



Australian Government
**Cotton Research and
Development Corporation**

SUMMER SCHOLARSHIP REPORT: 2015-2016 SEASON

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Summer Scholarship Report

Investigation of Lepidopteran communities within transgenic cotton varieties alongside refuge crops.

Miss Edwina Murray, Dr. Mary Whitehouse, Dr. Tanya Latty

1. Executive Summary:

The introduction of transgenic cotton within the cotton industry has allowed for increased yields due to decreased losses from insect activities. The main pests which have been targeted through the genetic modification are *Helicoverpa punctigera* and *Helicoverpa armigera*. The reduction in the presence of these two species could have several ecological implications, including an increase in other lepidopteran species more tolerant to Bt toxins that were previously suppressed by *Helicoverpa* spp. The results presented in this report are part of a Masters thesis which will look at the moth communities in Bt cotton and its refuges over four seasons. The results presented here focus on the 2015/16 season. During this season low numbers of moths were caught, which matched the low numbers of *Helicoverpa* caught in the same traps. There was no difference in the Lepidopteran communities between crops, and in particular Bt and non-Bt cotton. This was probably due to the low sample sizes, and may also reflect a finding in other cotton communities, that differences in Bt and non-Bt cotton communities are only found when there is high *Helicoverpa* pressure. There was a difference in the number of moths found in pigeon pea and cotton, with more moths found in cotton in January, and more moths were found in pigeon pea in February. This may reflect the phenology of the crops where cotton is flowering and probably more attractive in January than February, while pigeon pea tends to remain attractive and flowering later in the season.

2. Background:

In Australian cotton, the majority of economic damage can be traced to moth species within the Families *Helicoverpa*, *Heliathius* and *Earias* (Matthews and Tunstall 1994). These pest species cause immense damage due to their feeding habitat of consuming fruit structures, young leaves and apical meristems (Fitt 1994). Initial pest management of these species focused on the widespread usage of pesticides (Garis 2013). This system of protection led to the development of resistance to the pesticides, alongside other environmental consequences (Fitt 1994). Widespread resistance to insecticides led to the development of a different approach to manage these pests which involved transgenic crops.

The development of transgenic “Bt” cotton began when Perlak et al. (1990), inserted genes from the soil bacterium *Bacillus thuringiensis*, into cotton, enabling the plants to produce Cry1Ab or Cry1Ac proteins which are toxic to most Lepidopterans. Australian cotton varieties were transformed to include similar gene

constructs by Llewellyn et al. (1994). This led to the release of Bt cotton commercially in Australia containing the gene producing Cry1Ac in 1996 called Ingard in Australia (Olsen and Daly 2000). This release had the intentional purpose of providing protection against two main pests of cotton in Australia, *Helicoverpa armigera* (Huber) and *Helicoverpa punctigera* (Wallengren) (Olsen and Daly 2000). Ingard initially acted as a strong control method. It became apparent though that the efficacy of Cry1Ac declines over the plants life time (Olsen et al. 2005). This could enable larvae to survive on older plants. This eventuated in the creation and replacement of Ingard by Bollgard II™. Bollgard II expresses two *B.thuringiensis* genes, generating Cry1Ac and Cry2Ab toxins (Downes and Mahon 2012). The Cry2Ab toxin's expression was reported by Greenplate et al. (2003) to remain in relatively stable levels throughout the plants life. Thus, allowing for a greater industry adoption with the added benefit that it was also unlikely that resistance build ups could occur simultaneously to both toxins.

Bollgard II will be replaced by Bollgard 3™. Bollgard 3 has not been released as of yet for commercial usage, but its composition is known. Like the other Bollgards it produces the Cry1Ac and Cry2Ab toxins; however additionally it contains the capacity to also produce Vip 3A toxin (Monsanto, 2015). Stacking three toxins within this transgenic variety will enable the crop to be effective for a longer period of time: As each individual toxin has a differing mode of action, the development of resistance within Lepidopteran communities will occur separately for each toxin (Monsanto 2015). Initial testing of this transgenic variety by Monsanto (2015) indicates that it will be a very effective control method, especially against *Helicoverpa spp.*

Whitehouse et al. (2014), compared the invertebrate communities of Bt and non Bt communities. They found that there is a difference between Bt and non Bt invertebrate communities, but only when *Helicoverpa* pressure is high, resulting in high numbers of *Helicoverpa* in non-Bt cotton. When *Helicoverpa* pressure is high, there are higher numbers of invertebrates associated with frass and decay (commonly produced by *Helicoverpa* larvae) in non-Bt cotton; and higher number of invertebrates associated with flowers (which have not been destroyed by *Helicoverpa* larvae) in Bt cotton. This indicates that changes in the cotton ecosystem caused by the presence or absence of *Helicoverpa* could have a strong effect on other parts of the invertebrate community not focused on in the study, such as the lepidopteran community.

