The Role and Effectiveness of Refuge Crops in *Bacillus Thuringiensis* Cotton Production, to Reduce *Helicoverpa armigera and Helicoverpa punctigera* Numbers for Resistance Management.

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Publications by the Candidate Relevant to the Thesis

Tan, W.N., Mansfield, S., Whitehouse, M.E.A., Cross, D. 2013. Corelating refuge attractiveness with productivity (abstract) In: *Proc. Of the Association of Australian Cotton Scientists' Inaugural Cotton Research Conference, 8-11 September 2013, Narrabri, NSW. Association of Australian Cotton Scientists, Narrabri, p.73.*

Industry Reports

Tan, W. N. 2013. Correlating refuge attractiveness with productivity. *Cotton Research Development Corporation Summer Scholarship Program Progress Report* (10pp)

Awards

2013 Winner of best student poster for "Correlating Refuge Attractiveness and Productivity" at the *AACS Inaugural Australian Cotton Research Conference*.

1. Abstract

Bt cotton has been extremely effective at controlling moths of the genus *Helicoverpa*. In order to delay the development of resistance by *Helicoverpa* to the Bt toxins, moths emerging from Bt cotton carrying the recessive resistant gene need to mate with susceptible individuals who do not carry the resistant gene. Refuges are planted to produce these susceptible moths. As pigeon pea is regarded as twice as attractive and productive to Helicoverpa as cotton, growers only need plant half as much pigeon pea as cotton refuges. However, on commercial cotton farms where cotton and pigeon pea refuges don't synchronize flowering, and experience differing amounts of care, is pigeon pea still advantageous? To see if pigeon pea refuges are twice as attractive and productive as cotton refuges, we correlated attractiveness, productivity and plant parameters within and between cotton and pigeon pea refuges grown on commercial farms. We used egg counts to quantify attractiveness, and pupae digs to quantify productivity. Overall, pigeon pea refuges in our sample were twice as attractive and productive as the cotton refuges, while the larvae developing in cotton tended to have a higher survival rate. The attractiveness of the pigeon pea was correlated with the proportion of the crop that was flowering as opposed to forming pods. Results indicate that the pigeon pea is most effective when the pods are flowering, and hence timing is important when planting as you want this to synchronise with the resistant moth emergence in Bt fields. Pigeon pea was only effective when it was looked after and able to reach its flowering potential. These results show the importance of insuring that pigeon pea flowers at the appropriate time to be an effective and attractive refuge.

2. Introduction

The Pest

Helicoverpa belongs to the family Noctuidae, and *H. armigera and H. punctigera* have the common names ‘cotton boll worm’ and ‘native budworm’ (Grundy. T 2012). These are common pests of cotton and do damage to all vegetative and reproductive parts of the plant during their caterpillar stage and at all instar levels. They are not host specific to cotton, as they are also pests of sunflower, lupins, linseed, tomatoes, beans, peas, lucerne, maize and tobacco (Jones D 2012). *Helicoverpa* spp. is so successful worldwide because of high fecundity, mobility, facultative diapause and polyphagy (Fitt, 1989).

Lifecycle

(Figure 1) At 25°C, *Helicoverpa* spp. eggs take 3-4 days to hatch. During this time eggs turn from white to brown, and close to hatching, the black head capsule of the larvae is visible through the eggshell. Larvae develop through five or six growth stages (instars) and become fully grown larvae in 2-3 weeks. With the inclusion of the time from moth emergence to fertile egg production of around 5 days, a generation is estimated to be completed in about 40 days during summer, however this may vary depending on day degrees and day length (Knight 2013). Four to five generations occur per growing season. Final instar larvae move to the soil to pupate, usually not far from the base of the plant where they completed development. These larvae dig a tunnel leading to a chamber in which they enter the pupae stage. In this stage they undergo metamorphosis and emerge as adults. The final larvae build an emergence tunnel from the pupae chamber to the soil surface (usually <10cm). During summer pupae maturation takes about 16 days. However, late in summer, as day length shortens and temperatures fall an increasing proportion of these pupae will enter diapause and will spend the winter as pupae. Cultivation of the soil to a depth of 10cm during winter destroys the emergence tunnels, directly kills many pupae and brings others to the soil surface where they are eaten by predators such as birds (Mares 2012).

