae on Bt plant material from third instar.*

A colony was established from *H. punctigera* moths that had emerged from pigeon pea in the 2013/14 season (Sections 1 & 2). In the 2014/15 season (March) we raised neonates from this colony on diet until they reached third instar. From this cohort we set up 48 individuals on flowers and bolls (see Fig. 5.1, B & C); 30 on non-Bt flower and bolls. We also set up 100 neonates (in groups of 5) on flowers (Fig.5.1 B). We recorded how many survived to pupation.

Measuring the amount of Bt toxin in the plant material given to the larvae.

Relative concentrations of toxins January 2014

During the 2013/14 season we estimated the relative amount of toxin in the plant material we were feeding the larvae, by taking about 20 mg of wet weight from different plant structures (boll walls, leaves, boll contents, pollen & anthers, petals) weighing them, and then crushing them in distilled water, before sending them to CSD (Cotton Seed Distributors) for processing using EVO 200 luquid handling system, which uses EnviroLogix QualiPlate Kits to provide qualitative estimates of the amount of toxins in the samples. These kits are a "sandwich" Enzyme –linked ImmunoSorbent Assay (ELISA). The supernate from the plant material is added to the test wells of the kit, which are coated with antibodies raised against Cry1Ac or Cry2Aa toxins. Any residues present in the sample extract bind to the antibodies and are detected by the addition of enzyme-labeled Cry1Ac / or Cry2Aa antibodies. After washing the plate the results of the assay are revealed in a colour development step, where the amount of colour is proportional to the amount of toxin in the sample extract, which is read by a spectrometre.

This method enabled us to establish if different parts of the cotton plant had more or less toxin, but the difference could not be quantified because it was not possible to calibrate the spectrometre reading with its toxin concentration.

Relative concentrations of toxins March 2015

To try to get the most accurate readings of the Bt toxin concentrations of different plant structures we took 3 flowers, and 3 bolls, separated them into different constituents, and sampled about an "Eppendorf diameter" of material (the aim was to obtain 5 mg). We weighed the samples, freeze-dried them, weighed them, then crushed them, and where

necessary re-weighed them. Samples were then taken to CSD where they were ground in 500ul of extraction buffer, Sodium teraborate. The samples were then centrifuged down three times to get all the particulate matter at the bottom of the tubes. We used 3 serial dilutions (1, 10 100) for each sample with both Cry1Ac and Cry2Ab tests which were processed using EVO as above.

Preliminary tests indicated that between spectrometre readings of 0.2 and 3.5 was when the correlation between the spectrometer readings and the amount of toxin in the sample was most reliable. We compared samples by plotting the spectrometer readings for the three serial dilutions of each sample against their dry weights. Because we assumed a linear relationship between the serial dilution readings, we were able to use the same the dry weight for all samples. We chose the dry weight where most samples had spectrometer readings between 0.2 and 3 to give us the best qualitative comparisons of the amount of toxin in the different plant structures.

RESULTS

1. *Raising* Helicoverpa armigera *larvae on Bt plant material from neonates*

Over the course of the 2012/13 season, 3966 neonates were set up on Bt plant material over 6 months (from the 31st of October 2012 until the 7th of May 2013). Plant material was initially sourced from the glasshouse, and then sourced from the field. The second generation of 1239 neonates was set up on Bt plant material over 1 month from the 27th of May 2013 to the 25th June 2013. As the experiment was stopped after the cotton was killed by the frosts by the 24th of July, the last co-hort only had 28 days in which to pupate. The first generation took on average 36 days to pupate. Therefore, in order to compare the first with the second generations, we only analysed co-horts establish before the 19th of June, to enable them time to pupate (=1089 neonates).

Of the 3966 individuals set up in the first generation, only 50 pupated (1.3%) while 49 pupated from the 1089 neonates of the second generation (4.5%). Therefore significantly more larvae survived to pupation in the second generation (Fig. 5.2, Chisq = 44, $P<0.001$, df=1). Larvae took on average 36 days to mature.

In the same time frame that it took to complete two generations on Bt cotton material (31st of October 2012 until the 19th June 2013) four generations (1860 neonates) were completed on non-Bt plant material (Fig. 5.2). The survival rate was much higher (30-8.8%, Fig. 5.2) but still not very high. There was also a significant difference in survivorship between generations (chi sq= 58, $P<0.001$, df=3). Larvae took on average 34 days to mature.

As the second generation of larvae on both Bt and non-Bt cotton were more likely to survive, we compared the change in survivorship between the first and second generations of larvae on Bt cotton and non-Bt cotton. We found that there was a bigger increase in survival between generations on Bt cotton than generations on non-Bt cotton (log linear analysis; df=7, overall $P<0.001$; Crop x Generation x Survival: $P=0.031$).

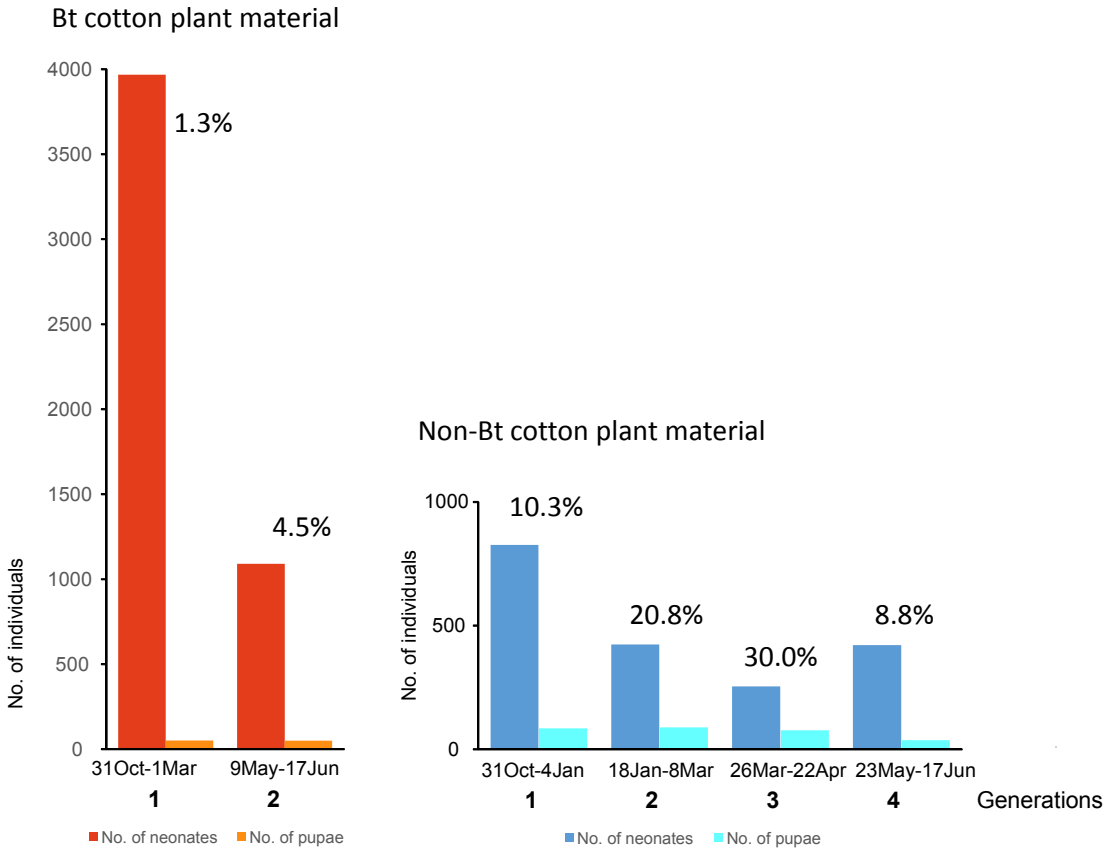

Fig. 5.2. The number of neonates (and resulting pupae) per generation set up on Bt and non-Bt cotton plant material between the 31 Oct 2012 and the 17th June 2013.

The highest survivorship of neonates on Bt plant material occurred latter in the season in March, April and May (Fig. 5.3). This was not reflected in the neonates raised on non-Bt cotton material, where the highest percentage reaching pupation occurred in January.

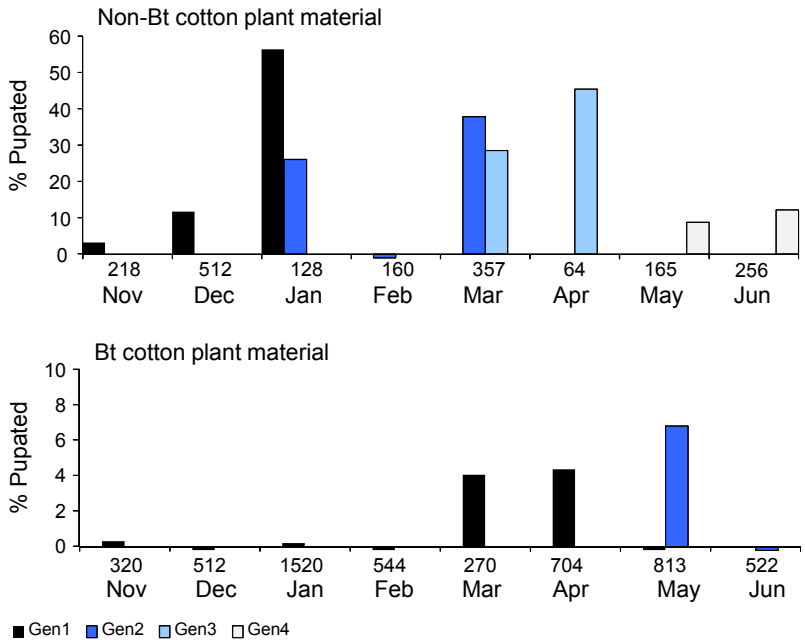

Fig. 5.3 The percentage of neonates (raised on Bt and non-Bt plant material) that survived to pupation for each generation per month. The number under the histogram is the number of neonates set up that month. Dashes under the line indicate cohorts where neonates were established but none reached pupation.

2. *Raising* Helicoverpa punctigera *larvae on Bt plant material from third instar.*

While none of the 100 neonates set up on Bt flowers survived to pupae, 15 of the 30 third instars set up on non-Bt cotton flowers reached pupation (50%), and 5 of the 48 set up on Bt cotton flowers (10%; Fig. 5.4). Thus third instar *H. punctigera* had a much higher chance of surviving on Bt cotton flowers than neonates (Fisher's exact test: P=0.002).

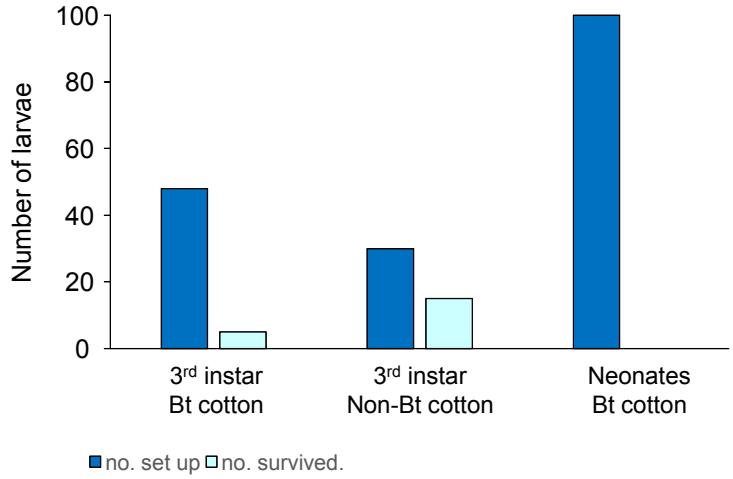

Fig. 5.4 Survival of *Helicoverpa punctigera* larvae set up as 3rd instars or neonates on Bt cotton or non-Bt cotton.

Measuring the amount of Bt toxin in the plant material given to the larvae.

2014 Relative concentrations of toxins

Comparative analyses of the toxin concentrations of different parts of the Bt cotton plant revealed that at the end of January, all structures tested (pollen & anthers, petals, boll skins, boll internals) had lower concentrations per wet weight of Cry1Ac than leaf samples, independent of whether they were sampled from the tail ditch end, the head ditch end, or the middle of the field (Fig. 5.5). This was also true for Cry2Ab toxin, except for petals, which seemed to have a higher concentration of Cry2Ab toxin.

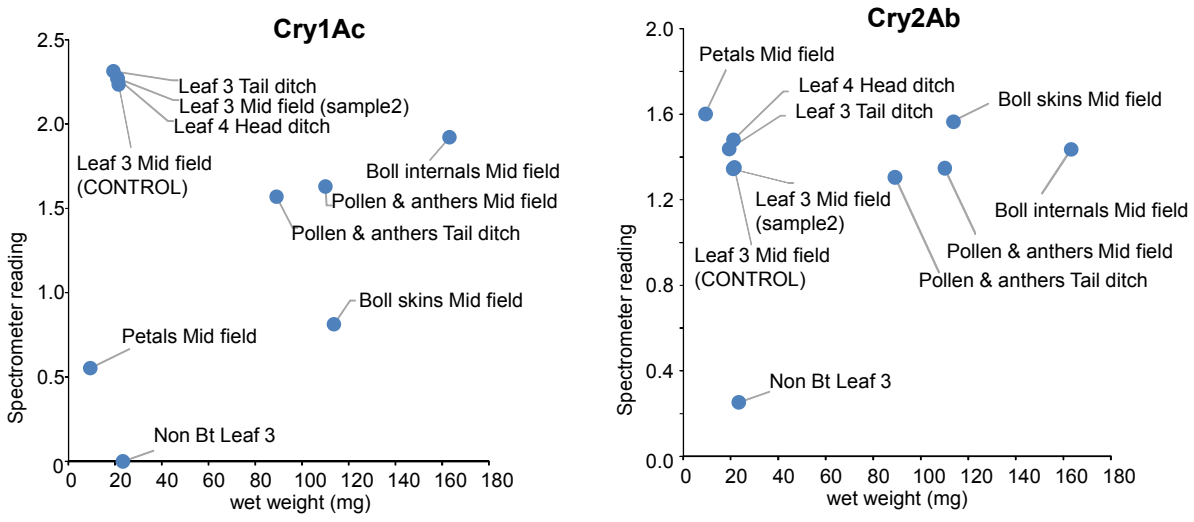

Fig 5.5 Relative concentrations of Cry1Ac and Cry2Ab toxins as measured by spectrometre per wet weight in different parts of the cotton plant sampled 31st January 2014.

2015 Relative concentrations of toxins

Plant samples taken in March 2015 produced similar results to those taken in January 2014 (Fig. 5.6). We found Cry1Ac levels in pollen+anthers, Bt boll internals, and Bt boll skin had lower levels of toxin than the leaves, but that Bt flower base internals had higher levels. In respect to Cry2Ab, in these samples (which were collected from the field in March) pollen+anthers, Bt flower internals, and Bt flower base skin had similar levels of toxin to the leaves, but Bt boll internals and Bt boll skin had lower levels.

The samples from different parts of the plant also reacted quite differently to the sodium tetraborate buffer (Fig. 5.6) emphasizing differences in the chemical composition of the different structures.

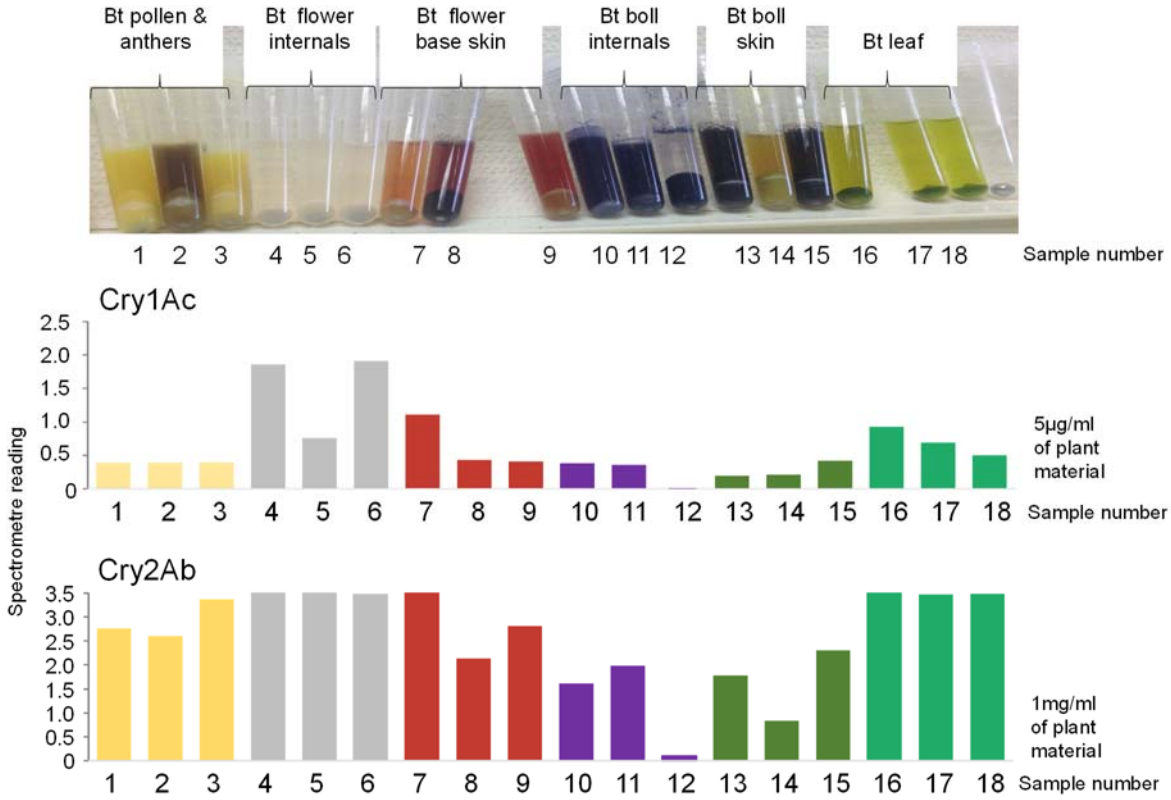

Fig. 5.6. Qualitative estimates of different levels of toxins in different plant structures in Cotton collected from the field in March. The top band shows the samples in the buffer.

DISCUSSION

These results show that both susceptible *Helicoverpa armigera* and *punctigera* can survive on Bt cotton, but at low rates. *H. armigera* in particular appears to be better able to overcome the toxins towards the end of the season, with very few surviving early in the season. This matches the common understanding that the efficacy of Bt cotton drops as the season progresses, however it doesn't match the findings from the field cage work (Section 1) where moths emerged from Bt cotton throughout the season and not just at the end of the season. Thus other factors, not just toxin efficacy, may influence *Helicoverpa* survival on Bt cotton.

There were also differences in survivorship between generations of larvae raised on non-Bt cotton, which could reflect changes in the palatability of the cotton material over time. The different reactions of the plant structures to the tetraborate buffer indicated differences in the chemical composition of different plant structures upon which the larvae could feed. How Bt toxins interact with these chemicals (which include tannins) in the cotton plant may be very

important in determining whether larvae are able to feed on those structures. Future work examining this interaction would be useful.

These results indicated that the offspring of larvae raised on Bt cotton (from neonates) are more likely to survive on Bt cotton plant structures than their parents. However, one of the problems with this work is establishing whether the difference between generations is caused by a change in tolerance levels, or simply caused by an increase in overall fitness. In Sharna Holman's work (Appendix A) she reported the offspring which had the highest tolerance level were moths that were only exposed to Bt toxins as late instars and experienced the lowest fatalities. If the increase in survival on Bt toxins was due to increased overall fitness, then in Holman's work the group exposed as neonates, where a higher proportion died, should have had the highest tolerance level. Therefore, Holman's results suggest that the larvae didn't increase their overall fitness but increased their tolerance levels. The results presented here also show that late instar *Helicoverap punctigera* larvae are more likely to survive to pupation on Bt cotton than neonates. The next step is to see if the offspring of late instar larvae are also more likely to survive on Bt cotton than their parents.