The work presented here is part of a study that will look at the lepidopteran species in Bt cotton and refuges over four seasons. In this report I will focus on the work undertaken during the 2015/16 season. The aim is to establish if there are differences in the ground emerging Lepidopteran species in Bt cotton and its refuges. The ultimate aim is to establish if there are any species that could be future pests given the reduced pressure from *Helicoverpa* on cotton.

3. Aims and Objectives:

To investigate if there is a difference in Lepidopteran communities between different genetically modified Bt cotton crops and their refuges.

4. Materials and Methods:

Cage set up:

Emergence cages were set up in a staggered method at least 20 meters from the tail ditch of the crop and spaced one meter apart (Fig 1). Each crop had two sets of fifteen cages. Firstly, Sample 1 was set up within the following crops over a one-week period: pigeon pea (dryland), conventional cotton, Bollgard II, pigeon pea (irrigated) and Bollgard 3. Sample two within each crop was set up the following week in a similar fashion. Cages were left in their positions for a two-week period, after which the set of cages was moved across two rows to begin another sample. The study consisted of eight samples, taken during January to mid- March.



Figure 1 – cage set up

Collection:

Collection of the emerging species occurred every second day initially, then proceed to occur daily. Lepidopteron species would emerge and fly up into the collection container, which was then removed and the cage and sample number was noted. Collected species were then placed in glass vials and received a sample number. The samples were killed in a -180°C freezer to preserve their form and genetic integrity.

Species determination:

During the 8 week summer scholarship period, moths from all four seasons (2012/13 = 300 moths, 2013/14 =301 moths, 2014/15 = 111 moths and 2015/16 = 74 moths) were examined. In this report only those from the 2015/16 season will be discussed. Species were initially divided into two groups: well known species and unknown species which required more detailed investigation. The method of species determination varied between each species. Some species were easily recognized by physiological traits - especially wing venation and markings. Others required more in-depth examinations. Certain species required dissections and analysis of genital claspers.

Statistical analysis:

Initial visual analysis was undertaken using Excel graphics. Community compositions were compared using ordination techniques via the program CANOCO 4.5. In particular, the direct effect of environmental variables (such as crop type and sample date) on the communities was tested using Canonical Correlation Analyses (CCA). To compare the effect of the different crop types on specific species we used a Friedman's non parametric ANOVA and chi-square tests of independence via the program GENSTAT 16.

Results:

A histogram of moths indicated that the crops differed in moth abundance during the 2015/16 season (Fig 2) with dryland pigeon pea producing the greatest number of moths. However analysis using a Friedmann non-parametric ANOVA indicated that the perceived difference was not significant over the course of the season, largely because of low numbers in dryland pigeon pea in week 2, when cotton numbers were high ($df=4,26$; test statistic = 6.97; $P=0.125$ NS). Indeed, the response of moth numbers to cotton and pigeon pea crops in January and February was different, with cotton January and February numbers significantly higher and lower respectively than expected; and pigeon pea the exact reverse, with January and February numbers significantly lower and higher respectively (Table 1, Chi-sq test of independence: $df=1$, $Chi-sq=20.07$, $P<0.001$).

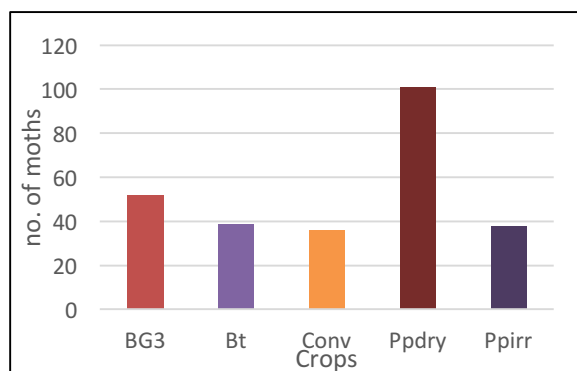


Figure 2 - Lepidopteran total samples from crops - 2015/16 season (BG3= Bollgard 3, Bt=Bollgard II, Conv = non-Bt cotton, Ppdry = Pigeon Pea dryland, Ppirr = Pigeon Pea irrigation)

Table 1. The number of moths collected from cotton and pigeon pea crops in January and February.