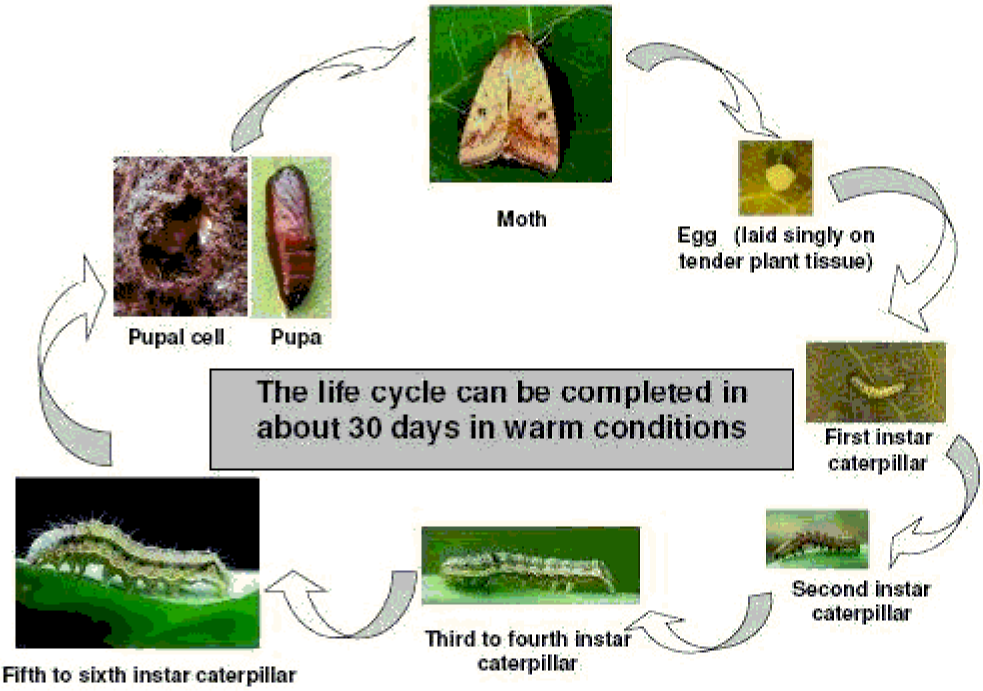

Figure 1. Lifecycle of a typical Helicoverpa spp. (Varela 2003)

Damage and Economics

H. armigera and *H. punctigera* are common pests of cotton, damaging all vegetative and reproductive parts of the plant. They are not host specific to cotton and feed upon other plants including sunflower, lupins, linseed, tomatoes, beans, peas, lucerne, maize and tobacco (Jones & Elliot 2012). These pests cause damage as larvae, and damage cotton bolls results from the feeding of later instar larvae. The larvae burrow into the immature fruit capsule and consume the white damp lint held in the fruiting body. These pests can cause either direct damage via consumption of cotton bolls, or indirect damage, when eating leaves and flowering parts by opening wounds for pathogens to enter, and reducing the quality of the cotton. A study was conducted on conventional cotton comparing the economic costs of *Helicoverpa* from 5 seasons leading up to 1996/1997 and the six years leading up to

2002/2003 (Fitt et al. 1994). The results were an averaged $325/ha of costs towards *Helicoverpa* control and an averaged $562/ha respectively and a combined cost of $282 million per annum on control and residual damage. This shows how much cost of sprays has risen over the years and the economic impact of *Helicoverpa*, Bt cotton however has since changed the cotton industry, making farming practices more environmentally friendly by reducing insecticidal sprays by up to 80% (Fitt et al. 2000). With the introduction of transgenic Bt cotton, the industry has been looking into long term sustainable farming practices as well as encouraging more environmentally friendly management of land.

Therefore with the introduction of Bt cotton, a producer's economic threshold has to be adjusted to account for new factors. One of these factors is the reduction of insecticide sprays, enabling farmers may to save money that would have been spent on sprays. However, growers now have to incorporate biological control and refuge management into their economical thresholds. Another factor is the cost of environmental contamination. Previously high levels of chemical waste built up when using conventional cotton due to sprays. Now there is a potential for Bt toxin to build up in the soil while growing the cotton (Helassa 2010). Along with this, habitat diversity becomes a large part of biological control, as attraction of beneficial insects to the farm will help with a lot of control over *Helicoverpa*. These include creating alternative prey sources for beneficials or increasing the local nectar sources via diversity of flora in the area (Fitt et al. 2000).

Integrated Pest Management

Management in Bt

Management of *Helicoverpa* spp. is usually done within the IPM (Integrated Pest Management) framework. IPM involves incorporating chemical, with cultural and biological practices to control the population of pest insects. Biological methods in the cotton industry rely heavily on parasitoids to control numbers, as parasitoids which exist naturally in the field are mainly parasitic wasps, and flies, these groups will lay their eggs either externally or internally in the caterpillar, and from there the parasitoid larvae will feed once emerged. Chemical control almost no longer exists now that Bt cotton is around as none is usually needed, however management practices need to be put in place to counter resistance. Cultural control involves the physical change of the environment, including cultivation to destroy pupae (pupae busting).

Biological

Biological methods of pest management is defined as the control of a pest by the introduction of a natural enemy or predator (Gullen 2010). Cotton fields typically contain a rich diversity of insects, and up to 450 different species can be found. Most of these tend to be beneficial in one way or another however it is difficult to prove this as their interactions are so complex on many different levels (Hearn 1992). A range of parasitoid and predatory insects attack *Helicoverpa* spp. These parasitoids and predatory insects are called beneficials as they benefit the farmers by controlling the population. The main insects which parasitise *Helicoverpa* spp. include, *Trichogramma*, *Netelia*, *Ichneumon promissorius*, *Tachinid* and *Heteropelma scaposum*. These parasitoids attack the *Helicoverpa* spp. either at the egg, larvae or pupal stages in its life cycle (DPI 2005). To find the host caterpillars they use smell, either detecting the odour of the host itself, or the path of its activity (leaf damage or faeces). Parasitoids such as the fly's and wasps attack the caterpillars via planting their eggs within or on the surface of the host stage they are attacking (DPI 2005).