SECTION 6.

Larval movement between Bt cotton and its refuges.

Contributors: MaryWhitehouse, Abbey Johnson

INTRODUCTION

Refuges are an important aspect of the Resistance Management Program, as they produce moths that have not been exposed to Bt toxins, which can then mate with any moths emerging from Bt cotton that carry resistant genes, thereby diluting the resistant genes. In order to be effective, refuges need to be large enough so that they attract laying moths, and close to Bt cotton to facilitate mating between moths emerging from both crops.

The current requirement for refuges is that they need to be at least 24 rows wide. But is this wide enough to be attractive to laying moths? An aim of this section is to see if laying moths differentiate between crops at a scale of 24 rows, and if a refuge close to Bt cotton and attracting high egg lays causes higher egg lays in the Bt cotton.

Section 5 demonstrated that larvae feeding on Bt toxin after third instar were more likely to survive than those initially exposed as neonates. This finding also supports the finding from the Summer Scholarship (Appendix A) that larvae exposed to Bt toxins from 3rd instar onwards had a better survival rate on Bt toxins, and that their offspring had higher tolerance to these toxins than those of larvae unexposed. Because refuges need to be close to Bt cotton, but only need to be 5-10% of the land area of Bt cotton, refuges are often the last 24 rows on the edge of a large Bt cotton field. Given that larvae are known to move between fields, an aim of this section is to establish if there is potential danger that larvae will walk from refuges into Bt cotton thereby gaining exposure to Bt toxins at the critical period of late instar.

METHODS

Sampling method

All sampling undertaken in this Section involved searching the plants for either larvae or eggs. For the 2012/13 season work, 1 metre samples were examined intensely for either eggs or larvae at the locations indicated by the crosses in Figs 6.1 (eggs) and 6.2 (larvae). For the 2014/15 season work, we searched plants for marked larvae near where they had last been seen. We had a time limit of 4 mins. If larvae were not found in this time period, we assumed they has moved further than our searching circle (1m radius).

Do ovipositing Helicoverpa *differentiate crops at 24 rows?*

In the 2012/13 season, Bt cotton plots were set up in between Pigeon pea and non-Bt cotton plots to test if more eggs were laid in pigeon pea than Bt plots, and if Bt in the rows next to the pigeon pea crops, also received heavier lays. We set up four groups of 3 plots; each plot was 24 rows wide and 23 m long (Fig. 6.1). In nine locations within each plot we undertook one metre visual searches, either in the outer or central rows (Fig. 6.1, black crosses). Each time we sampled (10th Jan, 7th Feb) we sampled the same rows, but changed the distance from the tail ditch, so we didn't resample the same locations.

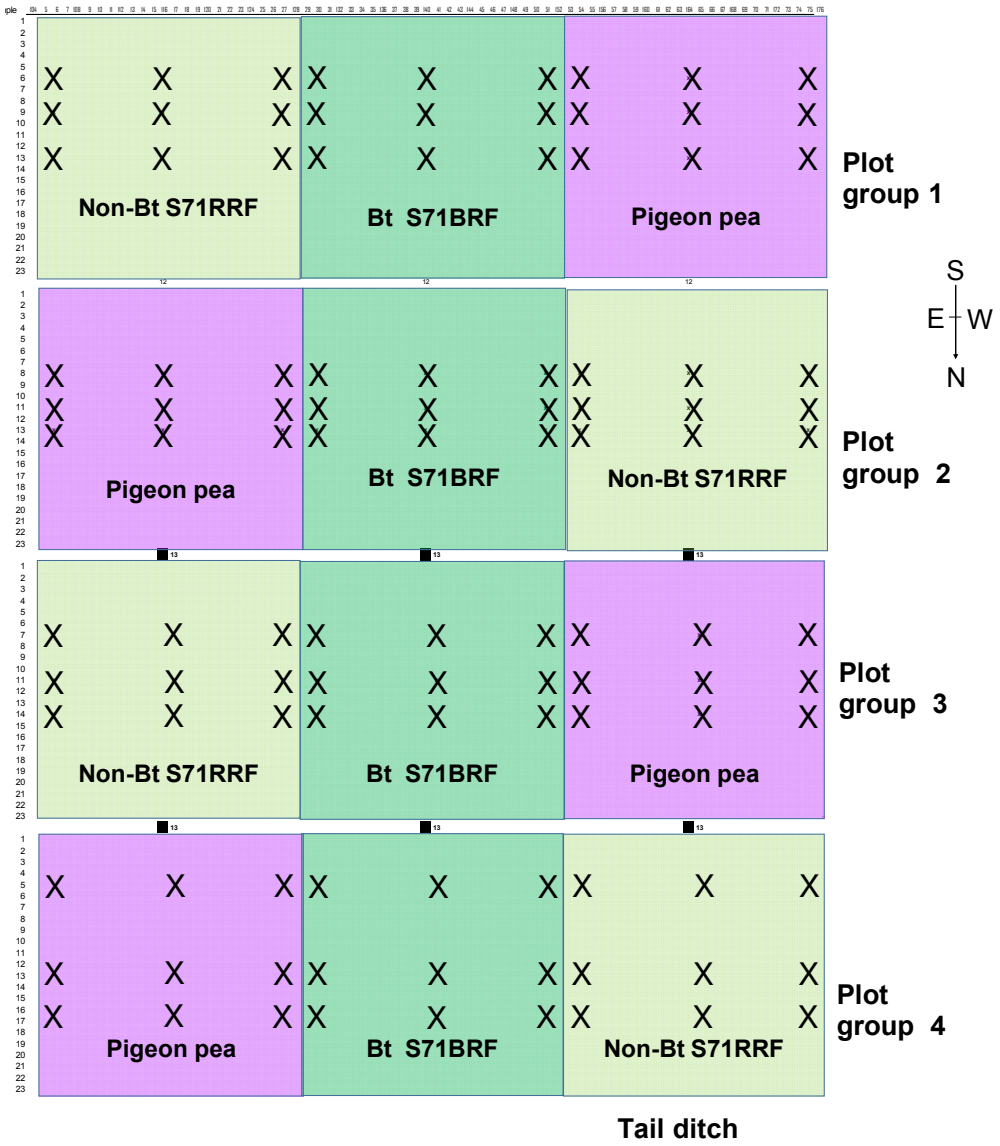

Fig. 6.1 The location of the first visual searches for *Helicoverpa* eggs. For each plot group the metre distance of the searches from the tail ditch changed each time the survey was undertaken, so no two surveys were undertaken in the same spots.

Do Helicoverpa move between crops?

In the 2012/13 season, we again used the 3 x 4 plots (Fig. 6.2) but this time we stacked the plots with 3rd -4th instar larvae. Larvae were marked by covering them in florescent powder (HCA Colours Australia, VM317 Yellow).

The marked larvae were placed on either the edge of refuges or edge of Bt cotton plots (the black dots in Fig. 6.2 indicate the position of the first release of larvae at the edge of refuges). All larvae were released in the outer row of the crop, but the distance from the tail ditch changed, so that we didn't release the larvae at the same location each time. The pattern of sampling around the release points moved with the release points. Larvae were released on the edge of the Bt plot on the 15th Jan (plots were searched for larvae 1 and 3 days after the larvae were released); and on the 20th of Feb (plots were searched on days 1 and 2). Larvae were released on the edge of refuge plots on the 11th Feb (plots were searched for larvae on days 1 and 2) and on the 15th March (plots searched on day 1 only).

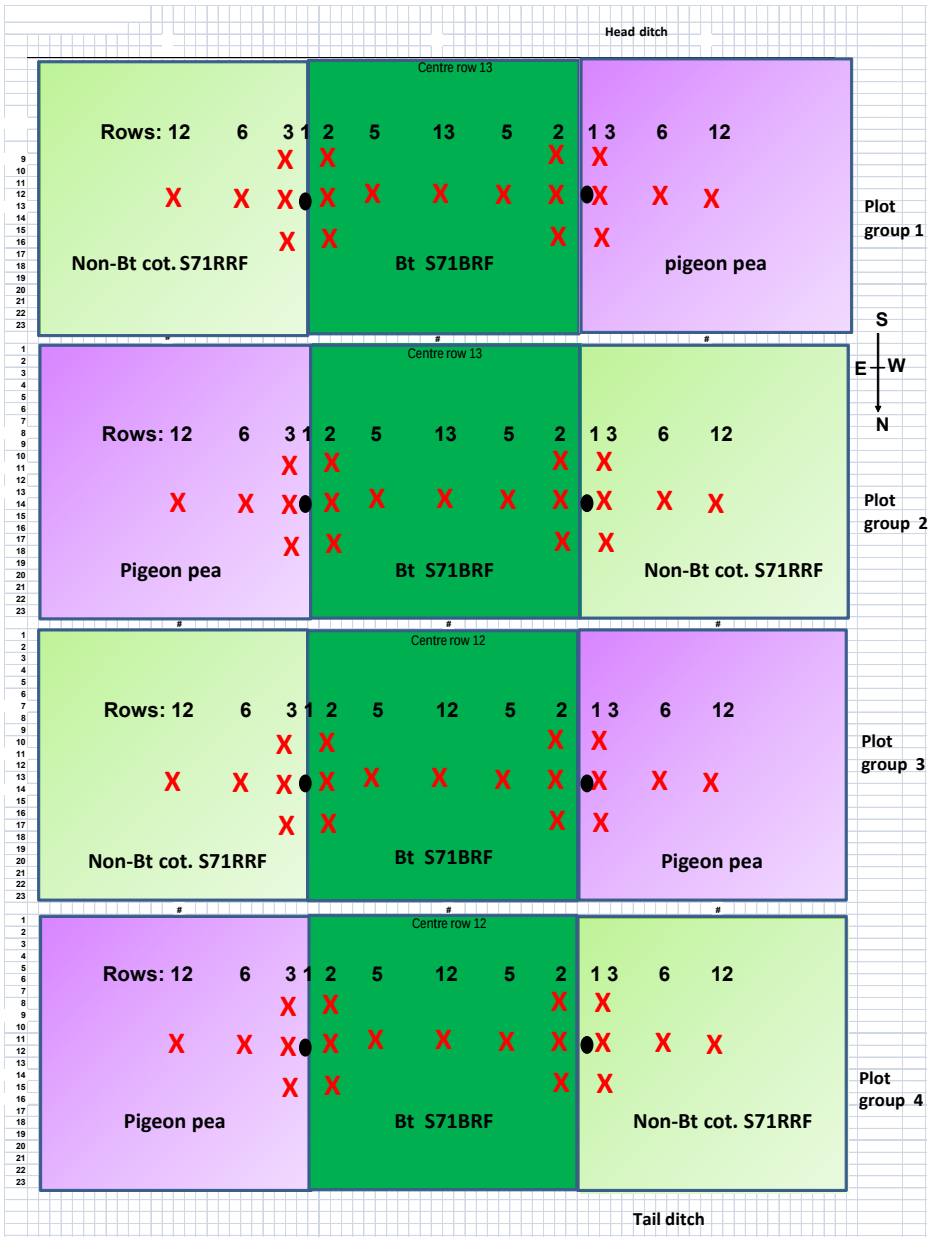

Fig. 6.2. The plot layout for experiments examining *Helicoverpa* movement between Bt cotton and either cotton or pigeon pea refuges. The black dot indicates where the larvae were released, the red crosses indicate where we searched for larvae, and row numbers indicate the rows in which those searches took place.

2014/15 experiments following individual larvae: The effect of plot type on larval movement.

To understand when larvae may move between Bt cotton and pigeon pea, we undertook a series of experiments on small pigeon pea plots (between Bt cotton plots) and followed the movement of individual larvae.

Four columns of pigeon pea plots (6 rows wide, 16 m long) were set up between four columns of Bt cotton (plots also 6 rows wide, 16m long, Fig. 6.3). There was a gap of 2 metres between the columns of cotton and pigeon pea plots and between each plot. The pigeon pea plots varied in quality, with columns B and D unirrigated, and columns A and C irrigated. Column D and its outer Bt cotton column were particularly poor quality and did not receive any pre-season nitrogen application to the soil (Fig. 6.3). All the other plots received 120 units of nitrogen pre-season. Some pigeon pea plots were also planted without inoculant, and in other plots flowers were removed from the outer 2 rows, or occasionally from the whole plot (16th Jan). On the 17th of January all fruiting bodies were counted from the central 2 metres in the second row of each plot.

All experiments took place in the early evening between 6pm and 10pm. Sunset occurred at about 7:45pm.

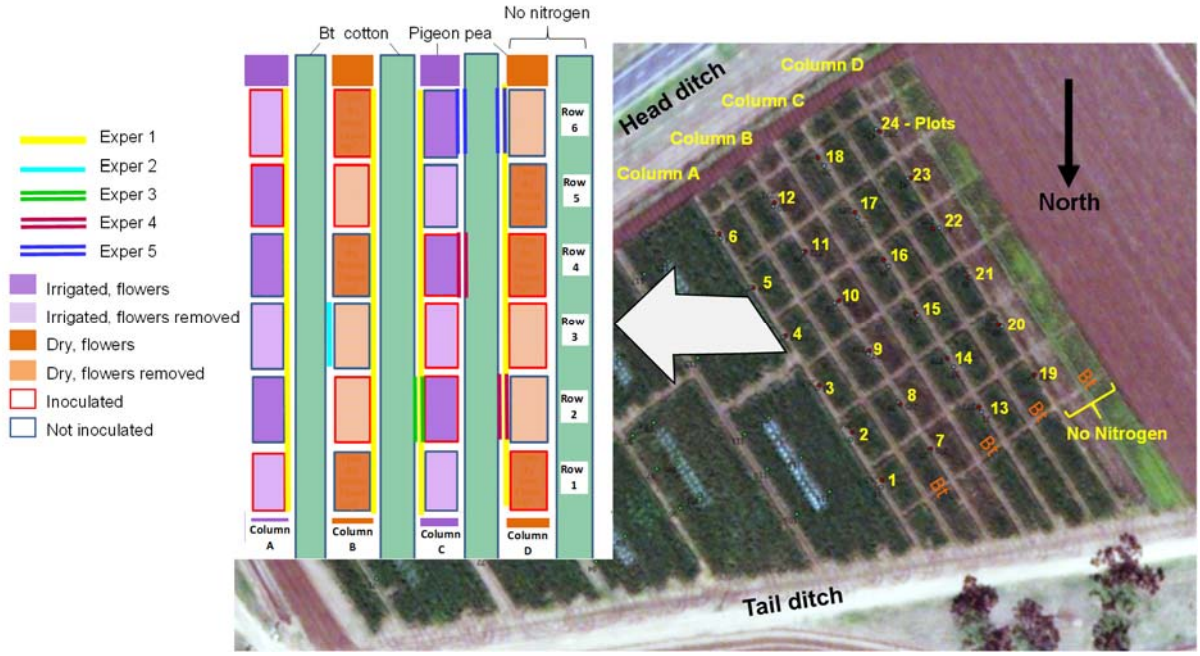

Fig. 6.3 Satellite image (from the tail ditch perspective) of pigeon pea plots used in the 2014/15 season. These are the same plots seen in Fig 1.3 and 8.3. The insert provides more detail on the treatments per plot, and also where among the plots the experiments were located.

Larvae used in these experiments were marked using white correcting fluid – Artline 400XF "paint marker". To identify up to 26 larvae individually (see experiments 2 to 5) we used a base 3 system, where we divided the larvae into three parts along its back; the head region (ones), the middle (threes), and the tail (nines). Fig. 6.4 shows how we marked larvae up to 25.

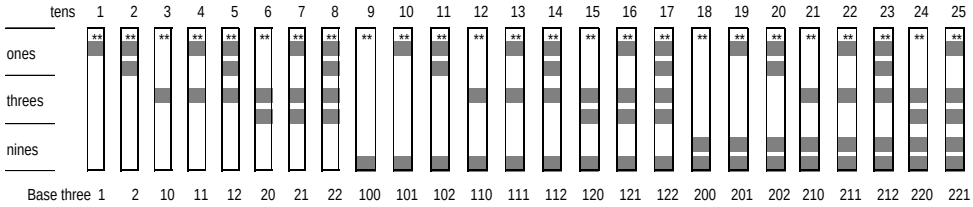

Fig. 6.4. Diagram showing how larvae were individually marked up to 25 using base 3, where the ones are marks in the head region, multiples of threes are marks in the middle regions and multiples of nine are marks in the tail region.

Exper.1, Are larvae on flowering pigeon pea less likely to move than larvae on non-flowering, poor quality pigeon pea?

On the 15th of Jan, at 6 -7 pm three *Helicoverpa armigera* larvae per pigeon pea plot were set up on the outer row on one side of each column facing Bt cotton (78 larvae in total). Larvae were marked with a dot of correcting fluid on either the dorsal surface of the head, the center of the body, or at the hind region, to distinguish between the 3 individuals per plot. A larvae were placed 5m from either end of the plot and the third in the middle of the plot. They were placed on a leaf in the top ¼ of the plant (about 20-40 cm from the top of the plant) and their location marked with a bit of masking tape. They were checked after an hour (7-8 pm) and then rechecked after an hour and a-half (8:30-9:30 pm). We allowed 3-4 minutes to search for each larvae.

Exper 2, larval movement on poor quality pigeon pea.

Results from Exper. 1 showed that larvae on some refuges disappeared within the 1 hour (once they move more than a metre away from their last known location they were very difficult to find). To observe *H. armigera*'s movements on poor pigeon pea, on the 22nd Jan 2015 we set up 17 individually marked (with correcting fluid) *H. armigera* larvae on Plot 9 (no-flowers, no irrigation, no inoculant; Fig. 6.3). Larvae were placed along the edge of the plot at 7pm, starting 1 m from the northern end and adding one every following metre. Larvae were checked every 10-20 mins and followed for one and a-half hours until rain stopped observations.

Exper 3, Are larvae on Bt cotton more likely to move than larvae on flowering (good quality) pigeon pea?

On the 29th of Jan (at 6 pm) six individually marked (with correcting fluid) *H. armigera* larvae (early 4th instars) were set up on the edge of an irrigated, inoculated flowering pigeon pea plot (Plot 14, Fig. 6.4) and six more were set up on the facing Bt cotton. As with the previous experiments, larvae were from the susceptible lab colony reared on diet. The larvae's movements were recorded every 10 minutes, and the larvae were watched for 2 ½ hours 6-8:30pm. We checked the larvae again the following morning at 8:30am, and in the afternoon at 4:20pm.

Exper 4 & 5. Are 4th instar larvae with experience on cotton more likely to move on Bt cotton than larvae on flowering (good quality) or non-flowering (bad quality) pigeon pea?

We were concerned that a lack of experience of plants may be influencing the larvae's behavior. To give the laboratory colony larvae plant experience, we put out 12 bags of 5 2nd - 3rd instar *H.armigera* on the terminal branches of field-growing non-Bt cotton, and left the larvae for a week until they were early 4th instar. We then collected the larvae, individually marked them with correction fluid, and stored them on diet for use that night. On the 5th of Feb at 5:45 pm we set up 6 larvae on both Bt cotton and healthy irrigated pigeon pea (Plot 16, Fig. 6.3) and then 20 minutes later, set up larvae on Bt cotton and poor quality dryland pigeon pea from where flowers had been removed (Plot 20, Fig. 6.3). As always, the location of the larvae were marked with masking tape.

This process was repeated (Exper. 5) with pigeon pea Plots 18 (good quality irrigated) and Plot 24 (poor quality unirrigated) on the 18th of Feb 2015.

RESULTS

Do ovipositing Helicoverpa *differenciate crops at 24 rows?*

We counted 132 eggs from 93 1m visual samples in the January survey and 104 eggs from 105 visual samples in the February survey (some visual samples weren't undertaken due to an infestation of nutgrass). The majority of those eggs were collected from the pigeon pea plots which were flowering (Table 6.1). Table 6.1 shows that moths showed a very clear preference for laying eggs on pigeon pea during these surveys (Kruskal-Wallis one-way ANOVA, $H=13.3$, $df=2$, $P<0.001$, mean rank: pigeon pea = 20, Bt cotton = 10, Non-Bt cotton = 8) and that within the pigeon pea crop there was no preference to lay either at the field edge, middle, or near the Bt cotton edge of the field (ANOVA, $P=0.7$, $F=0.2$, $df=50$).