	January	February
All cotton crops	100	27
All pigeon pea crops	73	66

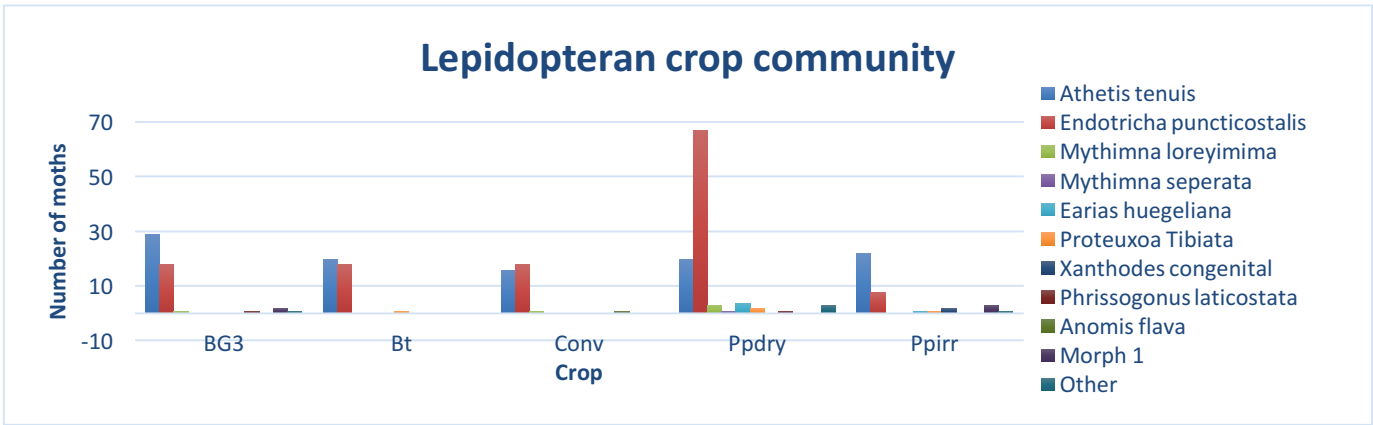


Figure 3 - Lepidopteran communities across examined crops (See Fig 2 for full crop names)

Nine species were identified from this season (Fig 3). These included Army worms (*Mythimna* spp, Rough Bollworm (*Earias hugeliana*) and The Cotton Looper (*Anomis flava*). In the 2015/16 season we did not catch any cut worms (*Agrotis* spp), even though they were common in other seasons. It was perceivable from Figure 3 that crop type may affect the diversity of species. Pigeon pea dry and irrigated produced 8 and 7 species respectively, while Bollgard 3, Bollgard II and Non-Bt cotton produced 6, 3 and 4 species respectively. More work using diversity indices is needed to test this observation. Figure 3 also shows that two species dominate the lepidopteran communities: *Athetis tenuis* and *Endotricha puncticostalis*.

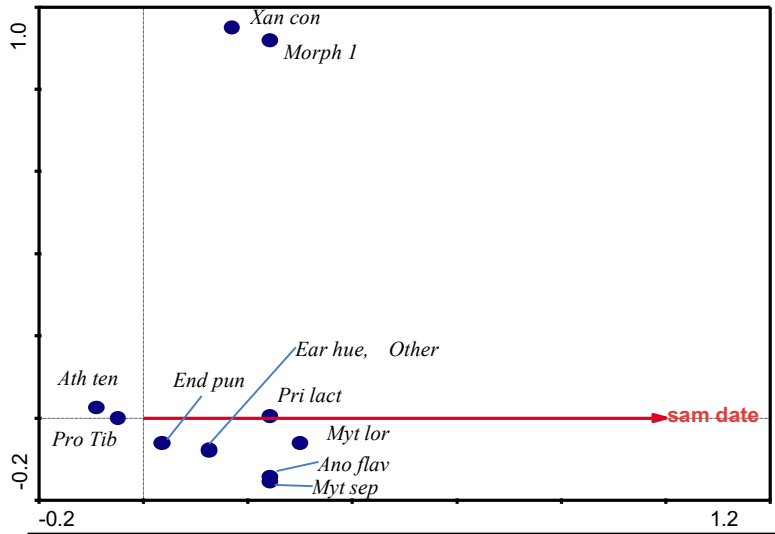


Figure 4 – The effect of Bt, cotton and pigeon pea on community (CCA) (See Fig 3 for full species names and Fig 2 for crop names)

To compare the communities in the different crops several direct ordinations (Canonical Correlation Analyses; CCA) were performed. Because of sparse data, moths collected per crop type were combined per week for the analysis (6 weeks per crop type). Figure 4 presents the results of a CCA illustrating the effect of Bt, cotton and pigeon pea on the communities. The two axes of the diagram explained 7.2% of the variance between samples, but this was not significant ($P=0.4$) indicating that Bt, cotton and pigeon pea had no significant affect upon the Lepidopteran community