Trichogramma are small yellow wasps which lay their eggs directly inside *Helicoverpa* spp. eggs. Along with *Telenomus* wasps they are <2mm in length, and once the egg has been parasitised the wasp will emerge after approximately 10 days (O'Brien 2010). *Netelia producta* are slender wasps that attack the larvae stage of the hosts by stinging them multiple times to paralyse the host. Once paralysed the wasp lays the eggs on the surface of the caterpillar near the head to prevent it being removed via the caterpillar's mandibles. The egg hatches when the caterpillar goes to ground to pupae. The wasp larvae emerges, and feeds on the caterpillar, completely consuming it (Johns 2004). Another major beneficial insect is the *Tachinid* fly, which attack its host while they are in the pupal stage. The fly will locate the chambers left by the pupating caterpillar, and from there plant its egg to develop inside of the pupae (DPI 2005).

Ichneumon promissorius is a wasp parasitoid which flies close to the surface of the soil (Figure 2). Once the wasp locates a silken cap which covers the *Helicoverpa* spp. chambers, it digs through, and lays an egg inside the pupae. Once the larvae is born, it feeds on the fluids which have leaked out from the puncture wound and it consumes the tissues surrounding it, crawling back up the chamber tunnel when fully developed (Short 2002). Another parasitoid wasp is *Heteropelma scaposum* (Figure 3), this is a slender black wasp, which attacks caterpillars at their third instar stage or higher. They parasitise the larvae which will continue to live out its larval stage and still pupate as per normal (DPI 2005). Once the

Figure 2. Ichneumon promissorius

Figure 3. Heteropelma scaposum

Heteropelma is fully developed it chews through the pupae casing and crawls out the chamber through the emergence tunnel. There are many benefits to reducing the reliance on sprays and making the change to IPM to control pest species. Once such example is the cotton production in Vietnam where farmers went from 15-20 sprays per season to only 1 or 2 and *Helicoverpa* spp. went from a major pest to a minor problem, due to the rise in numbers of beneficials (Fitt 2000).

Cultural

Cultural control is defined as, manipulating the environment and ecosystem to control a pest population. Several cultural control strategies are practiced in farming to reduce the numbers of *Helicoverpa* spp. and/or slow resistance within an area. These farming practices include; pupae busting, narrow planting windows, mandated cap on Bt acreage and biodiversity within a cropping field (Bates 2005). Early sowing (early October), proper use of nitrogen and water along with growth regulators, prevents any late maturing crops, thereby reducing late season problem with *H. armigera* (Fitt 1994).

Bt Cotton

Bacillus thuringiensis is named after the German town, Thuringia, where the virus was first identified. Bt toxins were first applied to crops as an insecticide spray, but the efficacy of the spray was inconsistent. A much more consistent control of key pests was achieved by incorporating the Bt toxin into the genes to produce the Bt cotton plant (Whitehouse, 2009). This therefore brought a higher efficacy to the control of pests, but also increased the risk of resistance. The current version of Bt cotton, Bollgard II, includes 2 specific Bt genes, Cry1Ac and Cry2Ab. Once proteins produced by these genes are consumed by the caterpillar, they bind to specific receptors in the gut wall forming holes, shown in figure 4. The activity of these proteins stops the feeding, and ultimately kills the caterpillar by allowing bacteria to enter the body cavity (Aroian R, 2012). Bt cotton is a perfect example of a successful genetically modified plant which is accepted globally. In order to keep this GM cotton effective certain measures must be put in place to prevent too much resistance build up quickly.

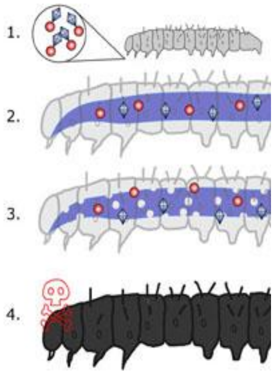

Figure 4. Steps illustrating how Bt forms holes in the gut wall of the

Refuge

The goal of refuges, is to produce non-resistant *Helicoverpa armigera* and *H. punctigera* moths that then mate with any resistant moths from the Bt cotton fields, thereby diluting the recessive resistant genes and producing phenotypically susceptible insects. In theory the idea behind a refuge, is to delay resistance by 20 generations by ensuring that at least 10% of the population is not exposed to Bt toxins.

There are a lot of regulations around the use and management of Bt cotton. These regulations are defined in Australia as the RMP (resistance management plans). As the name suggests the plan is in place for the sole purpose of reducing resistance in a population. This plan has 5 key components which contribute to the establishment and maintenance of refuges for cotton fields (Addison 2010). These 5 key components include:

1) Use of refuge crops (either non-cotton crops or non-Bollgard II cotton), to produce Bt susceptible moths on farm.
2) A defined planting window for Bollgard II to reduce the number of *H. armigera* generations potentially exposed each season.
3) End of season pupae destruction by cultivation and trap crops to remove any Bt selected individuals from the population between cotton seasons.
4) Control of cotton volunteers and stub cotton to remove the risk of creating mixtures of Bollgard II and conventional cotton that may allow survival of *H. armigera* larvae heterozygous for Bt resistance genes.
5) Spray limitations, including no use of Bt spray in refuges.