Jan10th 2013				12th Feb 2013			
Non-Bt 0	Bt 4	Ppea 40	Group 1	Non-Bt 1	Bt 0	Ppea 21	Group 1
Ppea 36 (nut grass)	Bt 6	Non-Bt 0	Group 2	Ppea 43	Bt 0	Non-Bt 0	Group 2
Non-Bt 1	Bt 1	Ppea 34	Group 3	Non-Bt 0	Bt 0	Ppea 35	Group 3
Ppea 1 (nut grass)	Bt 0	Non-Bt 8	Group 4	Ppea 4 (nut grass)	Bt 0	Non-Bt 0	Group 4

Table. 6.1 Total number of eggs collected from the 12 plots during the 2 sampling events. In some pigeon pea plots, not all the visual searches were undertaken due to an infestation of nutgrass.

We tested if a larger egg lay in pigeon pea was linked to a larger egg lay in Bt cotton. We found no link (spearman's rank correlation coefficient: r=0.436, t=1.16, df=6, P=0.28). The presence of an attractive pigeon pea plot did not increase the likelihood that the Bt plot would have a higher egg lay.

Because very few eggs were laid in Bt plots it was not possible to analyze if more eggs were laid near the pigeon pea crop or not. The raw data indicate no effect of crop positioning (Table 6.2).

Bt plot			
	Non-Bt	Centre	pigeon pea
Group1	2	2	0
Group2	1	3	2
Group3	0	1	0

Table 6.2 number of eggs laid on the non-Bt cotton side, the centre, or the pigeon pea side of Bt plots. There was no effect

There was no difference between the egg lays in Bt and non-Bt cotton (Mann-Whitney U test: U=0.38, df=1, P=0.44).

Do Helicoverpa move between crops.?

In the first **Bt release** (15 Jan) we released a total of 400 *Helicoverpa armigera* larva (50 larvae/release point) at 8 release points on the edge of the Bt cotton crop. We relocated 48 larvae after 1 day, but they were all found at the release site despite intensive searching elsewhere. After 3 days we found 8 individuals, again at the release site.

In the second **Bt release** (20 Feb) we released 65 *Helicoverpa armigera* on the west side of plots 3 and 4, and 75 *H.armigera* at the other 6 sites (=a total of 8 release sites and 580 larvae released). We relocated a total of only 6 individuals after one day at all the release sites.

In the first **refuge release** (11 Feb) we released a total of 400 *Helicoverpa armigera* larvae at 8 release points (50 per release point) on the edge of pigeon pea and non-Bt cotton refuges. These larvae were slightly larger (5^{th}-4^{th} instars). We relocated 35 larvae, 23 at the pigeon pea release sites, and 9 at the non-Bt cotton release sites. Three larvae from the pigeon pea release site were found 5 metres into the Bt plot.

Only one larvae was found on the second day, and that was at the release site.

In the second **refuge release** (15 March) we released a total of 400 *Helicoverpa armigera* marked larvae at 8 release points, and recaptured 80 larvae after 1 day, all at the release sites (48 on pigeon pea, 32 on non-Bt cotton).

These results indicate that 3rd and 4th instar larvae are unlikely to move if they don't need to. However, it is interesting how many stayed put on the Bt plants. Consequently, we did a series of experiments following larvae on Bt cotton, non-Bt cotton, and pigeon pea to measure how far they move on these crops, and whether crop type or quality influenced their movements. We were also interested to see if the larvae would move off unsuitable plants and travel across the 2 metres between crops.

2014/15 experiments following individual larvae

Exper.1, Are larvae on flowering pigeon pea less likely to move than larvae on non-flowering, poor quality pigeon pea?

There was no difference between watered and dry plots in either the likelihood of finding larvae after 2 hours, nor in the distance traveled by the larvae we found.

There was, though, a significant difference between the 4 columns of plots in the larvae's tendency to go missing after 1 hr, with larvae placed in column D distinctly more likely to go missing than larvae placed in plots in the other column (chi sq=10.92, df=3, p=0.012, Fig. 6.5). Column D was un-watered and did not receive pre-season nitrogen. A comparison of the fruiting bodies in the plots revealed a significant difference between plots, and that plots in column D had less florescences than those in the other columns (Kruskal-Wallis ANOVA; H=7.99, df=3, P=0.045; mean number of florescences: column A=11.7, B=7.7, C=11, D=1.8).

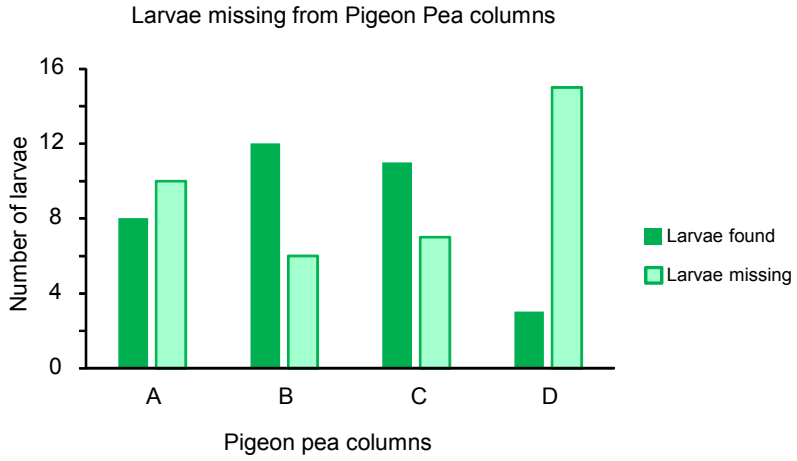

Fig. 6.5 Relocation of larvae placed in the 4 columns of pigeon pea after 2 ½ hours.

Exper 2, larval movement on poor quality pigeon pea.

Seventeen larvae were followed for up to after 2 ½ hours (5 observations each, n=71 total larval observations). However, not all larvae could be located at any time, and seven larvae went missing. Of the larvae we could locate, most (63% of observations) were found at the top of the plant. There were only 5 observations (7%) of larvae in the bottom third of the plant, and that included three observations of 2 larvae on the ground. One of these went missing, and the other one climbed up the neighboring pigeon pea plant. Larvae moved a maximum distance of 210 cm within the plants (mean=106cm, Fig. 6.6). Eleven larvae moved from their original plant to another, the maximum number of plants traversed was 5. No larvae we followed changed rows.

Larvae spent most of their time walking 36 observations (54%) and most of their time on leaves 38 observations (57%). and even though we had removed all fruiting bodies possible,

the larvae managed to find a few buds and a pod (8 observations). There were 10 observations (15%) of larvae feeding, and they were significantly more likely to be feeding on the fruiting bodies (6 out of 10 observations) than leaves (2 observations) or stems (2 observations) (chisq= 26.37, df=4, P<0.001).

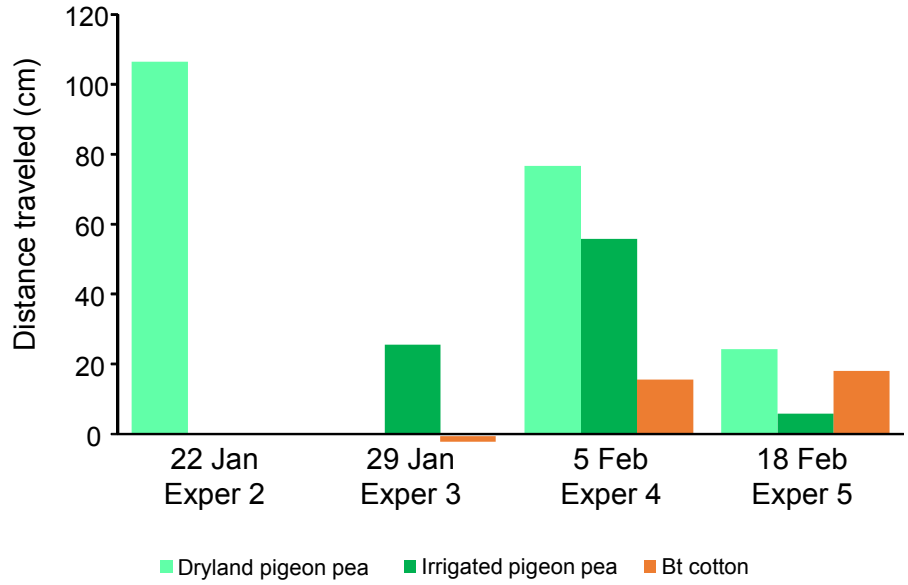

Fig. 6.6. The average distance traveled during the experiment by larvae on Dryland pigeon pea, Irrigated pigeon pea, and Bt cotton in the four experiments. The Bt histogram in Exper 5 is higher than expected because of one larvae crawled 189 cm to a boll. In Exper 3, no larvae on Bt cotton moved from where they were placed.

Exper 3, Are larvae on Bt cotton more likely to move than larvae on flowering (good quality) pigeon pea?

Twelve larvae were observed for 2-2 ½ hours on Jan 29th (10 observations for 5 larvae, 8 observations for 2 larvae) = 116 observations. There was a big difference in the behavior of the larvae on Bt cotton and healthy pigeon pea plants (for the location of this experiment, see Fig. 6.3, Plot 14). Larvae placed on pigeon pea were more likely to move than those placed on Bt cotton (Fig. 6.6). In fact none of the 6 larvae placed on a Bt leaf moved off the leaf (Fishers exact test, P=0.002). Five of the 6 took a few bites of the leaf, 3 vomited, and none further moved. One larvae placed on a Bt leaf walked around the leaf for 2 hours. The following morning all were in the same location (the one walking was still walking slowly). By the afternoon, the one walking had stopped, one was missing, and one had moved off the leaf, found a square and was feeding.

All six larvae on the pigeon pea readily moved off their leaf to a nearby florescence and stayed there feeding. They moved a maximum distance of 46 cm, and an average distance of 26 cm (Fig. 6.6). None moved to a new plant. The following morning they were all still feeding, by the afternoon, only one was still feeding, the rest were missing.

Exper 4 & 5. Are 4th instar larvae with experience on cotton more likely to move on Bt cotton than larvae on flowering (good quality) or non-flowering (bad quality) pigeon pea?

On Feb 5th (Exper 4) and Feb 18th (Exper 5) 20 and 30 larvae respectively were observed for 2-2 ½ hours (109 and 189 observations, removing missing data). Over the 2 ½ hours, Exper 4 larvae's activity was recorded six times, while in Exper 5, larvae on healthy pigeon pea and its associated cotton were recorded six times, while larvae on unhealthy pigeon pea and its associated cotton were recorded seven times.

In Exper 4, larvae on Bt cotton again did not change plants, nor move large distances (mean =15.6cm, Fig. 6.6). Of the 10 larvae set up on Bt cotton, one was eaten by a jumping spider, and only three clearly fed; two on leaves (one of these vomited) and one on a flower base. Of the non-feeders, three located bolls.

On the 18th of Feb, Larvae on Bt cotton again did not change plants. While most did not move large distances (mean=18cm, Fig. 6.6) and 11 didn't move at all, one of these did travel 189cm and located a boll. Of the 20 larvae on Bt cotton, 16 fed; 13 on leaves, one on a flower base and two on bolls. One larvae that didn't feed also located a boll. Of those that fed on leaves, none vomited, but seven stopped feeding and assumed an "arched pose", where they stood on their pseudopods, arched the anterior part of their body forward and slightly off the leaf and remained motionless.

On the 5th of Feb larvae on pigeon pea again moved larger distances than those on cotton (mean = 66.2 cm; Fig. 6.6) and 5 out of 10 changed plants, and one dropped onto the ground.

On the 18th of Feb larvae on pigeon pea moved a lot less. None changed plants, 3 of the 10 larvae didn't move at all (mean=15cm). While most larvae on Bt cotton didn't move at all, one traveled 189cm and found a boll.

Combined comparisons

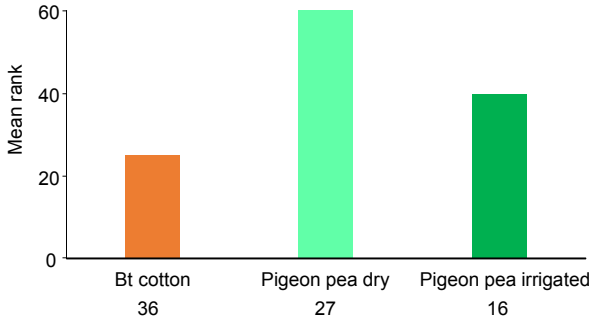

Fig. 6.7 Mean rank of all larval movement in the four experiments on Bt cotton, pigeon pea unirrigated, poor quality (dry) and pigeon pea irrigated, good quality. Numbers under the histogram are number of larvae.

Combining the results for Experiments 2,3,4 and 5 indicates that larvae are more likely to travel further on dryland pigeon pea than irrigated pigeon pea, and that larvae on pigeon pea travel further than those on Bt cotton (Fig. 6.7, Kruskal-Wallis non-parametric one-way ANOVA, H=36.22, df=2, P<0.001). Overall larvae on dryland pigeon pea were also more likely to change plants (14 changed, 13 didn't) than those on irrigated pigeon pea (3 changes, 13 didn't; Chi sq=4.6, df=1, P=0.032).

In experiments 2,3,4 and 5, larvae staying at the top of the pigeon pea plants for 90% of observations. There were only 3 observations of larvae moving or dropping to the ground.

Larvae on Bt cotton were unlikely to move once they had fed on Bt leaves, which made them sick. Nevertheless of the 53 larvae set up on Bt cotton in these four experiments, 9 (17%) found suitable parts of the Bt cotton on which to feed (3 flower bases, 6 bolls).

DISCUSSION

The refuge strategy adopted widely to delay resistance to Bt crops is based on the idea that susceptible insects produced on non-Bt host plants near Bt crops will mate with resistant pests surviving on Bt crops (Gould 1998). Previous work has indicated that edge effects from crops can extend 50m into a cotton crop. As refuges only need to be 24 rows wide, they could be subjected to edge effects which could compromise their attractiveness. The experiments undertaken here indicate that 24 rows is enough for moths to distinguish between pigeon pea refuges and Bt cotton. Moths actively laid in the pigeon pea plots, and there was no difference between their tendency to lay at the edge of the crop (near the Bt cotton) or in the

centre of the plot. However, this did not mean that there was any "spill over" and that more eggs were laid in Bt cotton rows next to the pigeon pea crop. These results also agree with Addison (2010) who found that *Helicoverpa* moths responded to boarders between Bt cotton and pigeon pea by only laying on pigeon pea. We also found no evidence of moths distinguishing between Bt cotton and non-Bt cotton, although the numbers of eggs laid on these crops was far too low for any strong comparison. Therefore, from a refuge attractiveness perspective, there is no disadvantage with putting refuges right next to Bt cotton in the same field.

However, from a larvae movement perspective, having no gap between Bt cotton and the refuge is not ideal. We found that larvae on pigeon pea will move large distances to locate their preferred food, fruiting bodies. On poor quality plots with fewer flowers (those in column D) larvae moved significantly greater distances than on healthy pigeon pea with a lot of fruit. However, larvae preferred to stay at the top of the plant, rarely venturing down to the bottom third of the plant. They more readily moved along rows, and few crawled along the ground. Thus it seems particularly important that there are no "branch bridges" between pigeon pea refuges and their Bt cotton crop.

There also seemed to be a seasonal affect in the amount of larval movement observed, as we observed less movement as the season progressed. Whether this was a factor of temperature, or characteristics of the plants is unclear.

Large larvae on Bt cotton do not move very much. If they sample the leaves, they are very sick, often vomiting, and do not move. However, in at least one case (Exper 3) a larvae recovered after feeding on a leaf, and then located a square. In fact 16% of the 53 larvae we followed on Bt cotton did locate low toxin structures (bolls and flowers) which could have helped them survive in Bt cotton.

Shelton et al 2000 argues that a separate refuge increases the survival of susceptible SS and SR, and therefore is more productive. He also argues that the use of a separate refuge is better in cases where insects can move between plants as larvae. In summary, the results of this section indicate that from the perspective of egg lays, 24 rows of pigeon pea next to Bt cotton poses no problems. There is no increase in egg lays on the Bt cotton, near the pigeon pea nor are less egg laid on pigeon pea that is near the Bt cotton. However, in terms of latter instar larval movement, we have shown that larvae will move to locate suitable food, but that they prefer to remain at the top of the plant, so "branch bridges" between pigeon pea and Bt cotton are a potential problem. If late instar larvae to reach Bt cotton, feeding on the leaves will make them sick and immobile, and probably more vulnerable to predators. Nevertheless many will be able locate fruiting bodies which could give them a chance of survival.

Therefore, our results indicate that a gap, such as a road or track, between pigeon pea refuges and Bt cotton would reduce larval exposure to Bt toxins, thereby reducing the possibility of more tolerant larvae and increasing refuge efficacy.

SECTION 7.

Comparing the attractiveness of commercially grown Bt cotton and its non-Bt cotton and pigeon pea refuges.

Contributors: Mary Whitehouse, Abbey Johnston

INTRODUCTION

Pigeon pea refuges are half the size of cotton refuges because they can attract and produce twice the number of eggs and moths as cotton refuges. In the 2010/11 season, as part of a CRDC summer scholarship awarded to David Harris, we undertook a survey comparing the refuges of 20 commercial farms in the Namoi/Gwydir valleys. We found no difference in the number of eggs laid on pigeon pea and cotton commercial refuges, and that more moths emerged from the cotton refuges. Because this finding was unexpected, we undertook another survey as part of a summer scholarship awarded to Will Tan in the 2012/13 season (Appendix C). The work showed that pigeon pea was more attractive than cotton in the latter part of the season (February), but not significantly different from cotton in January (although trending to be more attractive).

In the 2013/14 and 2014/15 seasons we repeated the work, focusing on fields at ACRI and Auscott where the results could be compared to satellite images captured using Worldview 2 (see Section 8). Here we report on the results of all four seasons combined. The aim is to establish: 1) if pigeon pea, particularly on commercial farms, is twice as attractive as Bt cotton, or at least significantly more attractive, 2) if pigeon pea is more attractive than cotton refuges; 3) if there is a difference between Bt and cotton refuges in attractiveness; and 4) if pigeon pea is more likely to be more attractive in February than January.

METHODS

In the 2010/11, the attractiveness of 26 fields (4 Bt cotton fields, 11 non-Bt cotton refuges and 11 Pigeon pea refuges) from 20 farms were measured in January (Fig. 7.1). In February the non-Bt cotton (11) and pigeon pea (11) refuges were re-measured.

In the 2012/13 season, the attractiveness of 22 refuges (9 non- Bt cotton refuges and 13 Pigeon pea refuges) from 21 farms were measured in both January and February. (Fig. 7.1).

In the 2013/14 season, the attractiveness of 16 fields were tested in January (Fig. 7.1). Of these 7 Bt cotton fields were paired with either non-Bt cotton refuges (2), pigeon pea refuges (3), or both (2). In February a further 33 fields were tested. Of these, 15 Bt cotton fields paired with non-Bt cotton refuges (2), pigeon pea refuges (10), or both (3).

In the 2014/15 season the attractiveness of 16 fields were tested in January. Of these, 8 Bt cotton fields were either paired with non-Bt cotton refuges (2), pigeon pea refuges (4), both (1) or not paired (2); while in February 18 fields were tested. Of these, 10 Bt cotton fields were paired with pigeon pea refuges (8), or not paired (2).

In each crop six one metre samples were undertaken. At each sample we recorded plant height, number of: plants, bud/squares, flowers and pods/bolls. We then visually searched the metre recording any *Helicoverpa* eggs and grubs we found.