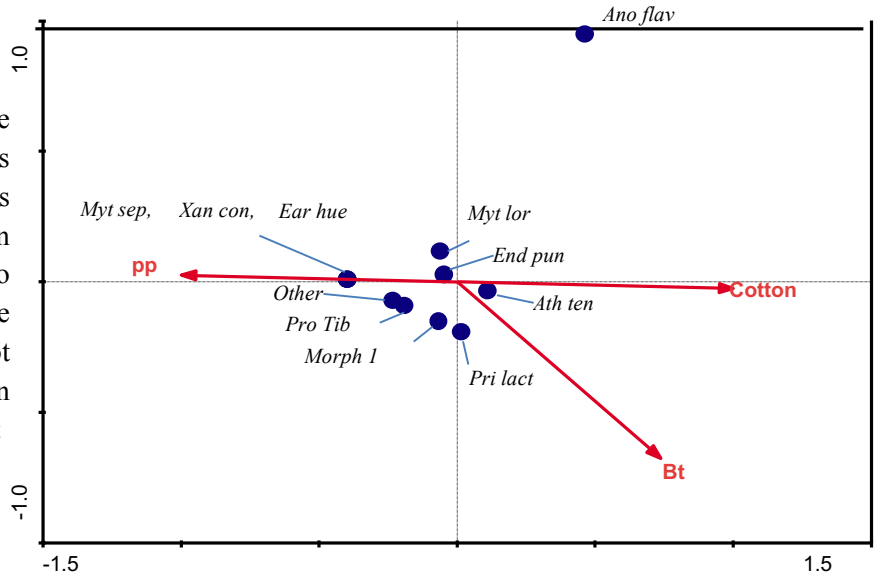


Figure 5 – The effect of sample date on community (CCA) (See Fig 2 for full crop names)

The effect of the sample week on community composition was also tested using a CCA (Fig 5). This test indicated that the community changed significantly over the six weeks ($P=0.014$) with the variance between samples explained by sample date equaling 11.3%.

Lastly another CCA was conducted testing the effect of cotton and pigeon pea crops on community with sample date used as a covariate (Fig 6). This ordination produced a P value of 0.39. Therefore, the addition of the significant covariate (sample date) still had no affect on the community found within the different tested crops.

Figure 3 also indicated that the dominant species present within the study are *Athetis tenuis* and *Endotricha puncticostali*. Friedman non-parametric ANOVAs were undertaken to test if the abundance of *Athetis tenuis* and *Endotricha puncticostali* varied between the crops. While there was no difference between crops in the abundance of *Athetis tenuis* (df=4, Friedman's statistic = 2.63, P=0.542) *Endotricha puncticostali* did significantly vary between crops (df=4, Friedman's statistic = 9.73, P = 0.020). This is further illustrated in Figure 7 which shows the mean ranks of *Endotricha puncticostali* in the different crops. The figure indicates that dryland pigeon pea (Ppdry) had the highest concentration of *E. puncticostali*, while irrigated pigeon pea (Ppirr) had the lowest ranking

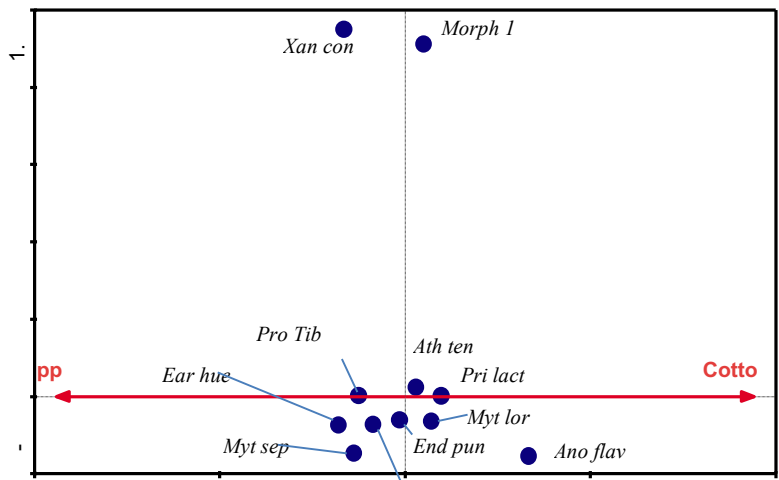


Figure 6– The effect of cotton and pigeon pea crop on community with sample date as a covariate. (CCA) (See Fig 2 for full crop names)