This can be a large cost to growers, and hence a lot of research has gone into the minimum requirements required to grow different types of plants. These factors which add to the cost are water costs for irrigation, labour, land space and all are needed in order to keep refuges attractive. In this study we will focus on the role of refuges.

The effectiveness of refuges is compromised if growers do not look after the refuges by either skipping irrigations or allowing weed infestations, as this neglect stresses the refuge plants and may affect flowering and their attractiveness to *Helicoverpa* moths, as seen in Figures 5 & 6 (Whitehouse 2009). As pigeon pea is known to need less water than cotton, growers are tempted to reduce the number of pigeon pea irrigations for economic reasons. Unfortunately, the lower levels of water may reduce the attractiveness of the pigeon pea crops to laying moths, and reduce its ability to produce healthy *Helicoverpa*. Refuge productivity is just as important as refuge attractiveness, as the more moths produced, the higher the chance of reducing resistance within a population (Fitt & Cotter 2005). As can be

Figure 5. Pigeon Pea farm with poor germination rate

Figure 6. Pigeon Pea farm with high weed infestation

seen in figure 5 and 6, refuges are not always watered or have proper weed management, this could affect their ability to attract egg lays by *Helicoverpa* spp. Pigeon pea can sustain many generations in one growing season while cotton usually has a maximum of 2-3. Pigeon pea also tends to have a lot of sub populations growing in and around the plant, allowing for more caterpillars to be produced (Fitt & Cotter 2005). Pigeon pea however can be flawed, as the plant may produce a high level of pupae, but the number that live on through to become moths are low (Bisane & Katole 2008). Cotton may produce more moths per pupae, but support fewer caterpillars. Whether pigeon pea are more attractive and productive than cotton, is becoming a controversial issue.

In a previous study conducted by a summer scholarship student; no difference was found in either the number of eggs laid (attractiveness) or moth produced (productivity), between pigeon pea and cotton on commercial farms. As these results are controversial, the comparison was repeated in this current study, with the inclusion of additional measurements (such as correlations between vegetative features and egg lays) as laboratory results indicated that these parameters affect refuge attractiveness and productivity.

Resistance Management

Since 2002, the method agronomists have used for measuring resistance is the F2 screen, which monitors field populations of *H. armigera* and *H. punctigera* for alleles conferring resistance to the Cry2Ab. This method generates isofemale lines that produce a proportion of individuals that are homozygous for haplotypes present in their field-derived parents. Through the use of specific crosses and bioassays this method determines whether the gene is present in a population and to what extent (Andow 1998). A study was conducted in China using this method, to test the amount of resistance build up of *H. armigera*, in a Bt cotton field with no refuge crop. In China, it is not mandatory to plant a refuge crop which is non-transgenic, however crops such as corn, soybean and peanut which are planted nearby act as refuge. This study showed that there was a high frequency of resistant alleles in the population (0.0146%/year on average from 2003-2005) compared to when Bt cotton was first commercially used (0.0058% in 1998). Although this number falls within the 95% confidence interval, it is possible that the actual resistance allele frequency in these populations may be even higher (Xu 2009). Using a similar method another study was done within Australia, this time however refuges and were used and resistance in both *Helicoverpa* spp., was found in low numbers. (Downes 2009). The populations of *H. armigera* which was found to be resistant was extremely low (frequency of <0.0005). This was due the better resistant management practices used in Australia (RMP) including the combination of high dosages of Bt toxins in the plant and the use of refuges to delay resistance.

The F2 screening method for rare resistant alleles in *Helicoverpa armigera* is shown below in Figure 7. The process involves collecting parents in the field, usually as eggs or larvae, and crossed to produce the F1 progeny. Here the F1 are sibmated to produce the F2, where there is a 1/16 chance to get a homozygous recessive resistant larvae (RR). The remaining larvae will be killed off by the toxin (Downes 2007).

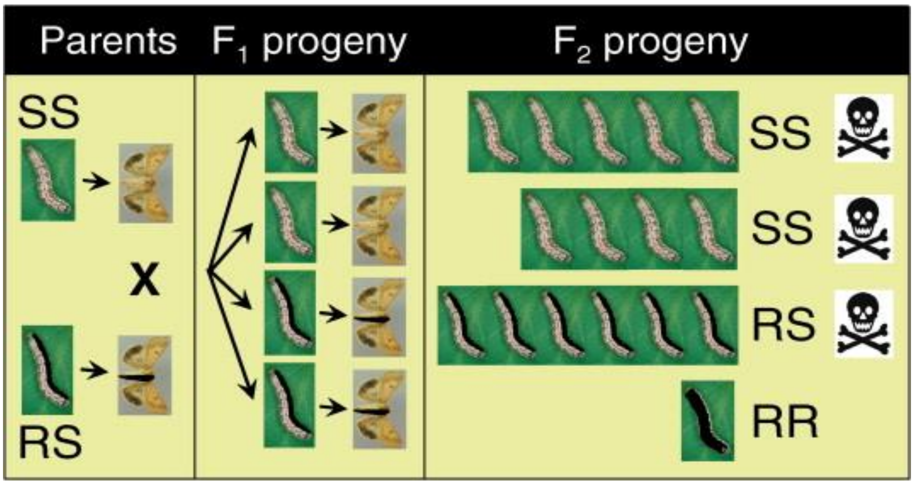

Figure 7. F2 Screening method for rare homozygous recessive resistant alleles (Downes 2007)