In the first two seasons we compared the number of eggs in pigeon pea and cotton refuges in January and February separately. In the last two seasons we compared Bt cotton with either their associated cotton refuge, or pigeon pea refuge; analysing the samples in January and February separately. Analysis was undertaken using Kruskal Wallis one way ANOVAs on eggs per metre.

RESULTS

Helicoverpa eggs were sampled from 34 farms and 131 Fields over 4 years. Fig. 7.1 summarizes the number of eggs per metre per field, separated into samples taken in January and February. There was no difference in the number of eggs attracted by Bt crops or their cotton refuges, although the numbers are very low, particularly in February. In January 2014/15, four eggs were found in Bt cotton, while only one was found in cotton refuges (all samples were taken at the end of January). In February 2013/14 only 1 egg was found in 60 metres of Bt cotton or cotton refuge. Pigeon pea in January tended to attract more egg lays than cotton refuges or Bt cotton crops while in February pigeon pea attracted significantly more egglays (Table 7.1).

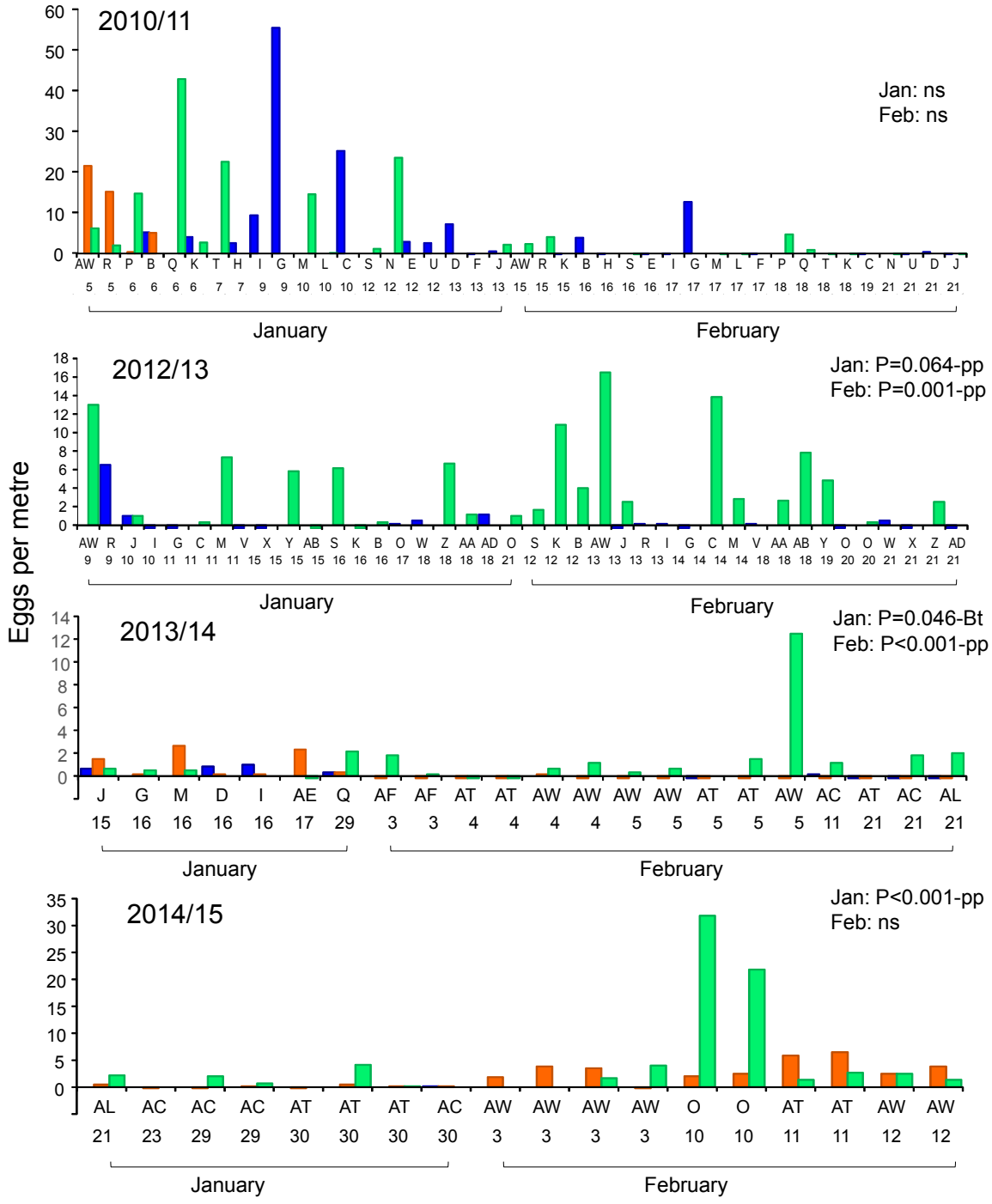

Fig. 7.1. *Helicoverpa* eggs per metre collected from 34 farms and 116 Fields over 4 years. Each farm is represented by a letter. The numbers under the letters are the dates the samples were taken. Orange=Bt cotton, Blue= non-Bt cotton, Green = pigeon pea. Coloured strips under the x axis indicate where samples were taken, but no eggs found. NB in the first 2 seasons Bt cotton was rarely sampled, while in the last 2 seasons, Non-Bt cotton was rarely sampled.

Month	Season	Comparison between crops				Statistic	P-value	n	Fields	Farms
		Mean eggs/m		Mean eggs/m						
January	2010/11	Pigeon pea	12	Cotton refuge	10.5	0.98	0.2	132	22	22
January	2010/11	Pigeon pea	7.6	Bt Cotton	12.3	0.9	0.34	36	6	3
January	2012/13	Pigeon pea	2.25	Cotton refuge	0.49	2.67	0.064	114	19	19
January	2013/14	Pigeon pea	0.8	**Bt cotton**	1.4	3.5	0.046*	60	10	5
January	2014/15	**Pigeon pea**	1.83	Bt cotton	0.27	12.3	<0.001**	60	10	4
January	2013/14	Cotton refuge	0.71	Bt cotton	0.54	0.3	0.54	48	8	4
January	2014/15	Cotton refuge	0.06	Bt cotton	0.22	0.73	0.15	36	6	3
February	2010/11	Pigeon pea	1.07	Cotton refuge	1.53	0.77	0.38	132	22	22
February	2012/13	**Pigeon pea**	3.7	Cotton refuge	0.05	8.35	0.001**	114	19	19
February	2013/14	**Pigeon pea**	1.8	Bt cotton	0	23.8	<0.001**	156	26	5
February	2014/15	Pigeon pea	8.4	Bt cotton	3.23	0.04	0.84	108	18	3
February	2013/14	Cotton refuge	0.02	Bt cotton	0	n/a	NS	60	10	3

Table 7.1 Comparative analysis of eggs lays in Bt cotton, pigeon pea refuges and Cotton refuges. Statistical test = Kruskal Wallis non-parametric ANOVA. n= number of 1 metre samples. Crop in bold had significantly more eggs.

DISCUSSION

Pigeon pea refuges are half the size of cotton refuges because they are expected to be twice as attractive as Bt cotton. Results here showed that while pigeon pea could be a lot more attractive than cotton, it was not consistently more attractive and therefore it was not performing its role within the RMP as well as expected. This seems to be the case particularly earlier in the season. In January in the 2013/14 season, Bt cotton was even significantly more attractive than pigeon pea. However in February, towards the end of the season, pigeon pea was more likely to be more attractive than cotton. This follows the pattern outlined in the previous project (CRC 1.01.52) which also showed that pigeon pea was more attractive latter in the season.

To stop the development of resistance to Bt, the cotton industry uses a number of approaches. One is to dilute the resistance genes during the season ("Genetic Dilution"). Refuges are the main tools used in this approach. Another approach is to stop resistance genes transferring from one season to the next ("Seasonal Quarantining"). Pupae busting is the main tool used in this approach. Unfortunately, the relative importance of Genetic Dilution and Seasonal Quarantining is unclear, and actions that support one approach can work against the other approach.

For example, following Genetic Dilution, refuges should be left in situ until the following season because the *Helicoverpa* pupating in refuges were not exposed to Bt cotton. Therefore, when they emerge as moths, they would dilute any resistance genes in moths emerging from Bt cotton. However the direct ancesters of *Helicoverpa* pupating in these fields could have been exposed to Bt cotton within the preceding three generations. Therefore the current generation could be carrying resistant genes selected for in previous generations. To stop the chance of any resistance genes transferring from one season to the next, their pupae should be destroyed when the cotton field is destroyed. This follows the Seasonal Quarantining approach.

Our results show that pigeon pea refuges are not twice as attractive as Bt cotton during January, the critical month for fruit production, and are more likely to be attractive in February after the bulk of the flowering has finished. In addition, the number of moths emerging out of Bt cotton is much higher than expected, so that during the course of the season, half the moths in the Bt /refuge complex in the Namoi valley could have been exposed to Bt cotton. Thus pigeon pea are not diluting resistance genes as expected, but in their current form, they could be acting more like a trap crop.

The current BMP recommends that growers maintain their refuge crop until the next season, following the Genetic Dilution model. While some growers will do this, many will plough in their refuges when they plough their cotton crop. Given that the dilution effect of pigeon pea refuges is compromised, this may be the best approach as it is likely that a reasonable proportion of *Helicoverpa* pupating in the refuges could have been exposed to Bt cotton in the previous three generations. Given the widespread exposure to Bt toxin (Section 1) and the effect that this exposure has on the survival of larvae in Bt cotton (Section 4, 5, 6) an approach following Seasonal Quarantining may be best. Thus our work indicates that utilizing pigeon pea's de-facto behaviour as a trap crop may be more effective than treating them as refuges at the end of the season. That is, destroying pigeon pea refuges when the cotton is destroyed would be more effective than letting them stand, as it could reduce gene flow from Bt-exposed *Helicoverpa* to the next season.

SECTION 8

Testing the ability of remote sensing to identify characteristics of Bt cotton and its refuges.

Contributors: Mary Whitehouse, Peter Verwey, Ian Rochester

INTRODUCTION

A major challenge in using refuges to counter the development of resistance by *Helicoverpa* to Bt cotton is maintaining refuge quality so that both pigeon pea and non-Bt cotton refuges produce enough moths to dilute any resistance genes emerging from Bt crops. Currently refuges are assessed by Monsanto using subcontractors, who are also likely to be agricultural suppliers to the growers. This approach is time consuming, and could be counter-productive as the subcontractors could have a conflict of interest. A desktop method to monitor refuges, using current satellite imagery, would save time, money and quickly identify potentially underperforming refuges that could be assessed by ground staff. Thus refuges that need assistance to increase performance, or locations where additional anti-resistance mechanisms may need to be deployed, could be quickly identified and assisted.

The rationale behind refuges is based on modeling that states that if 10% of *Helicoverpa* have been exposed to non-Bt crops, then this will delay the development of resistance by 20 generations (Roush 1998). This equates to 10% of the egg lays on non-Bt crops, which is why non-Bt cotton refuges are 10% of the Bt cotton crop. The model assumes that non-Bt cotton is as attractive as Bt cotton; that is, that moths are just as likely to lay on Bt cotton as non-Bt cotton. Because pigeon pea is thought to be twice as attractive as cotton, moths are assumed to lay twice as many eggs on pigeon pea refuges as they do on Bt cotton

In order to measure the attractiveness of a refuge crop, we need to know what the moth perceives as attractive. We also need to decide if a refuge crop's attractiveness should be re-assessed throughout the season, or whether attractiveness is a quality that is maintained throughout the crop's life. Because both Bt cotton and non-Bt cotton follow the same developmental pattern, their attractiveness is correlated (see CRC1.01.52) and it is likely to remain constant throughout the season. Therefore, the attractiveness of cotton refuges in relation to Bt cotton is likely to be maintained throughout the crop's life.

Unfortunately there is no such correlation between the attractiveness of pigeon pea and cotton (see CRC 1.01.52). Therefore, to ensure pigeon pea was attracting significant egg lays to counter those in Bt cotton crops, we suspect that the attractiveness of a pigeon pea refuge may need to be tested throughout the season, particularly when the cotton is

3 most attractive. Previous work (CRC 1.01.52) indicated that the ratio between pigeon pea flowers and pods were correlated with egg lays (attractiveness). If this ratio is correlated with a specific spectral signature that could be picked up from the satellite, then this could be used to estimate the attractiveness of a pigeon pea crop at any key time during the season. Therefore an aim of this work is to identify if a spectral signature could be used to estimate the attractiveness of a pigeon pea refuge.

While there has been no work on satellite imagery in pigeon pea crops, there have been comprehensive studies on satellite imagery in cotton, particularly using the NDVI (Normalized Difference Vegetation Index). The NDVI is calculated by subtracting the red light from the near Infrared (NIR) and dividing this by the NIR plus the red light, thus giving a ratio between these two bands of light. This calculation indicates the fraction of absorbed, photosynthetically active radiation. Thus the NDVI is a measure of photosynthetic activity.

If NDVIs are a reliable indicator of cotton yield, and cotton yield is good indicator of the healthiness (and therefor attractiveness) of Bt cotton, then NDVIs could be a good measurement of cotton health for cotton refuges. There has been a lot of work with remote sensing in cotton (eg Plant et al. 2000, Iqbal et al. 2013, Motomiya et al. 2014) but the use of NDVIs is not widespread due to the variability of its success (Gutierrez et al. 2012, Gwathmey et al. 2010, Gonzalez-Dugo and Mateos 2008). Some authors claim that the most accurate readings occur at peak flower (Iqbal et al 2013; Gutierrez et al. 2012, Zhou et al. 2007) particularly with some minor modifications for plant height (Zhou and Yin 2014) or through considering diurnal changes (Oliveira and Scharf 2014). But others report these correlations are still not very high (47%, Gutierrez et al 2012). While other workers have found that the correlation between relative lint yield and reflective indices were strongest at the early flower stage (Zhao et al. 2007; with an R^2 of 0.56-0.89). NDVIs have also been used in cotton to measure nitrogen status (Motomiya et al. 2014) again with mixed success (Raper et al. 2013). Given that there may be limitations to the usefulness of NDVIs, it may be more informative to use a larger spectral range to assess the healthiness of cotton refuges, and cotton crops in general.

An aim of this work is to see whether a more holistic analysis of remote spectral information is better at estimating the overall health (as indicated by cotton yield) of cotton than NDVIs. In addition, we tested if the NDVI or a combination of spectral readings were correlated with key nutrient deficiencies in cotton crops, such as nitrogen. We also tested if real time factors which may influence a cotton crops attractiveness to *Helicoverpa*, such as the presence of flowers, were correlated with a spectral pattern.

One of the problems with remote sensing is scale. Many characteristics we test (such as flowers and egg lays) are easier to measure at the scale of metres, while free satellite imagery is at the scale of tens of metres. While it is possible to buy high resolution satellite images accurate to 1-2 metres, these are expensive and impractical when the aim is to monitor refuges industry wide. Therefore another aim was to test if correlations obtained using high resolution imagery could be applied to low resolution, free imagery.

METHODS

GIS image capture and analysis.

This study was carried out during the 2013/14 and 2014/15 growing season on 100 km2 section of a cotton growing region near Narrabri, New South Wales, Australia (33ºS, 149ºE) which included the Australian Cotton Research Institute (ACRI), and the Auscott cotton farm (Fig. 8.1). Geoimage provided satellite photographs that were captured on the 12th of February 2014, the 17th of January 2015 using Worldview-2 which captured 8 colour bands ranging from 400 to 1040 nm and a pan image from 400-800nm (Fig. 8.2). Resolution was 2m and 0.5m for the colour and panchromatic bands respectively in 2014, and 1.6m and 0.4 m for the colour and panchromatic bands respectively in 2015.

Images were also captured from the Landsat 8 satellite which flew over on the 12th of February 2014 and the 17th of January 2015 and the 12th of February 2015. These images captured quite different wavelengths. In the visual spectrum there was no yellow, and there was only one near infrared (NIR), which covered a more narrow range than the two NIRs captured by Worldview 2. Landsat also captured two short wave infrared bands which are recommended for measuring the moisture content of soil and vegetation. These 8 bands ranged from 400 nm to 2290 nm with a $30m^2$ resolution; while the pan (black and white) image (500 to 680 nm) had a $16m^2$ resolution. Three additional bands (Cirrus, TIRS1 and TIRS 2) were even longer with much less resolution ($100m^2$) and not used in this study.

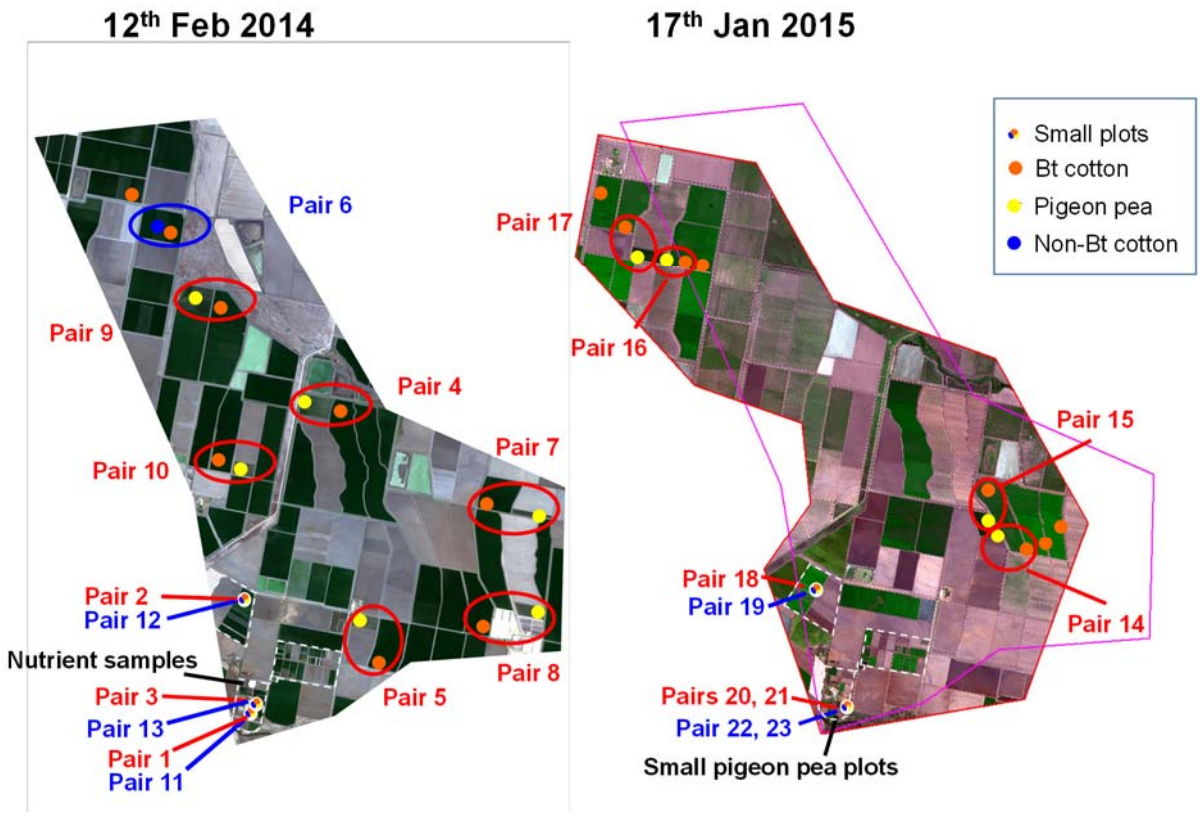

Fig. 8.1. Area photographed by the Landsat 8 satellite in the two seasons (c. 100 km^2) which covered all managed by ACRI (dashed white outline) and most of Auscott. The relative position of the 2014 photograph is outlined in purple over the 2015 map. The maps indicate the location of sampled Bt cotton, non Bt cotton, pigeon pea, and smaller fields which contained all three crop types. Sample pairs are indicated by red circles and lettering (Bt paired with pigeon pea refuges) or blue circles and lettering (Bt paired with non-Bt cotton). The location of the nutrient samples (2014) and small pigeon pea plots (2015) is also indicated.