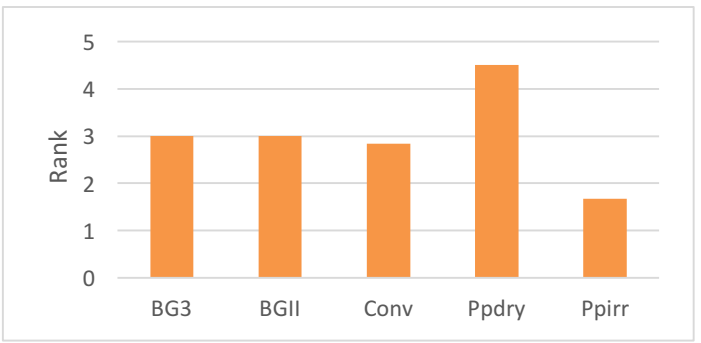


Figure 7 – Mean ranks of *Endotricha puncticostali* numbers in crops (P value 0.020) (See Fig 2 for full crop names)

5. Discussion and Conclusions:

The results presented here indicate that during the 2015/16 season, Lepidopteran communities in this study did not differ with respect to Bt toxins. There is also no significant difference in the communities of cotton and pigeon pea crops, although there were relatively more moths in cotton earlier in the season, and in pigeon pea later in the season. This may reflect the phenology of the crops where cotton is flowering and probably more attractive in January than February, while pigeon pea tends to remain attractive and flowering later in the season.

This study was completed in the growing season of 2015/16. However, Lepidopteran samples were examined from three seasons prior. These examinations are not finalised so have not been included within this study. The lack of significance results could be attributed to the small sample size which was made by just including one season's data. The collection of samples was sometimes disturbed which should also be considered. For example spiders and rainfall destroyed specimens or disrupted the collection of samples.

In previous studies, there were no difference in invertebrate communities in Bt and non-Bt cotton unless there was high *Helicoverpa* pressure (Whitehouse et al 2014). Interestingly, only eight *Helicoverpa* moths were collected from these same traps over this season, indicating that in these crops *Helicoverpa* pressure was extremely low. *Helicoverpa* pressure may also affect the lepidopteran community, so very low pressure may partly explain why there was no difference between Bt crops and non-Bt crops in this study.

The only moth that clearly responded to the different crop types was *E. puncticostali*. This moth is normally found in Australia, Selayar Islands, Christmas island, Philippines, Sumba, Java and Sulawesi (Whalley, 1963). The species lives in a web among detritus near the base of a food plant and are considered a minor agricultural pest (Butterfly House, 2014). *E. puncticostali* was most common in dryland pigeon pea, and least common in irrigated pigeon pea. This may be a reflection of the unusual weather conditions this season, with a lot of rain early in the season which favoured dryland pigeon pea. Irrigated pigeon pea in this study may have received too much water early on, rendering it less attractive.

While during the 2015/16 season we did not find a difference in Lepidopteran communities between crops, the study has greatly increased our understanding of the non-*Helicoverpa* moths in cotton systems. During this year I will be analysing seasons in which there was high *Helicoverpa* pressure, and I will be looking in depth and the food preferences of a common Lepidopteran pest in these samples, the cutworm, to see if it could be a future major pest.

6. Highlights:

Studies:

- In a low pressure *Helicoverpa* system there was no difference between Bt and non-Bt cotton Lepidopteran communities
- There were more moths in cotton early in the season, and in pigeon pea later in the season
- Sampling date significantly affects the makeup of the Lepidopteran community
- *Endotricha puncticostalis* has significantly different numbers between tested crops
- Increased knowledge of non-*Helicoverpa* Lepidopterans in cotton
- The time spent investigating the Lepidopteran communities and learning identification methods is an experience which will forever be enriching to my scientific career. This experience certainly sparked an excitement for a future within the entomology research area.

7. Future Research:

This study has provided evidence that Lepidopteran communities and diversities can be significantly affected by the crop environment provided. It further indicates that the diversities and differences are being influenced by the creation of ecological niches. Further research should be employed to investigate these niches. Understanding the drivers which make the niches accessible to other species and the ecological aspects that are creating the niches, will clarify how potential pest species could penetrate ecological niches and exploit them.

This year as part of my Honours project I will analyse the Lepidopteran communities collected in other seasons, and undertake a preliminary study looking at the behaviour of cut worms to see if, in the absence of *Helicoverpa*, they become a bigger pest in Bt cotton.

8. Presentations and Public Relations:

- Honours final presentation (University of Sydney)

9. Reference List:

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