Attractiveness is defined by the amount of eggs found in a refuge, and productivity is defined as the amount of ‘live’ pupae emerged. Together these define whether a refuge is serving its purpose or not. In order for a refuge to serve it purpose, the pigeon pea pupae which emerges must be around the same time as the Bt cotton pupae that emerge (Baker 2008). This allows for interbreeding between the two to reduce the resistance of *Helicoverpa* spp. The aim of this work is to test if pigeon pea grown on commercial cotton farms is twice as attractive and productive as cotton refuges. The second aim is to identify what parameters indicate an attractive and productive refuge.

3. Materials and Method

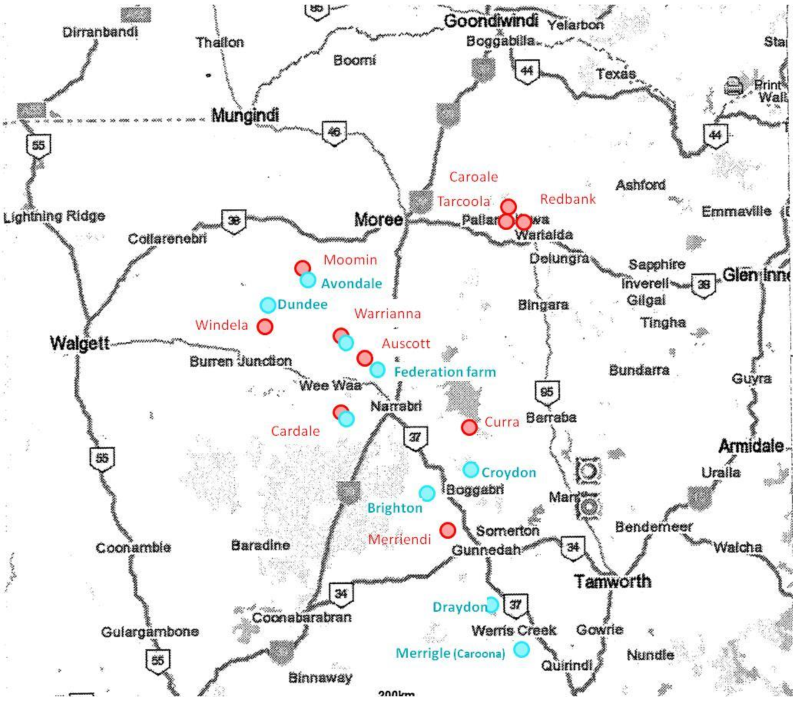

Figure 8. Map showing the distribution of cotton (blue) and pigeon pea (red) refuges used in this study

Site Description

The experiment was conducted within the Namoi valley, 19 refuges, (9 cotton and 10 pigeon pea), were sampled (Fig. 8). While pigeon pea refuges were very common and easy to secure, we had difficulty finding enough cotton refuges. Because of this our sample included one sprayed cotton refuge. We tried to ensure that the pigeon pea and cotton refuges were located in the same region within the valley to reduce the effect of any macro/micro-climatic variations over an 8 week period. Each farm planted the Bt cotton at approximately the same time, with pigeon pea plantings differing from farm to farm. There was a period of rain and slight flooding during the January month which may have allowed natural conditions to kill off many of the pupae.

Visual counts/ Pupae dig method

Attractiveness is defined by the amount of eggs found in a refuge, and productivity is defined as the amount of 'live' pupae emerged. Together these define whether a refuge is serving its purpose or not. In order for a refuge to serve it purpose, the pigeon pea pupae which emerges must be around the same time as the Bt cotton pupae that emerge (Baker 2008). This allows for interbreeding between the two to reduce the resistance of *Helicoverpa* spp. Visual egg counts are conducted by carefully searching between the leaves, flowers, stems and petioles for any eggs of the *Helicoverpa* (Figure 9), being careful not to count any rough bollworm eggs. In order to count the vegetative features of the plant, within the metre marked out, number of pods/bolls, flowers, and squares/buds were counted and compared. Pupae digs are conducted by carefully removing the soil on both sides of the row within the 1 metre plot of cotton, by scraping aside to a depth of 20cm gently. Any entrances to pupae chambers found were excavated further, hopefully locating a pre-pupae or pupae.