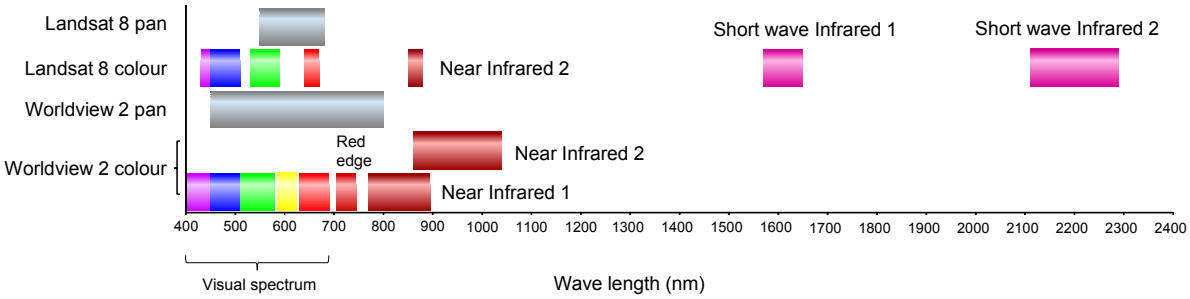

Fig. 8.2. The Spectral ranges of Satellite images from Landsat (free, course-grained) and Worldview (purchased, fine-grained). The NDVI was calculated using Red and Near Infrared 1 from the Worldview image, and Near Infrared 2 and Red from the Landsat image.

Images were captured and analysed in collaboration with Mr Peter Verwey (NSW DPI) using the programs ArgGIS desktop and ArgGIS Pro.

In both seasons there was a discrepancy between the GPS reading from the handheld recorders and the satellite imagery. In 2014 we used a Garmin eTrex10 to record the GPS location, and we discovered the discrepancy upon analysis. Because we could not accurately locate exactly where each of the ground samples were taken, we took 6 spectral readings for each plot in the vicinity of the ground samples, but were unable to completely match the spectral readings with the 1 m samples. In 2015 we used a more sophisticated GPS recorder, a Leica CS10, with thanks to the Narrabri Shire Council. We also had a known reference point for each time we took samples, so that we knew the error in the GPS recording and could record the spectral readings of pixels that covered our 1 m samples. For each sample we took two spectral readings in the two closest pixels and averaged the readings.

Cotton field analysis

Three types of analyses of cotton field crops were undertaken:

1. The relationship between high resolution satellite imagery and nutrients, water stress, and yield.
2. The relationship between high resolution satellite imagery and egg lays and vegetative measurements (such as height, number of flowers/bolls/squares).
3. Comparing fine-scaled to course-scale imagery.

1) The relationship between high resolution satellite imagery and nutrients and yield.

This work was done in collaboration with the late Dr Ian Rochester. In the 2013/14 season 120 plots 8 rows by 16 metres were planted on the 20th of October 2013. Each plot (referred to here as "Rockies plots") followed either a crop of vetch, wheat or faba, and were treated in a randomized plot design with either 0, 40, 80, 120, 160, 200, 240, 280, 320 units of nitrogen. All plots were watered in accordance with best practice. In the centre of each plot four spectral readings were taken from images captured by Worldview 2 on the 12th of February 2014. On the 7th of February 2014, samples were taken for mineral analysis from each plot. At the end of the season the yield for each plot was calculated.

2) The relationship between high resolution satellite imagery, egg lays and vegetative measurements in cotton

Six 1m samples were taken in 28 cotton fields (which included 12 Bt fields and 4 non-Bt fields in 2014; and 11 Bt fields and 3 non-Bt fields in 2015). For each sample, we recorded the number of plants, their height, number of bolls, flowers and squares; and the number of *Helicoverpa* larvae and eggs, and where possible the yield. These were compared with the spectral range as described above.

The spectral readings and NDVIs of non-Bt cotton and Bt cotton in each of the 7 pairs were compared.

3) Comparing fine-scaled to course-scale cotton imagery.

The spectral readings of the 17 largest fields analysed in 2) above were compared to the spectral readings obtained using Landsat 8.

Pigeon pea analysis

Two types of analyses of pigeon pea crops were undertaken:

1. The relationship between high resolution satellite imagery, egg lays and vegetative measurements (such as height, number of flowers/pods/buds).
2. Comparing fine-scaled to course-scale imagery.

The effect of flowering and pod count on image scores and yield

In 2015, 4 rows of 6 plots (each plot was 8 m wide by 16 m long) of pigeon pea were set up between strips of Bt cotton 8 m wide (Fig. 8.3). Half were irrigated, half were inoculated, and half had their flowers removed. So among the 24 plots there were three reps of each treatment combination, laid out in a randomized block design. In each plot 2 metre samples of number of plants, their height, number of pods, flowers, buds, *Helicoverpa* eggs and larval load were recorded around the 17th of January, and the 12th of February 2015. The Worldview 2 image of these samples were compared with these factors using ordination techniques.

In 8 fields in 2014, and 7 fields in 2015, six 1m samples were taken in which the number of plants, their height, number of pods, flowers, buds, *Helicoverpa* eggs and larval load were recorded. Again the location of each sample was recorded using GPS recorders and matched to satellite imagery as reported above. The Worldview 2 image of these samples was compared with these factors using ordination techniques.

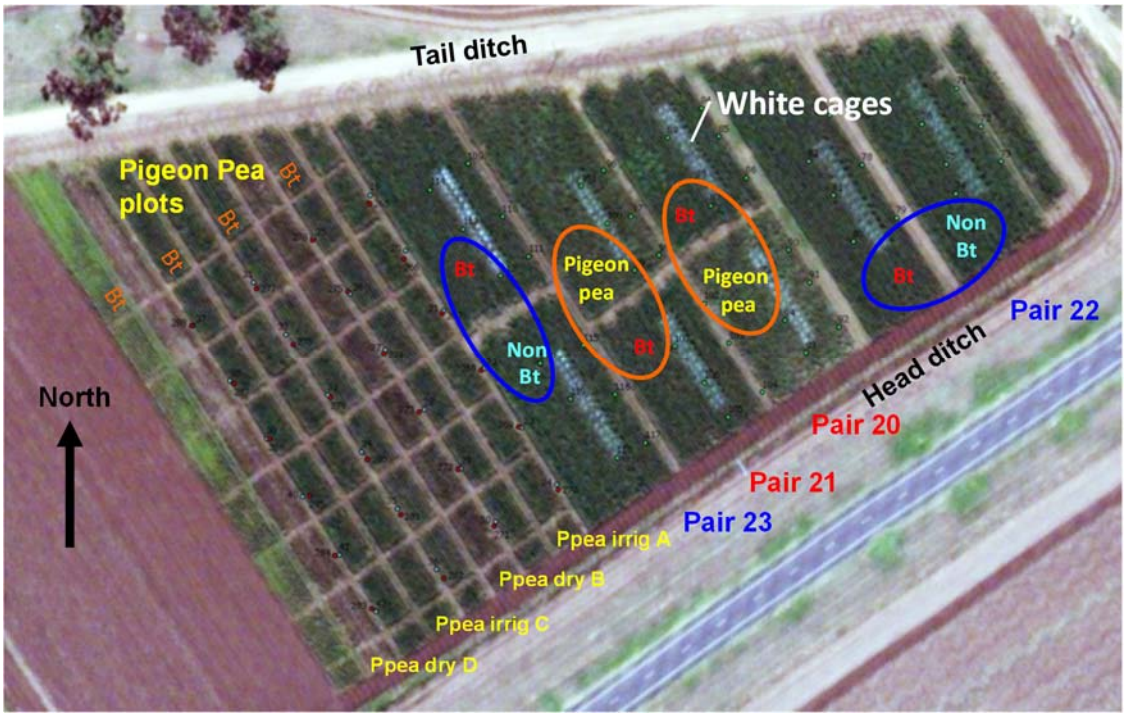

Fig. 8.3 Satellite image (17th Jan 2015) showing detail of pairs 20-21, and the location of the Pigeon pea plots which included both irrigated (Ppea irrig) and non-irrigated (Ppea dry) plots between plots of Bt cotton (Bt). These plots are the same as those used in Section 6. The white patches are the location of the cages used in Section 1 and 2.

Comparing fine-scaled to course-scale imagery to predict efficacy

This work is only at the preliminary stages. With data collected during the 2015 season, we compared spectrums collected using Worldview 2 and Landsat 8.

Statistical Analysis

General statistical analyses were conducted using Genstat 16 (Payne 2000). Community analysis of beatsheets and suction samples was undertaken using ordination techniques available in the program CANOCO 4.5 (Ter Braak and Simlauer 2002). In all cases, an indirect technique (Detrended Correspondence Analysis; DCA, where the analysis is not forced to be linked to any environmental variables preconceived to be of importance) was used to look for associations between samples and species, before the effect of any treatment variables was considered. The Detrended Correspondence Analysis (DCA) highlights similarities and differences between "species" and environmental variables. In these analyses "species" were the spectral readings for the 8 bands of colour, or the chemical analyses, while the environmental variables were the treatments or yield. A DCA assumes that "species" have a Gaussian or bell-shaped (rather than a linear) response to environmental variables. This is presented graphically and the spread along the first ordination axis (x), which illustrates most of the differences between samples, while the second ordination axis (y) uses the residuals of the first axis to further separate samples. Samples close to one another in the resulting diagrams have communities with more similar species compositions than those far apart.

NDVIs were calculated following the formula: (NIR1-R)/(NIR1+R).

RESULTS

Cotton field analysis

The relationship between high resolution satellite imagery and nutrients, water stress, and yield.

Degree days are a measurement of cotton crop stages. The nutrient testing site had been planted on the 20th of October thus the plots were photographed on about 1630 degree days. This is after the peak of cotton flowering, and is just after when the bolls start to open. From the literature, the ideal time to taken the image would have been at peak flower, which is at 1300 degree days.

Nevertheless the DCA of the spectral readings indicated that most of the variance (97%) was explained in the two dimensional graph, with the majority (88.6%) explained by the first axis. This indicates that the relationships between the spectral bands were very consistent (Fig 8.4 A).

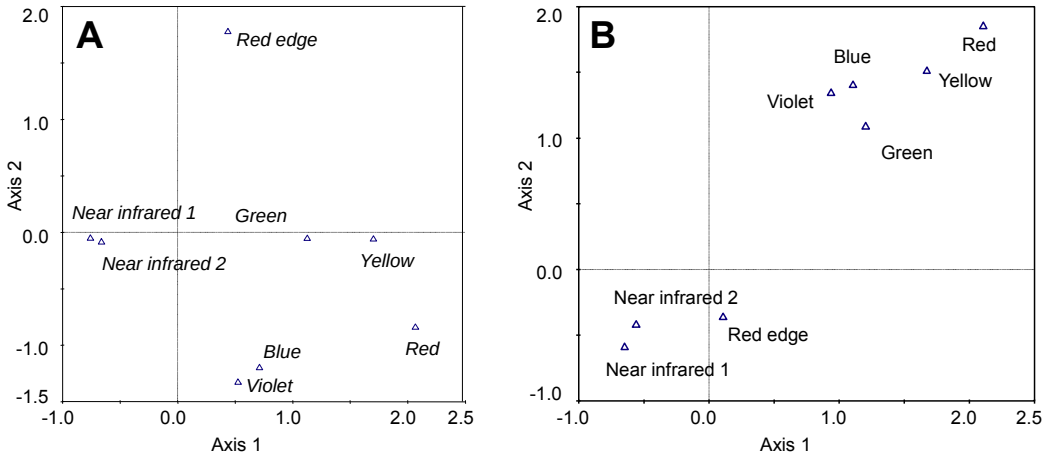

Fig. 8.4. DCAs of Rockies plots (A) and the cotton fields (B) in the 2014 season. In both cases the variance explained was 97%, indicating that relations between the spectral bands within each group was very consistent.

Relevance to yield. In a regression analysis, the NDVI explained 79% of the variance in bales per ha (v.r.= 1793; df=1,478; P<0.001; % var=78.9). This was supported by a strong correlation between NDVI and bales per ha (0.889, n=480, Fig. 8.5). A generalized linear model revealed that satellite spectral bands explained 83.2% of yield (v.r.= 33.5, df=73,406, % var.=83.2, P<0.001). When this association was explored using a direct ordination analysis (CCA) with only one environmental variable (bales /ha). It is clear that Near Infrared 1 was the band width most strongly associated with high yield, and the red band width the least associated (Fig. 8.6, which explains 70.4% of the variance in bales /ha, F=1137, P=0.002, n=480). This supports the use of NDVI to explain bales /ha variance.

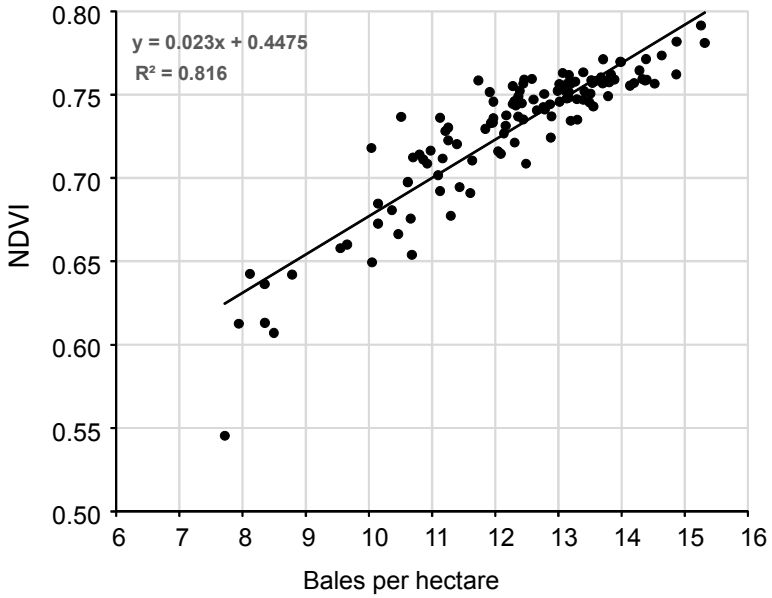

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Fig. 8.5 The relationship between bales per hectare and NDVI per plot, using the NIR1 and Red bands (provided by Worldview 2) showing a very strong correlation in Rockies plots.

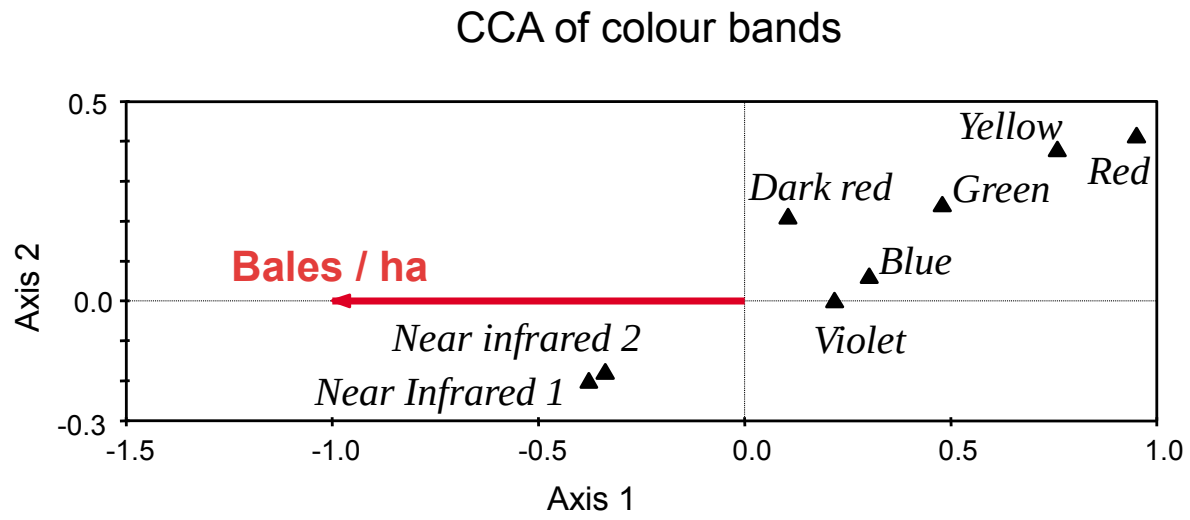

Fig. 8.6. CCA of colour bands from the Worldview 2 2014 (12th Feb) image in relation to bales/ha in Rockies plots. There was a strong association of the near infrared band widths to bales /ha. 70.4% of variance explained, F=1137; P=0.002, n=480).

When the average readings for each band width are plotted out for the highest and lowest yielding plots (those above 14 bales /ha, and those below 10 bales/ha, Fig. 8.7) it is clear that the main difference is in the infrareds, with the intensity of "Coastal" (violet) and blue not changing at all.

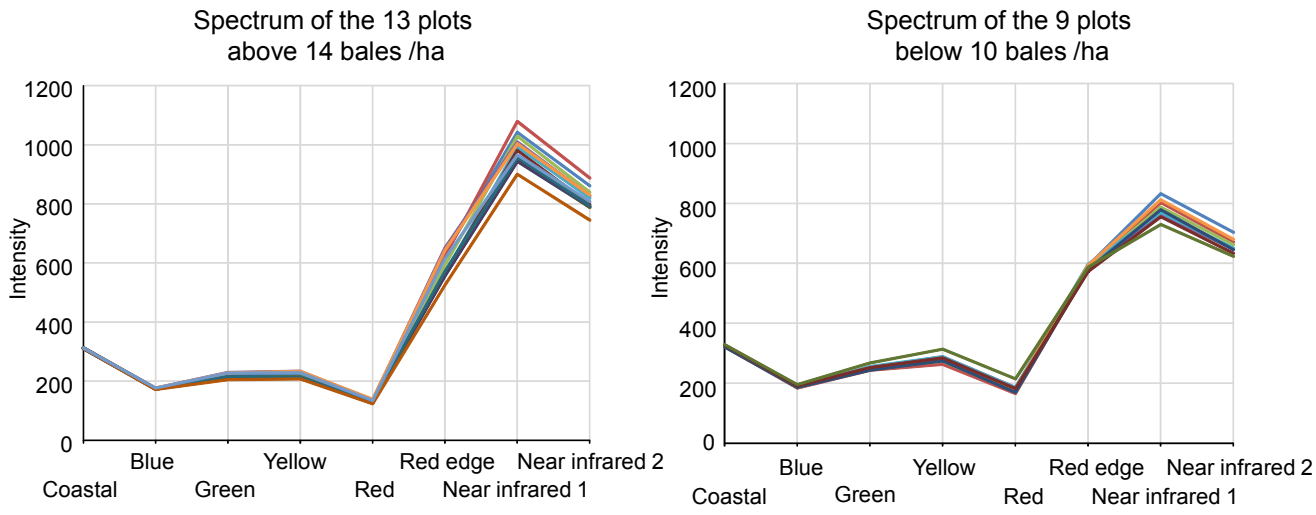

Fig. 8.7. Differences in the spectral intensity of the 13 best and 10 worst performing plots in Rocky's plots. The main difference is a large change in Near infrared 1 and 2, as indicated by the CCA.

If satellite imagery is modified to show only NIR 1, NIR2 and red (Fig. 8.8) it is clear that well performing and poorly performing plots become very apparent, and these colour images could be a useful tool to quickly check fields remotely.

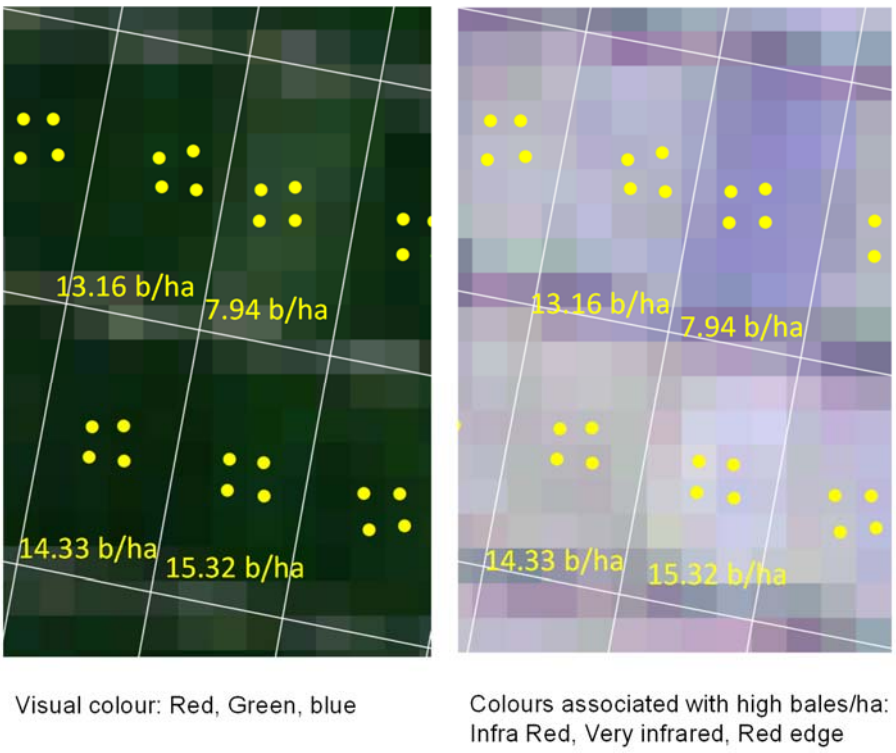

Fig. 8.8. A comparison between colour seen by humans, and colour bands useful in predicting bales per hectare in four of Rockies plots. The significant colours identify the differences between 14.33 and 15.32 bales /ha, and clearly reveal the 7.94 bales /ha plot.

We tested to see if any of the variance in the colours could be explained by different concentrations of minerals. We compared the mineral content (and uptake of Nitrogen) in plant material with bales per ha, dry matter and plant density. We found that these variables did influence mineral content, but the strongest influence was due to plant biomass (Fig. 8.9 A). Larger plants contained proportionally more Sodium, Magnesium, Manganese, Copper and took up more Nitrogen than those with less biomass. Sodium was the mineral most associated with bales per ha.

The mineral composition of the cotton could not be identified from the colour spectrum of the crop (fig. 8.9 B) as all minerals were strongly associated with the Near infrared bands.

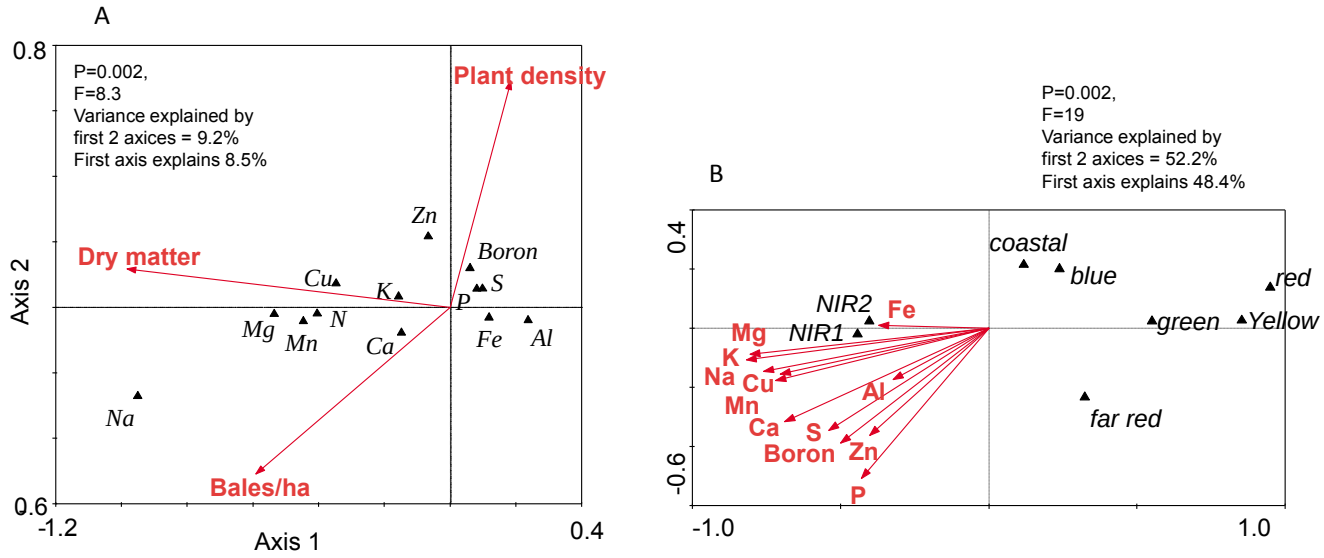

Fig. 8.9 A the relationship between mineral content and Nitrogen uptake of the cotton plants and Bales /ha, Plant dry matter, and Plant density. Dry matter had the biggest effect on mineral uptake. B. The spectral bands did not differentiate between the different minerals in the cotton, but all were strongly associated with the Near infrared bands.

The relationship between high resolution satellite imagery and cotton crop characteristics 2014

Crop characteristics were compared with the high resolution spectral bands in 10 Bt crops and 6 non-Bt cotton crops in 2014. Within the cotton crops the relationships between the spectral bands was very consistent (Fig. 8.4 B) with 97% of the variance in the spectral bands explained in the image, and the Near infrared and Red edge bands forming a group separate from the visual spectrum bands (Fig. 8.4 B). The separation of the Near infrared bands and the visual bands occurred in all the cotton analyses.

Crop characteristics explained a significant amount of variation in the spectral bands, with plant height and flowers strongly associated with the near infrared bands (Fig. 8.10). Near infrared bands are associated with plant metabolic activity. As the samples were taken in February, only one egg was present, therefore egg lays were not included in the analysis.

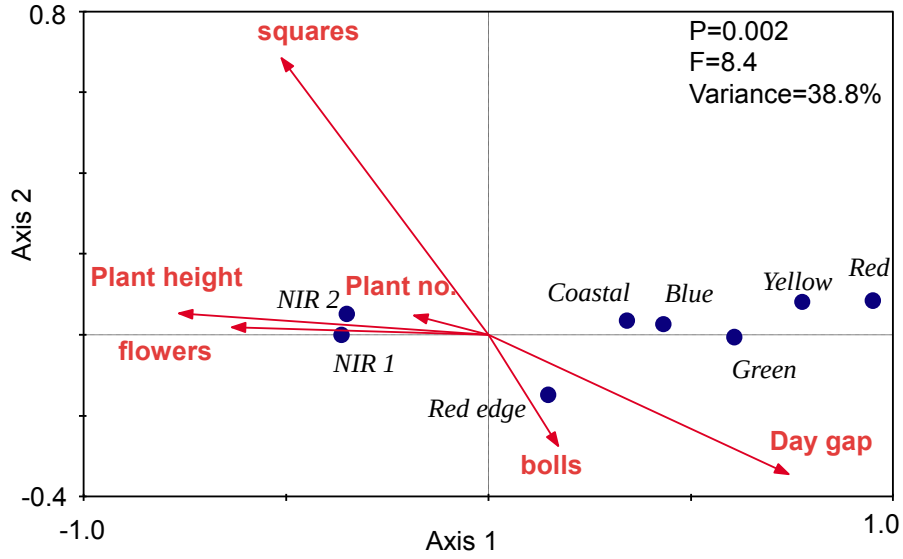

Fig. 8.10. The CCA of the relationship between cotton plant characteristics and the spectral bands sampled in 2014. Plant height and flowers are associated with the near infrared bands, which tend to be associated with plant metabolic activity.

In the fields where yield was recorded, there was no association between NDVI and crop yield (Fig. 8.10 A) although yield did explain some of the variance in the spectral bands (22%, Fig. 8.11 B). The near infrared bands were again positively associated with yield. However, it is clear that other factors are having a strong effect on the visual spectral bands, particularly the red band, given their strong association with the second axis.

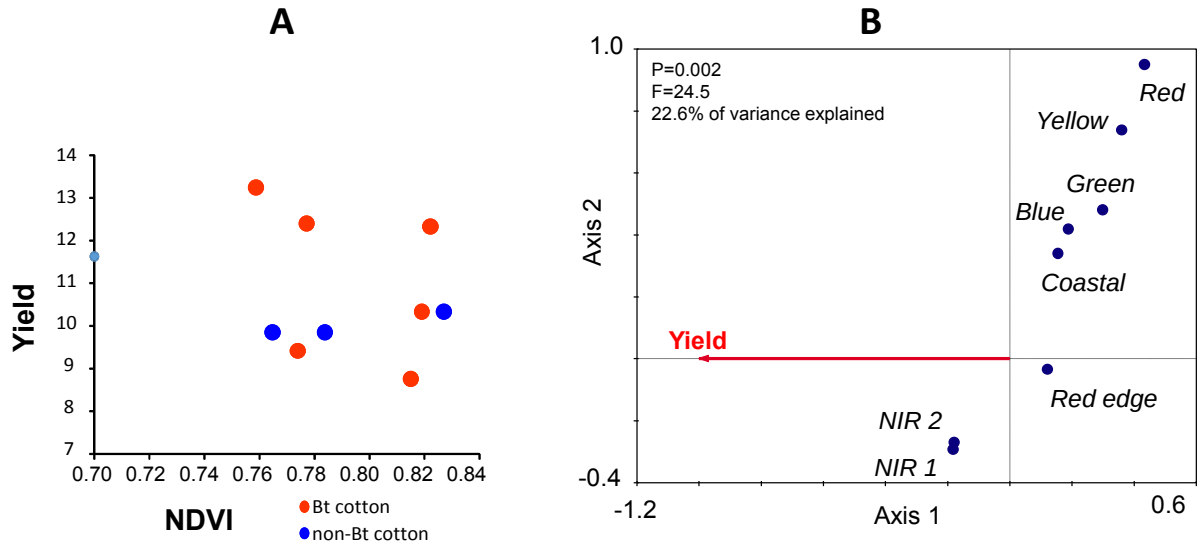

Fig. 8.11 showing no relationship between the NDVI and yield (A), and the CCA of the relationship between spectral bands and yield (B) in the cotton fields 2014.

A comparison of the spectral bands associated with Bt cotton and non-Bt cotton revealed no difference (DCA, P=0.187, NS, 2% variance explained). When the spectral bands are graphed, the Bt and non-Bt spectrums overlap (Fig.8.12) again indicating that in these samples there was no difference in the reflectance of these two crop types.

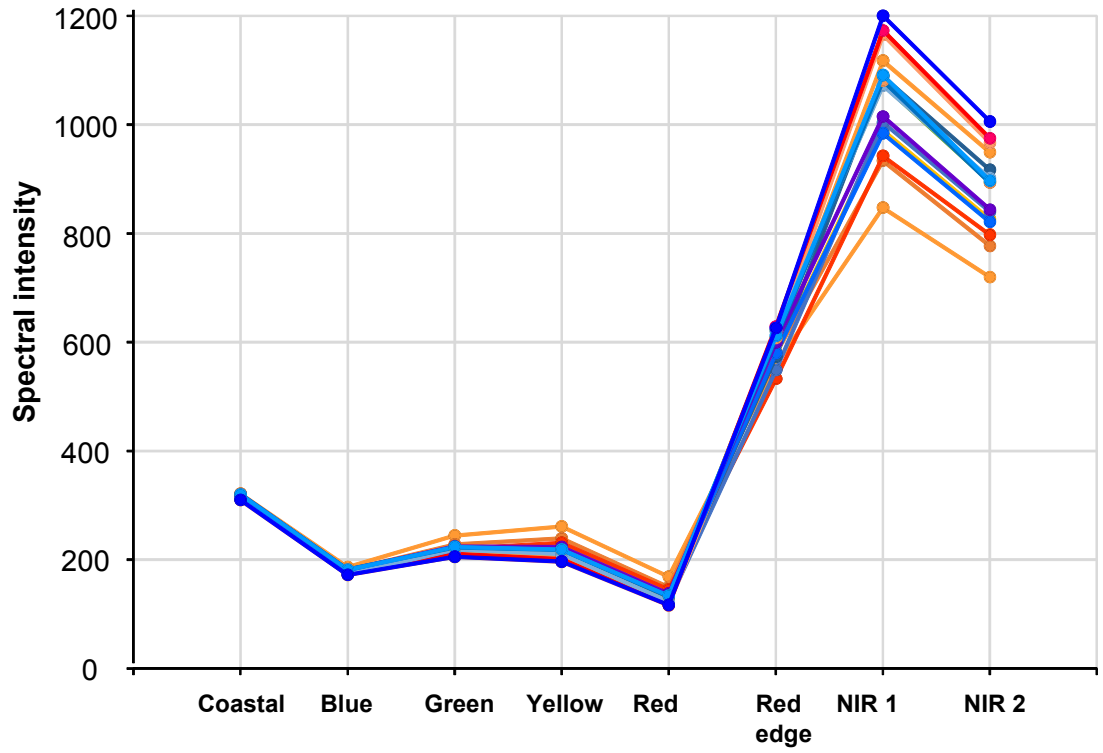

Fig. 8.12. The spectral bands of cotton crops in 2014. Orange== Bt cotton, Blue = non-Bt cotton. There was no difference in the spectral bands of these two crop types.

The relationship between high resolution satellite imagery and cotton crop characteristics 2015

In 2015 the satellite flew over a month earlier on 17th Jan 2015. Again an indirect ordination (DCA, not shown) explained 97% of the variance, again indicating very consistent relationships between the band widths.

Crop characteristics explained a significant amount of variation in the spectral bands (43.2%) but most of this was in the first axis (42%), with plant height and non-Bt cotton strongly associated with the near infrared bands, which tend to be associated with plant metabolic activity (Fig. 8.13). Again fruiting bodies (flowers and squares in 2014, squares in 2015) were positively associated with Near infrared bands. The time difference between sampling the crop and capturing the image ("days since image taken") seems to have influenced the relationship between plant characteristics and spectral bands.

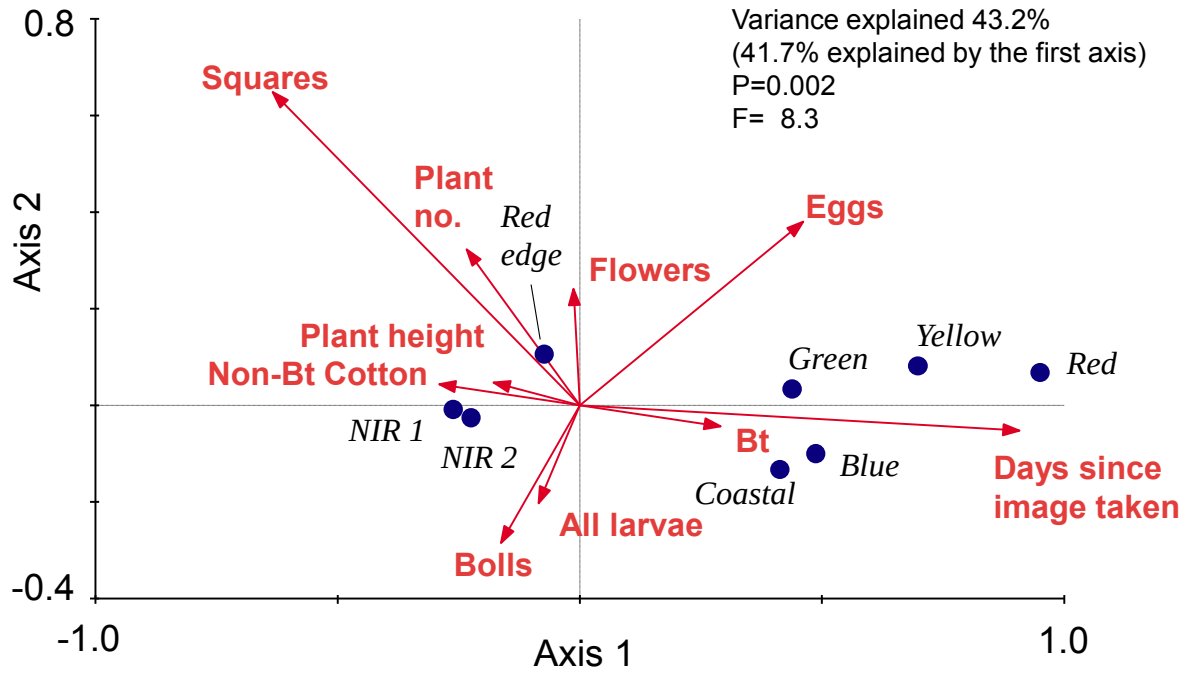

Fig. 8.13. Direct ordination of 2015 cotton samples comparing plant characteristics to spectral bands.

The spectral bands of the Bt and Non-bt cotton in 2015 (Fig. 8.14) show more variability than those taken in 2014 (Fig. 8.12). Whether this is an effect of timing or variability between the seasons is unclear. While Bt and Non-Bt cotton crop type was aligned in opposite directions with axis 1 in Fig 8.12, a separate direct ordination revealed crop type only explained 3% of the variance in the spectral bands. When the spectral bands of Bt and Non-Bt cotton are graphed, it is apparent that the association of Non-Bt cotton to high NIR readings is due to one field (Fig. 8.14) and that the readings on Bt and Non-Bt crops overlap, suggesting that both crops are equally as healthy and therefore equally as attractive.

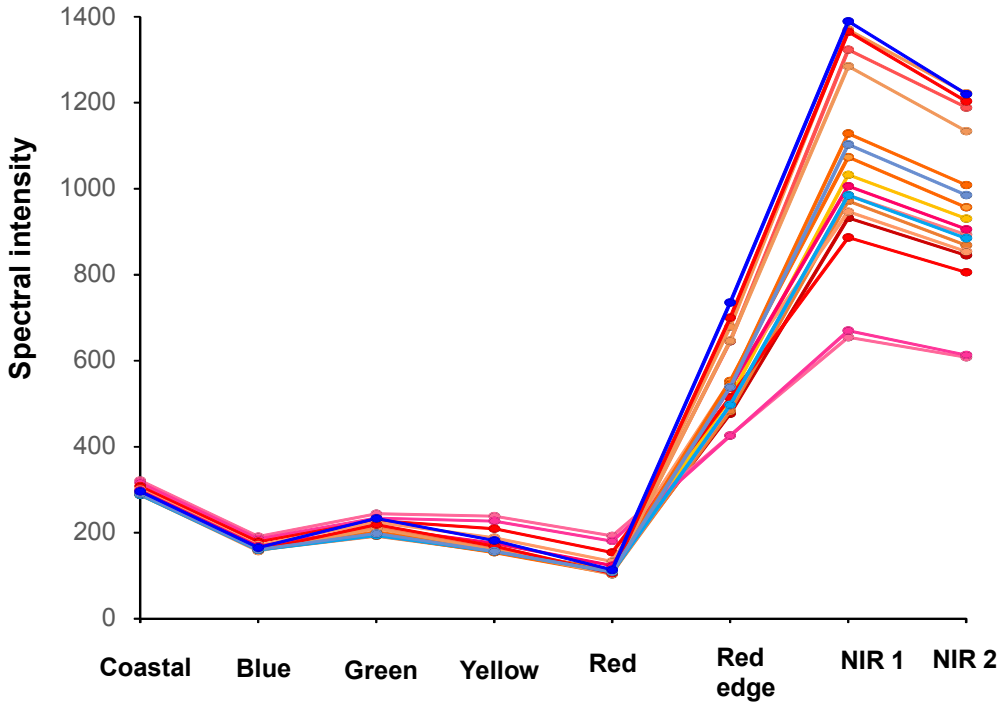

Fig. 8.14. 2015 spectral bands of Bt and non-Bt cotton captured with Worldview 2. The three non-Bt cotton fields are blue, while the Bt fields are different shades of red.

Pigeon pea analysis

The relationship between high resolution satellite imagery, nutrients and water stress in pigeon pea.