Experimental Design

This experiment was broken up into assessing attractiveness (egg lays), productivity (number of pupae), and whether attractiveness (egg lays) could predict productivity (pupae numbers). To do this 3 samples were taken; Sample 1 consisting of visual egg counts only, Sample 2 consisted of pupae digs only, and Sample 3 consisted of both visual egg counts at new locations and pupae digs at the same location as the first visual egg counts. Attractiveness was compared using the visual egg counts in sample 1 and 3; Productivity was compared using the pupae digs in sample 2 and 3; and predictability was tested by looking for correlations between the egg counts in sample 1, and the pupae collected in sample 3

Sample 1

This consisted of visual surveys for eggs (Fig. 9). Six random one metre plots at each refuge on each farm were marked with a standard fibre glass flag. At each plot, GPS coordinates were recorded to allow for pupae dig sampling in the exact same spot 4 weeks later (sample 3). Visual surveys of each spot consisted of carefully inspecting the one metre plot of plants and counting how many eggs were present. During our visual egg counts, we were careful not to mix up rough boll worm eggs with cotton boll worm eggs. While conducting this egg count all parts of the plant were looked at, however it was noticeable that the eggs are consistently located in certain areas only. Vegetative data, such as the number of squares, bolls or pods were also recorded per metre. This data is used to find any correlations between any egg locations, numbers and type of vegetation. The first sample took approximately 2 ½ weeks.

Figure 9. (Left) Undertaking visual counts of eggs and recording precise GPS mapping and visual data. (Middle) Helicoverpa eggs of cotton. (Right) Helicoverpa eggs on pigeon pea.

Sample 2

Approximately 2 weeks after the first sample we returned to the refuges to undertake 6 pupae digs at previously unsampled locations within the refuge field (Fig. 10). These samples were used to measure productivity. Any pre-pupae or pupae found were collected and taken back to the lab where they were housed at room temperature, reared, and any emergences in the following weeks were recorded.

Figure 10. Pupae sampling. Left: Conducting a pupae dig; Middle: Entrance into a pupae chamber; Right: Maintaining a pre-pupae in the laboratory.

Sample 3

At about 4-5 weeks after the first visual sample, we returned to the exact meter which was first sampled in week one, and repeated sample 1's process of plant measurements and egg counts in order to compare the attractiveness of the crop as the crop aged over time. The egg & pupae counts also included the analysis of attractiveness and productivity of each site and crop.

Statistical Analysis

Parametric statistics (ANOVA/ Regressions) were performed where valid, on raw or transformed data, using Genstat (Genstat statistical package). Where percentages were present and not abiding by the parametric assumptions, Non-parametric tests were conducted (Kruskal-Wallis one way ANOVA, Chi squared, and Spearmans Rank).

4. Results

Attractiveness

Figure 17 shows the comparison between all the farms containing both pigeon pea and cotton refuges throughout the 8 week period. Both sample 1 and 3 results are compared and based on the Kruskal-Wallis analysis, there is no significant difference between the number of eggs laid in the pigeon pea and cotton refuges in January (the first sample). However in February (the second visual egg count in the third sample) there is significant difference, with most pigeon pea refuges out performing the cotton refuges (figure 17).

There is a strong positive correlation between egg counts in non-transgenic cotton and Bt-cotton which is nearly one-to-one (figure 11), that is *Helicoverpa* spp. find both the refuge cotton and Bt cotton equally attractive. Figure 13 shows the comparison between pigeon pea refuge and Bt-cotton fields. There was no significant correlation between the egg lays in the pigeon pea refuge and corresponding Bt crop (Regression analysis: accounting for 3.7% variance, P value=0.185, standard error=4.67). However as pigeon pea is producing at least twice as many eggs as the Bt/ cotton refuge, it is performing as predicted by the theory. To understand if any vegetative factors were correlated with egg lays, we compared the number of buds/squares, flowers, and pods/bolls to the number of eggs laid per metre. Figures 13 and 14 show any relationships between egg counts and their respective vegetative data for cotton and pigeon pea samples. Both graphs show a positive relationship with buds/squares, however when the pigeon pea refuge egg count, is compared with the % of flowers to pods located in the metre at the time of visual counts, it was found that there was a strong positive correlation (Figure 15). Spearmans rank was used here as the results are a percentage and therefore do not follow a normal distribution for a parametric test.

Productivity

Figure 16 shows the comparison between pigeon pea and cotton pupae which either; emerged, managed to make it to moths, or were parasitised. It demonstrates that much higher number of moths emerged from pigeon pea that cotton refuges. A Chi Square test was conducted to compare the different numbers found in both cotton and pigeon pea survivals. There is no significant difference in survivorship of pupae removed from cotton and pigeon refuges, although there was a trend for *Helicoverpa* pupae in cotton to have a higher survival rate than those in pigeon pea (Chisq = 3.09, df=1, P=0.079).