An indirect ordination (DCA) of the pigeon pea plots with different levels of irrigation, inoculation and flowers showed that in pigeon pea there is also a very high level of homogeneity, with 99% of the variance in the spectral bands explained in the image. Again, Near infared (NIR) bands and the Red edge band grouped separately from the visual spectrum (Fig. 8.15A).

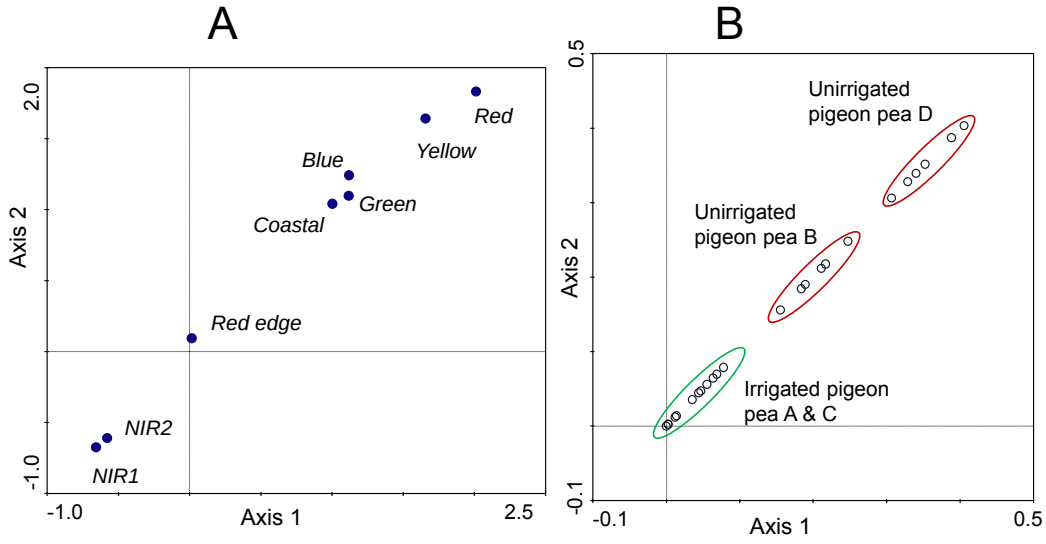

Fig. 8.15. DCA of the pigeon pea plots (see Fig. 8.3) designed to compare well performing and poorly performing pigeon pea. The plots fell along the spectral gradient, and the two unirrigated rows of plots fell out as separate groups.

The pigeon pea plots aligned themselves along the spectral gradient, with the irrigated plots more aligned with the NIR spectrums (Fig. 8.15 B). The two unirrigated rows of plots fell out

as separate groups, indicating a strong row effect on the spectral range of the plots. This is reflected in the spectral bands (Fig. 8.16) where the irrigated plots showed much higher readings of the NIR bands than the unirrigated plots.

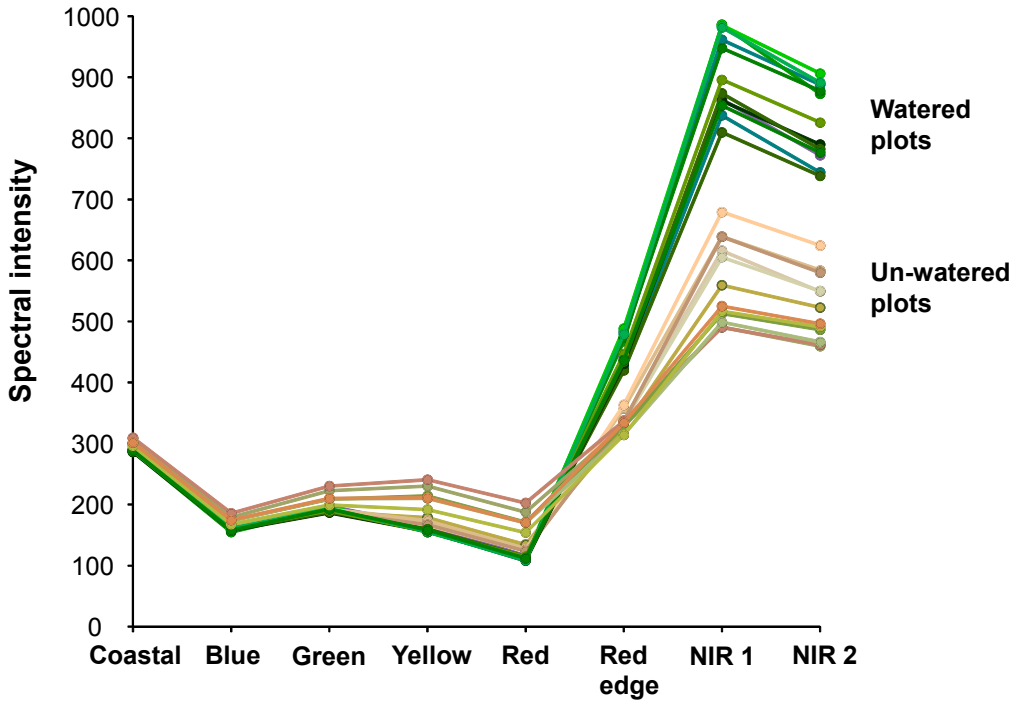

Fig. 8.16. The spectral bands of the pigeon pea plots, showing a strong differentiation between the watered and un-watered plots.

When a direct ordination (CCA) is undertaken on the plots, all tested variables measured in the day of the satellite reading aligned with the NIR bands (Fig. 8.17) and explained a high amount of variance in the samples (88%, P=0.002, F=11) indicating that high readings in the NIR bands correlated with well-watered plots, plant height, buds, florescences, higher larvae and egg numbers, and pod numbers. The only variable that did not correlate with the NIR readings is adding inoculant. This could be because the field (except for unirrigated pigeon pea row D) had been inoculated with 120 units of nitrogen, so adding inoculant to the seed was redundant.

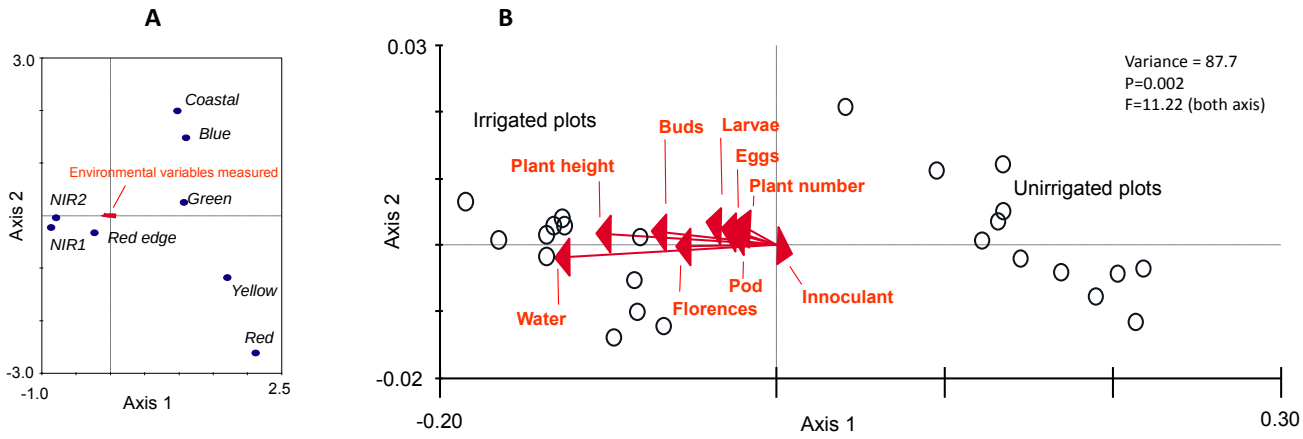

Fig. 8.17. Direct ordination (CCA) of the spectral bands (A) and the pigeon pea plots (B) in relation to a range of variables measured on the day of the satellite reading in the 2015 pigeon pea plots.

More readings were taken a month later (12th of February) and the variables again aligned towards the NIR plots (not shown) again explaining the same amount of variance in the plot's spectral readings (90%, P=0.002, F=14).

The relationship between high resolution satellite imagery and pigeon pea crop characteristics 2014

Nine pigeon pea crops in 2014 and 2015 were analyzed using worldview 2 satellite imagery. In both seasons the indirect ordination (DCA) again explained nearly all the variance (98% in both cases, not shown) with the bands arranged in a similar way to those in Fig. 8.17A: the NIR and red edge bands were separated from the visible bands along axis 1 (the x axis) and the visual colours separated along the second axis (y axis). In both seasons the NIR bands were positively correlated with plant height, flower, pod and bud numbers, while plant number was associated with the visible bands. In the samples to date, egg numbers showed no clear consistent pattern.

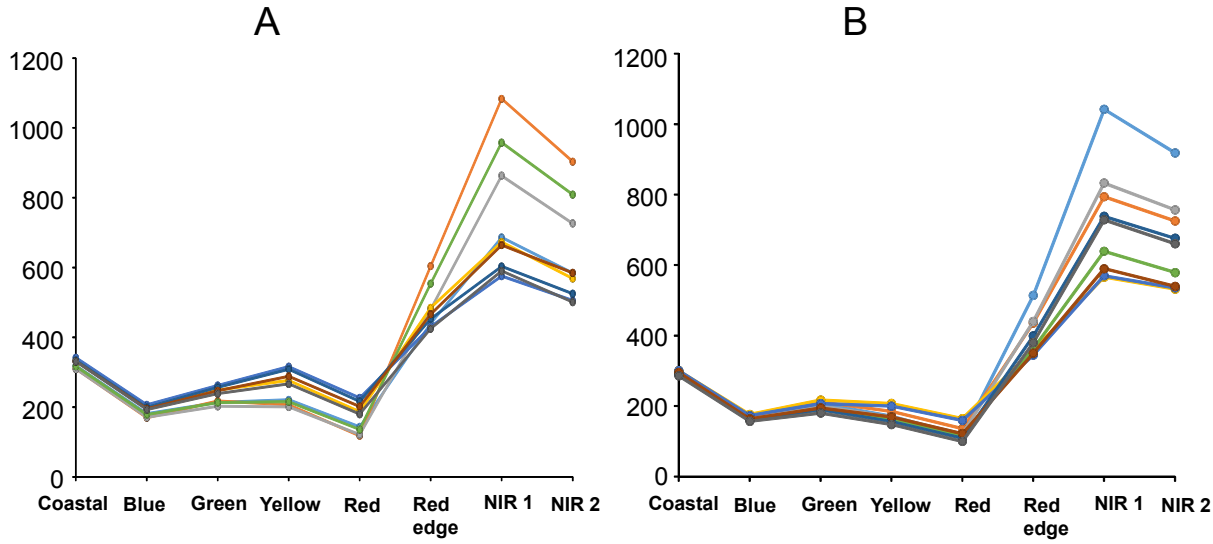

Fig. 8.18. the spectral bands of nine crops in 2014 (A) and 2015 (B).

When the spectral bands (Fig. 8.18) are compared with those from the pigeon pea plots (Fig. 8.16) is appears that some fields, with NIR bands below 600, may be suffering stress.

Comparison between high resolution and low resolution images.

Work in this area is preliminary. First we compared the spectrums of 2015 cotton fields for which we had yield data (Fig. 8.19). We found that bands obtained from Landsat ranked the cotton crops in the same order as those from worldview 2. However the Landsat data does not seem to differentiate between the crops to the extent of World view 2. In neither group did the NIR readings correlate well with cotton yield.

The spectral bands of pigeon pea fields captured by World view and Landsat were not so well correlated (Fig. 8.20). Again we found that the Landsat data does not seem to differentiate between the crops to the extent of World view 2, and that the NIR readings placed the crops in a different order, although the best performing refuge remained constant.

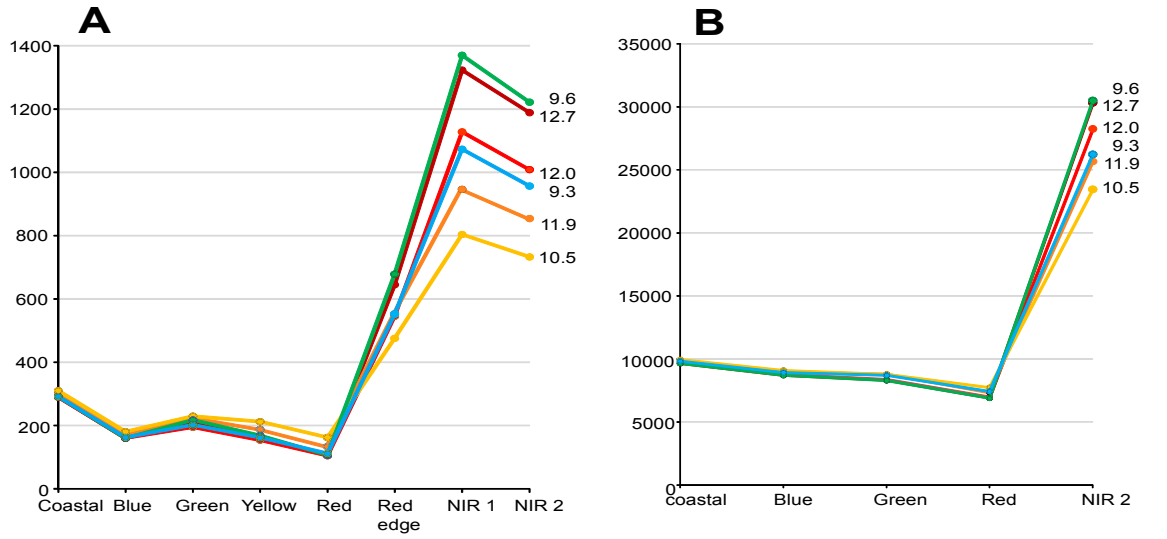

Fig. 8.19. The spectral bands in Worldview 2 (A) and Landsat 8 (B) of cotton fields with yield data (Bales /ha in black lettering; fields identified by colour).

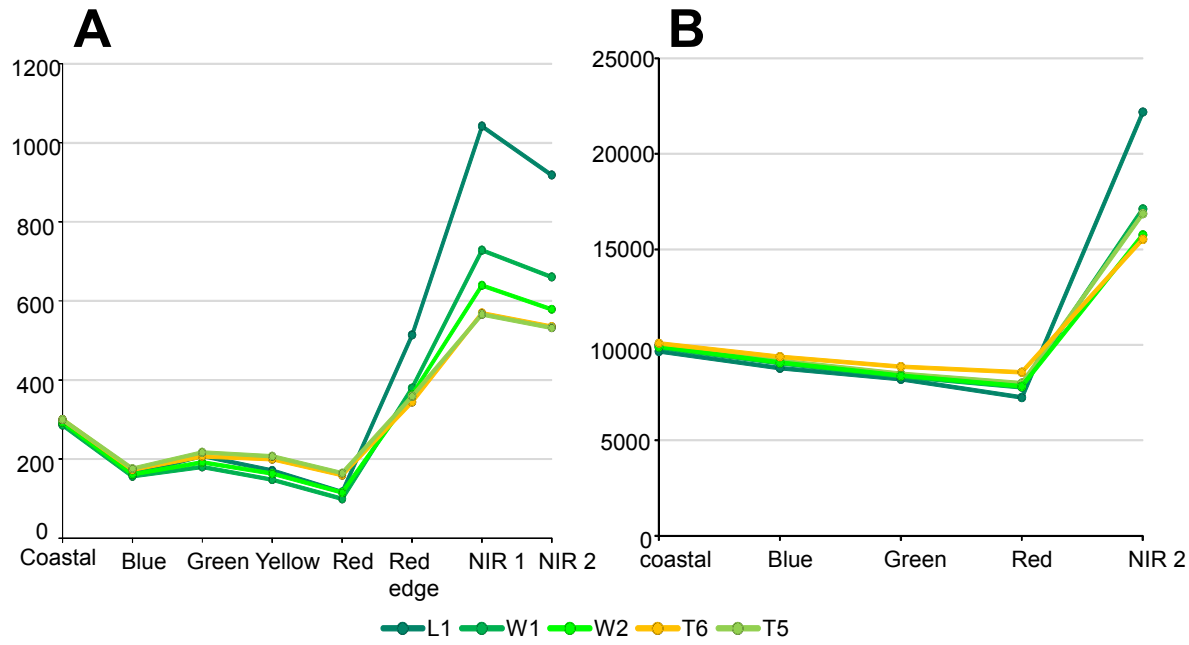

Fig. 8.20. The spectral bands in Worldview 2 (A) and Landsat 8 (B) of pigeon pea crops. Fields are again identified by colour.

DISSCUSSION

This work is only at the preliminary stages, and more analysis is required of this data to fully assess the usefulness of satellites in monitoring refuges. The results support the findings of other studies which showed that while NDVIs are useful for assessing quality and productivity in small uniform plots, they are less reliable for comparisons at the large scale, where other factors, such as soil type (Huang and Han 2014) and time of day the image was taken (Oliverira and Scharf 2014) can affect readings. Nevertheless satellite monitoring of refuges may be useful at a course scale.

Our results showed that satellite imagery may not be useful for short term analysis (whether at any point in time a refuge is underperforming) but may be useful for the long term assessment of a crop's health. For both cotton and pigeon pea, the visible spectral ranges were not correlated with attractiveness or yield of the crop. Instead, plant growth and fruit

were associated with the non-visual spectrum (NIR1 and 2) which seem to be the most useful in assessing long term crop health.

Within cotton crops we found that NIR readings were generally correlated with crop yield, (although not always so) plant height, squares, and to a lesser extent flowers. As NIR readings are correlated with photosynthetic activity, this is understandable – a more active plant would grow taller and produce more fruit. In addition there was no difference between Bt cotton crops and non-Bt cotton crops in their NIR readings. Therefore, if cotton refuges were managed the same way as the Bt cotton, the NIR readings should be similar. Thus NIR readings potentially could be used to assess if cotton refuges are being maintained as well as the Bt cotton they are trying to protect.

However, more work is needed to establish when the readings should be taken, and how reliable these reading are when taken over the whole cotton industry. Previous work suggests that peak flower is the best time to assess cotton crops for yield (Iqbal et al 2013, Gutierrez et al 2012) and this may be the best starting point from which to test the robustness of this potential assessment system.

Our work with Rockies plots showed a very strong correlation between yield and both NIR readings and NDVIs. However, they did not reveal much about mineral content of the plants, as all minerals measured were associated with high NIRs. More subtle analyses excluding NIRs may be necessary to see if satellite imagery can be helpful in identifying specific mineral deficiencies.

As far as we know this is the first attempt to assess pigeon pea remotely using satellite imagery. As with cotton, we found that variables such as plant height, buds and florescences were most strongly associated with higher NIR readings, again reflecting the higher photosynthetic activity associated with these structures. We suspect that, as with cotton, satellite imagery may be most useful in measuring overall plant health, rather that pigeon pea productivity at any point in time.

A large difference between cotton and pigeon peas is that pigeon peas can have more than one burst of flowers during a season. All the detailed work on pigeon pea plots reported here was based on only one photograph when most of the plots were flowering. Those plots where flowers were removed may still have had the photosynthetic activity (and therefore the NIR) of flowering plants. More work is needed to understand if and how changes in pigeon pea flowering is reflected in changes in its photosynthetic activity and therefore changes in its spectral image.

Within the analysis of the pigeon pea plots, and also to a lesser extent within the cotton plots, the variables we measured aligned with NIR readings, but the visual spectrum bands were strongly responding to variables we were not measuring. More work is needed to fully understand what wave lengths collected from satellite imagery actually reveal about crop condition.

One clear message from the analysis was the effect of irrigation on pigeon pea readings. In unirrigated plots, the spectral intensity of NIR wave lengths peaked at 600-500, while fully watered plots peaked at 1000. Thus it seems that NIR could be used to assess pigeon pea health. However, if we want to correlate pigeon pea health to the health of the associated cotton crop, how to do so is not so clear and requires more work.