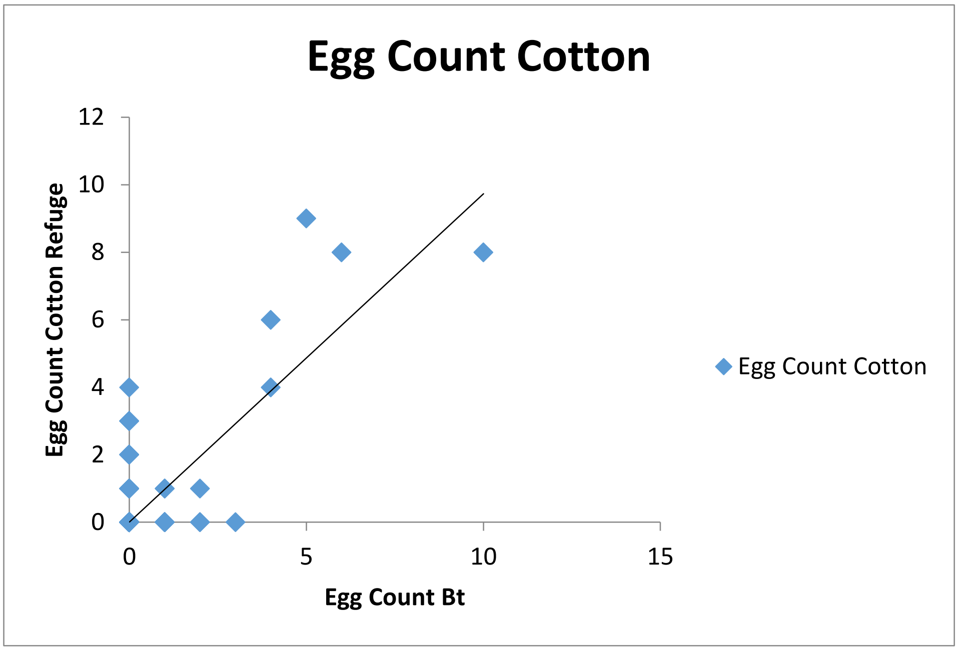

Figure. 11. The cotton refuge egg counts vs the Bt crop egg counts. The counts show both are equally attractive. Regression analysis: accounting for 91.2% variation in the data, P value=<0.001, Standard error=0.452. Due to the low P value, there is a significant positive correlation between the amount of eggs found in a cotton refuge and in Bt cotton. Line indicates expected relationship between egg counts in cotton refuges and the corresponding Bt crop

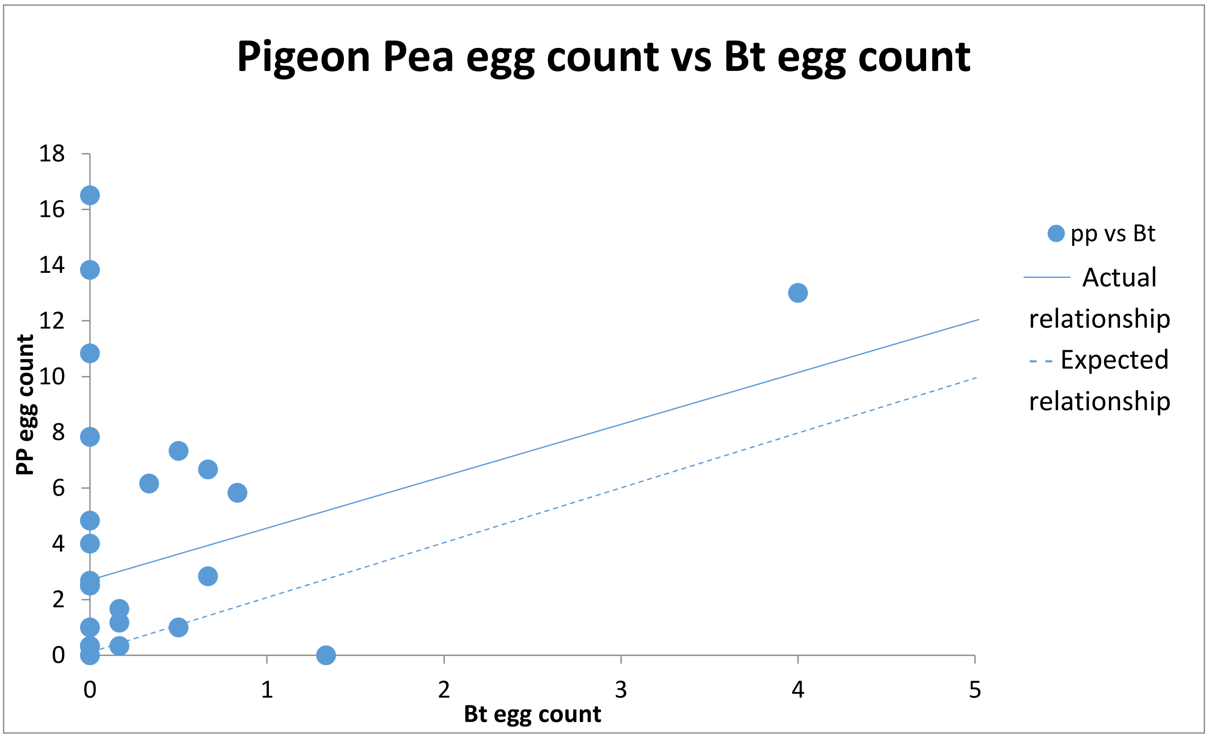

Figure 12. Egg counts in pigeon pea crops in comparison to the Bt egg counts. Pigeon pea is almost twice as attractive as cotton. Regression analysis: accounting for 3.7% variance, P value=0.185, standard error=4.67. Due to the high P value, there is no correlation between the pigeon pea and Bt cotton egg counts. Line indicates expected and actual relationship.