Data collected from Landsat 8 correlated with that collected from Worldview 2, but the variance between samples was reduced. In particular, Worldview has 2 NIR bands that cover nearly 300 nm of wave lengths, whereas Landsat 8 has only 1 narrow NIR band. The NIR bands were those most associated with plant productivity, so information from Landsat 8 is more restricted, but may be enough for assessing refuges.

In addition, Landsat 8 samples have much less resolution (30m) than Worldview 2 (less than 2m) and this is a problem when assessing refuges which may be only 24 m wide. As image resolutions are decreasing all the time, this may be less of a problem in the future.

In conclusion, measuring the NIR could be an easy way to identify underperforming refuges, but this needs further assessment to assess its limitations.

Acknowledgments

We would like to thank all the growers and consultants who allowed us to work on their land, provided us important information about their refuges, and gave us their survivors. These include, George Bernie, Bill Black, Phil Firth, Matt Norrie, Dale Smith, Hugo Weissen, Jamie Street, Nick Schneebeli, Ray Fox, Nick Gillingham, Michael Josh, Ken and Judy Stump, Gary Coulton, Chris Humphries, Ken and Robert Harris, Mike Carberry, David Palato, Dallas King, and John Phelps. We would also like to thank technicians, associates, and summer scholarship students who helped us with data collection and/or gave us valuable advice. These include Shannon Hamilton, Suzie Thompson, Shanna Smith, Tracey Parker, Norm Winters, Donna Jones, Lisa Bird, Lewis Wilson, Simone Heimoana, Sharna Holman, William Tan, and David Harris.

Outcomes

1. **Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.**

The objectives of this project were to test the assumptions underlying refuges, test if tolerance could be a potential threat to Bt cotton efficacy, and develop better refuge management and benchmarking techniques to improve refuge governance. The project has achieved these objectives.

First, we have shown that while some assumptions have been met (that moths from Bt and non-Bt refuges readily mate, and that the populations are not segregated) others have not. We have shown that proportionally more moths emerge from Bt cotton than expected under refuge modelling, therefore proportionally more moths in the Bt cotton /refuge complex are exposed to Bt toxins than expected.

How susceptible larvae are surviving in Bt crops is unclear. We know they exploit low toxin producing plant structures, and that they can develop low level tolerance to Bt toxins, which, in the case of Cry1Ac, can significantly increase after one generation of exposure. In addition, the offspring of *H. punctigera* moths emerging from Bt cotton appear to be more tolerant to Cry1Ac. Increases in tolerance to toxins could assist larvae surviving on low toxin producing plant structures.

Increasing tolerance within the population could assist resistance genes. If RS individuals that have only a slight advantage against Bt toxins then increase their level of tolerance, then the additive effect could enable the individual to survive on more Bt plant structures than it otherwise could. Consequently the on-going monitoring program is very important to check that resistance genes are not increasing.

Our results also suggest differences between *H. armigera* and *H. punctigera* in how they respond to toxins. Both species develop resistance and tolerance, but there may be differences between the species in the importance of these methods to overcome Bt toxins. *H. armigera* populations in our studies readily developed resistance to Cry2Ab, which supports long standing work that it is more likely to develop resistance than *H. punctigera*. However our work also indicated that *H. punctigera* may be more likely to develop tolerance to Cry1Ac. The ability of *H. armigera* to develop resistance to Cry1Ac toxins in laboratories is well known (see Akhurst et al. 2003). Yet in both species Cry1Ac resistance is very rare in the field. Perhaps induced tolerance is the easier path by which *Helicoverpa*, particularly *H. punctigera*, can counter this toxin in the field.

Our results suggest modifications to the RMP, but we suggest further scrutiny of our findings before their adoption. First, we found that if populations were no longer exposed to Bt toxins, then the levels of tolerance dropped. Thus refuges are not only important in delaying

resistance, but can help delay the development of tolerance. Therefore refuges have been confirmed as an important RMP tool.

Second, we confirmed findings that pigeon pea attractiveness is higher at the end of the season when cotton crops are less attractive, indicating that pigeon pea refuges have an important role as a trap crop at the end of the season. Given this role, we suggest that rather than maintaining pigeon pea refuges for as long as possible (as recommended given their refuge role) we should consider if pigeon pea refuges should be destroyed after harvest (in support of their trap crop role). We suggest submitting this recommendation to the TIMS committee for discussion.

Third, we found that the biggest chance of larvae surviving on Bt cotton may be from late instar larvae moving into Bt cotton. To inhibit late instar larvae walking from the refuge into the Bt cotton crop, our results indicate avoiding any "branch bridges" between Bt cotton and its refuges, and maintaining a gap, preferably a road or track, between refuges and Bt cotton. This important but subtle change should also be submitted to the TIMS committee for discussion.

In terms of improved governance and benchmarking techniques, our work shows that satellite imagery can identify long-term crop health. This could be developed as a broad brush to identify refuges which may need assistance. Once an underperforming refuge has been identified, then staff could be sent to check the refuge by measuring specific variables and suggesting improvements as required. This work needs further development before its viability can be assessed.

2. Please describe any:-

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

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

c) required changes to the Intellectual Property register.

Our results indicate that satellite imagery could be developed into a quick method to identify which refuges may need assistance. Currently, Monsanto pay industry reps to check refuges. Because the reps would like to sell products to the grower, the reps have no incentive to potentially lose a customer by identifying poor refuges. Checking refuges remotely removes this problem. When potentially problematic refuges are identified, and then checked on the ground, a list of quantifiable tests (*Helicoverpa* eggs/metre in both the Bt cotton and refuge; number of plants/metre; the irrigation records for the refuge) could be undertaken to identify problems and improve poor refuges.

Conclusion

3. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?

1. Much higher numbers of both *Helicoverpa armigera* and *punctigera* emerged from Bt cotton than expected. Therefore a high proportion of moths in the Bt cotton / refuge complex (about 50%) have been exposed to at least low levels of Bt toxins. Exposure to low levels of toxin increases induced tolerance to Bt toxins, particularly in *H. punctigera,* and in *H. armigera* can lead to the spread of highly recessive resistance genes. Thus *Helicoverpa* species have both genetic resistance and induced tolerance tools that can be used independently or combined to counter Bt toxins. These findings reiterate the

importance of on-going resistance monitoring work, and that there is no room for complacency when trying to maintain Bt cotton efficacy.

2. While pigeon pea refuges are used as trap crops in the north, they may be also performing this role in more temperate regions, as at least in the lower Namoi where they are more attractive than cotton at the end of the season. If these results hold more broadly, there may be a case for destroying pigeon pea refuges at the end of the season.
3. A heightened risk for the development of tolerance is the exposure of late instar *H. punctigera* larvae to low levels of Cry1Ac toxin. Late instar larvae readily move in search of food, particularly over "branch bridges". While refuges need to be close to Bt cotton to maximise mating opportunities between refuge and Bt cotton moths, there needs to be a gap, preferably an irrigation channel on road, to deter the movement of late instar larvae into Bt cotton.

Extension Opportunities

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

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

Satellite work. While satellite imagery is not useful in identifying field attractiveness at a particular point in time, it does indicate season-long vitality. As such, it could be used to identify refuges with low vitality which need further investigation. Focusing scout activity to where it is particularly needed would save time and money in the quality control of refuges, while concurrently increase overall refuge efficacy (because of the focus on improving poorly performing refuges).

In addition, satellite imagery could be used to assist growers in the management of their fields by identifying cotton fields which may be underperforming. If growers could gain a direct management benefit from the technology, monitoring of refuges using satellite imagery would be more readily accepted.

Changes to the RMP. Some of the recommendations here should be presented to the TIMS committee for discussion.

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

The focus for the next 12 months will be on turning the sections presented in this report into papers. Passing the peer review process is a measure of quality assurance on the work and will help fine tune the findings of the sections.

The project suggests some changes to the RMP, such as ensuring a gap between refuges and Bt cotton and destroying pigeon pea when the season finishes. These need to be reviewed by the TIMS committee before adoption.

Many of the points here lead themselves to articles in industry magazines, and this will also be a focus in the coming months.

The industry is reluctant to talk about refuges because the RMP has been so successful, and growers are operating in difficult conditions. Developing satellite monitoring so that it assists growers could help lift the discussion and profile of refuge management

(c) For future research.

Induced tolerance /Genetic relatedness. This project has emphasized that *Helicoverpa* may use multiple techniques, not just genetic resistance, to overcome Bt toxins. Non-genetic factors, such as increased tolerance to toxins, may help explain why so many moths are able to develop in Bt toxin. Changes in tolerance levels could indicate a potential problem, particularly as induced tolerance could enhance genetic resistance. Consequently, it is important to understand how other strategies such as induced tolerance may overcome Bt toxins both independent of genetic resistance, and how they may interact with genetic resistance to enhance its spread. Understanding how strategies such as induced tolerance works would improve our defence against losing efficacy of Bt toxins.

Relatedness of moths in the refuge and Bt cotton complex. *Helicoverpa* moths collected in this study are unique because we know when they emerged, where in the crop they emerged, and from which crop they emerged. If we analysed the relatedness of all moths collected in this study, particularly those from pigeon pea refuges and associated Bt cotton, then we would understand more about gene flow between *Helicoverpa* developing on cotton and pigeon pea, and the flow of genes spatially.

Differences between H. armigera *and* H. punctigera*.* These two species appear to vary in their response to developing both genetic resistance and induced tolerance. For example we know that the offspring of *H.punctiger*a exposed to low levels of Cry1Ac toxin as late instar larvae are more tolerant of Cry1Ac toxin than controls, but is this also the case with Cry2Ab toxin? Could *H. armigera* develop tolerance in the same manner? More work is needed to understand differences in the interactions between the toxins and the different species, and the significance of this to resistance management.

Quantifying Bt toxin. We need to have better access to methods for quantifying toxin levels in different parts of Bt cotton plants. If we could learn more about the amount of toxins in different parts of the cotton plant, and how they interact with other tannins to be palatable or not to *Helicoverpa*, then we would understand how larvae survive on Bt cotton, and possibly how to reduce this survival.

Moth emergence in Emerald. The little work done in Emerald was very significant. The high numbers of moths that emerged from Bt cotton need further investigation. More cages need to be set up in Bt and its refuges in Emerald to check if these high numbers are characteristic of the region or just a result of small sample sizes. If they are characteristic, then we need to know how the moths are surviving.

Revealing potential future lepidopteran threats. From this work we have a unique collection of other species of moths that have emerged from Bt cotton and its refuges. This group could indicate potential future cotton pests. Our aim, with the help of a Summer Scholar, is to identify these moths to see if any are emerging as potential future pests.

Using satellite imagery to monitor refuges remotely. More work is needed to fully develop satellite techniques so that it can be beneficial to the cotton industry. The work presented here is the first assessment of pigeon peas using this technique, and also needs further investigation.

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

Publications

Rendon D., Whitehouse M. E. A., Hulugalle N.R., Taylor P.W. 2015. Influence of Crop Management and Environmental Factors on Wolf Spider Assemblages (Araneae: Lycosidae) in an Australian Cotton Cropping System. Environmental Entomology: 1-12. DOI:10.1093/ee/nvu025

Whitehouse M., Rahman M., Walsh T., Tay W. & Downes S. 2014. Relevance of *Helicoverpa* emerging from Bt crops. Proceedings of the 17th Australian Cotton Conference 2014.

Rahman M., Roush R. & Whitehouse M. 2014. Tolerance to Bt toxins in *Helicoverpa* species. Proceedings of the 17th Australian Cotton Conference 2014.

Ceeney S., Cross D., Tann C. & Whitehouse M. 2014. When being attractive counts. Spotlight magazine Spring 2014

Ceeney S., Cross D. & Whitehouse M. 2014. Well-watered refuges provide strongest support to resistance management. Spotlight magazine, Summer 2014-15

Wilson, L., Downes, S., Khan, M., Whitehouse, M., Baker, G., Grundy, P., Maas, S. 2013. IPM in the transgenic era: A review of the challenges from emerging pests in Australian cotton systems. Crop & Pasture Science. 64(8): 737-749.

Whitehouse, M. 2013. Maintaining and Improving Refuges. Spotlight magazine

Whitehouse M. 2013. A dynamic contribution to Australian cotton research. The Australian cottongrower

Whitehouse M. 2013. When do your pigeon peas flower? Spotlight magazine

Ceeney S., Downes S. & Whitehouse M. 2013. Volunteers in Bollgard II Refuge Areas. Spotlight magazine, Spring ed, 2013

Ceeney S., Baker G., Whitehouse M., Gregg P., Tann C., Leven T., Downes S. & Wilson L. 2013. Refuge Crops – Investing in Cotton's Future. Australian Cottongrower , Dec-Jan 2012

Whitehouse M.E.A., Mansfield S., Harris F.D., Cross D. 2012. Variability in Moth Production from Pigeon Pea and Cotton Refuges on Commercial Farms. Proceedings of the 16th Australian Cotton Conference. 85-88.

Reports to growers

2013 Aug Report to Warrianna

2014 Aug Report to Warrianna

2014 Dec Report to Auscott

Talks

2015 The response of Helicoverpa to Bt toxins and refuges: the role of tolerance and the loss of efficacy. 2nd Australian Cotton Research Conference.

2015. The ramifications of oviposition and foraging decisions on resistance management and induced tolerance. 34th International Ethological Conference (Australia).

2015. The response of *Helicoverpa* to Bt toxins and refuges: the role of tolerance and the loss of efficacy. CSIRO Presentation.

2015. Refuges and *Helicoverpa* resistance to transgenic Bt cotton. The University of Adeliade.

2015. Spider adaptation and diversity. Narrabri West Public School, years 5 and 6.

2014. The role of refuges in Bt resistance management: current work and future directions. CSIRO Presentation

2014. Managing refuges and the possible role of remote sensing. CSIRO Cotton group presentation.

2014. Moths emerging out of Bt cotton. Presentation to the TIMS committee, February.

2014. Refuge variability, Attractiveness, and Productivity. Presentation to the TIMS committee, July.

2014. Refuge productivity and the potential use of satellites to improve refuge management. 2014 REFCOM.

2014. The importance of Refuges. Macquarie Farm walk presentation.

2014. Lecture on Invertebrate diversity in Australian cotton: patterns and significance. University of Sydney ENTO4004 Insects, taxonomy & systematic.

2014. Secondary pests in cotton. Brasilian Research and Grower group.

2013. Maintaining and improving refuges in cotton production systems. 1st Australian Cotton Research Conference (Australia)

2013. Maintaining and improving refuges in cotton production systems. 2013 IPM forum.

2013. Refuges and *Helicoverpa* resistance to transgenic Bt cotton. The University of Adelaide.

2013. Maintaining and improving refuges in cotton production systems. 2013 REFCOM.

2013 Lecture on Spider diversity in Australian cotton: patterns and significance. University of Sydney. ENTO4004 Insects, taxonomy & systematics.

2013. Maintaining and improving refuges in cotton production systems. Moree Aeroclub.

Publication plan

We hope to develop each of the eight sections presented here into peer-reviewed research papers over the next few years:

Whitehouse M., Johnston A., Rahman M., Walsh T. & Spargo G. In prep. Comparing field moth emergences from Bt cotton, its refuges (non-Bt cotton and pigeon pea crops) and the ramifications to IPM. TBA

Whitehouse M., Rahman M., Walsh T. Rahman F., Mathew L., James B., Piper M., Johnson A., Harden S. & Downes S. In prep. Resistance and tolerance in *Helicoverpa* moths and larvae emerging from Bt cotton, non-Bt cotton and refuges. TBA

Whitehouse M., Tay W. & Piper M. In prep. The relatedness of *Helicoverpa* moths emerging from different crops in two locations. TBA

Rahman M., Rahman F., Whitehouse M., Mathew L. & Walsh T. In prep. The ability of induced tolerance in *Helicoverpa armigera* to overcome Bt toxins. TBA

Whitehouse M., Johnson A., Saafi F., Liddle T. Holland N., Ross B. & Downes S. In prep. Raising susceptible *Helicoverpa* larvae on Bollgard II (Bt) cotton plants. TBA

Whitehouse M. & Johnson A. In prep. The movement of *Helicoverpa* larvae on Bt cotton and its refuges. TBA

Whitehouse M., Johnson A., Cross D., Harris D. & Tan W. In prep. Comparing the attractiveness of commercially grown Bt cotton and its non-Bt cotton and pigeon pea refuges. TBA

Whitehouse M. & Verwey P. Testing the ability of remote sensing to identify characteristics of Bt cotton and its refuges. TBA

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

No

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Part 4 – Final Report Executive Summary

Helicoverpa resistance to Bt toxins is a threat to the profitability of the cotton industry. Refuges are a tool to counter resistance, but in order for refuges to be effective, the assumptions upon which they are based must be correct and refuges must be well maintained. In this study we tested assumptions of refuges; tested if tolerance, in addition to resistance, could be a potential threat to Bt cotton efficacy; and looked at ways to improve refuge governance.

The main assumption of refuges is that they produce enough moths to dilute the genetic influence of any moths emerging from Bt cotton. We found that much higher numbers than expected emerged from Bt cotton, to the extent that half the moths in the Bt cotton / refuge complex would have emerged from Bt cotton. However there was no difference in the proportion of resistance moths emerging from Bt cotton or refuges. Thus Bt cotton was less efficacious than expected, but this was not due to high level genetic resistance.

We suspect that larvae were able to develop on Bt cotton by tolerating low levels of toxin found in the bolls and flowers of Bt cotton. We found that *H.punctigera* exposed to low levels of Cry1Ac toxins in later instars produced offspring with higher tolerance to Cry1Ac toxin; and those emerging from Bt cotton had higher tolerance to Cry1Ac. Exposure to both Cry1Ac and Cry2Ab concurrently lead to an increase in tolerance levels. Tolerance may also assist the survival of RS individuals, thereby assisting the spread of resistance, but this needs more investigation.

The offspring of *H.armigera* neonates that were fed plant material expressing lower levels of toxin were better able to survive on low expressing Bt plant structures than their parents. In addition *H. punctigera* were more likely to survive on Bt cotton flowers and bolls when exposed as latter instars. Latter instar *H. armigera* larvae that were put on Bt cotton leaves in the field were able to locate and feed on bolls and flowers. Thus susceptible *Helicoverpa*, especially in latter instars, could survive in Bt cotton by feeding on plant structures with low levels of toxin. Their offspring could have higher levels of tolerance, increasing their chance of survival in Bt crops. Fortunately, we found that tolerance can also decrease over generations when larvae are no longer exposed to toxins, so refuges may help to reduce the impact of both tolerance and resistance.

Narrow refuges do not detract from the attractiveness of pigeon peas to *Helicoverpa* egg lays, nor do attractive pigeon pea crops increase egg lays in neighboring Bt cotton. However, *H. armigera* larvae will move off poor performing pigeon pea refuges; and both *Helicoverpa* species at latter instars showed evidence of avoiding toxin or seeking out lower levels of toxin. Thus *Helicoverpa* could move off poorly performing refuges into Bt cotton at latter instars, particularly if the crops were adjacent. To inhibit this movement, we recommend maintaining a gap, preferably a road or track, between refuges and Bt cotton.

As pigeon pea attractiveness was higher at the end of the season when cotton crops were less attractive, pigeon pea refuges may act as a trap crop rather than a refuge at the end of the season. Given this role, we suggest that rather than maintaining pigeon pea refuges for as long as possible (as recommended given their refuge role) pigeon pea refuges should be destroyed quickly after harvest (in support of their trap crop role).

In terms of improved governance and benchmarking techniques, our work showed that satellite imagery can identify long-term crop health and could be used to check for problematic refuges remotely.

In conclusion, many non-resistant *Helicoverpa* larvae survive in Bt cotton, and this could lead to an increase in tolerance to the toxins, which in turn would enhance larval survival on plant structures that produce low levels of toxin. Refuges could counter both the development of resistance and tolerance, but they do not produce enough moths to counter the numbers emerging from Bt cotton. Maintaining refuge governance could be enhanced by using satellite imagery to identify underperforming refuges.