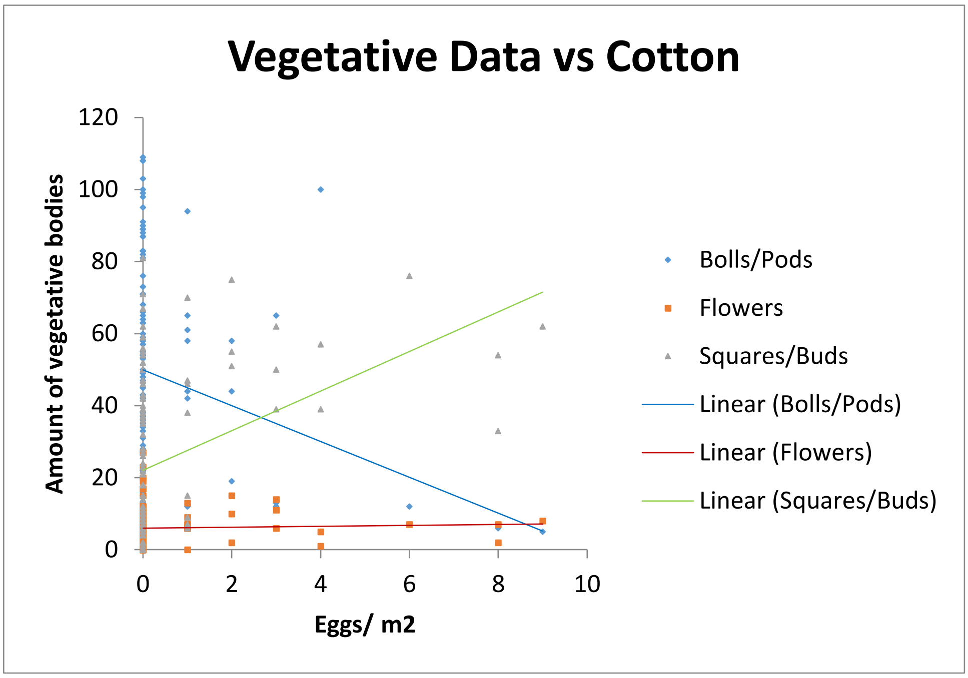

Figure 13. The correlation between vegetative data and egg counts in cotton. Counts of flowers, bolls and squares were compared.

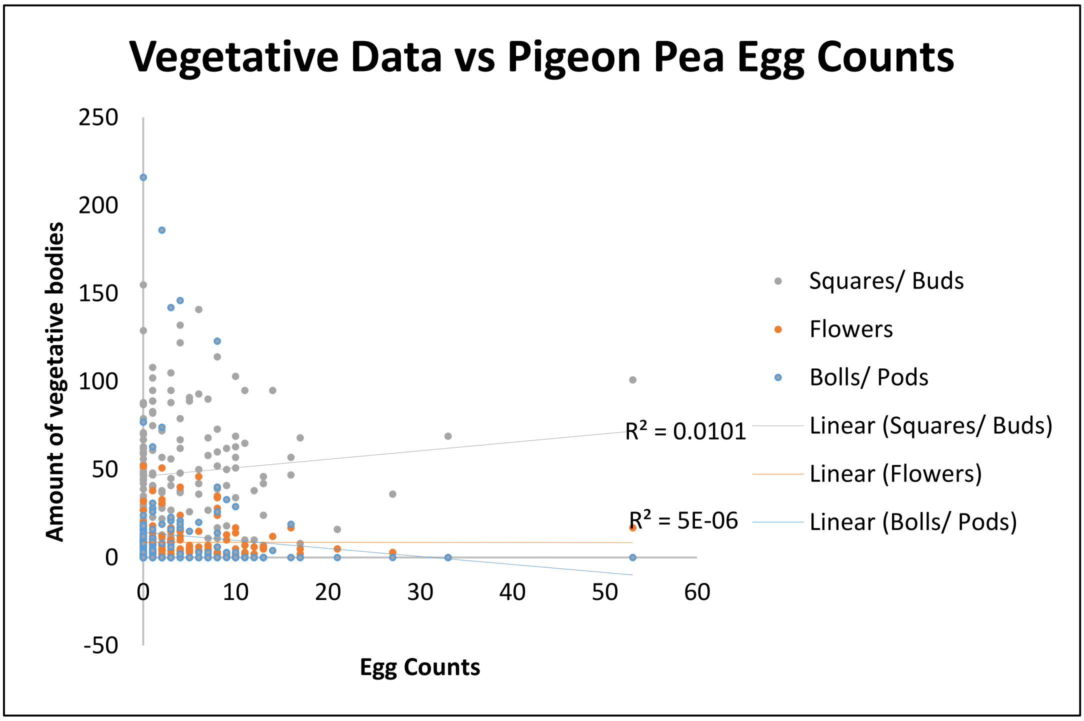

Figure 14. The correlation between vegetative data and egg counts in pigeon pea. Counts of flowers, pods and buds were compared.

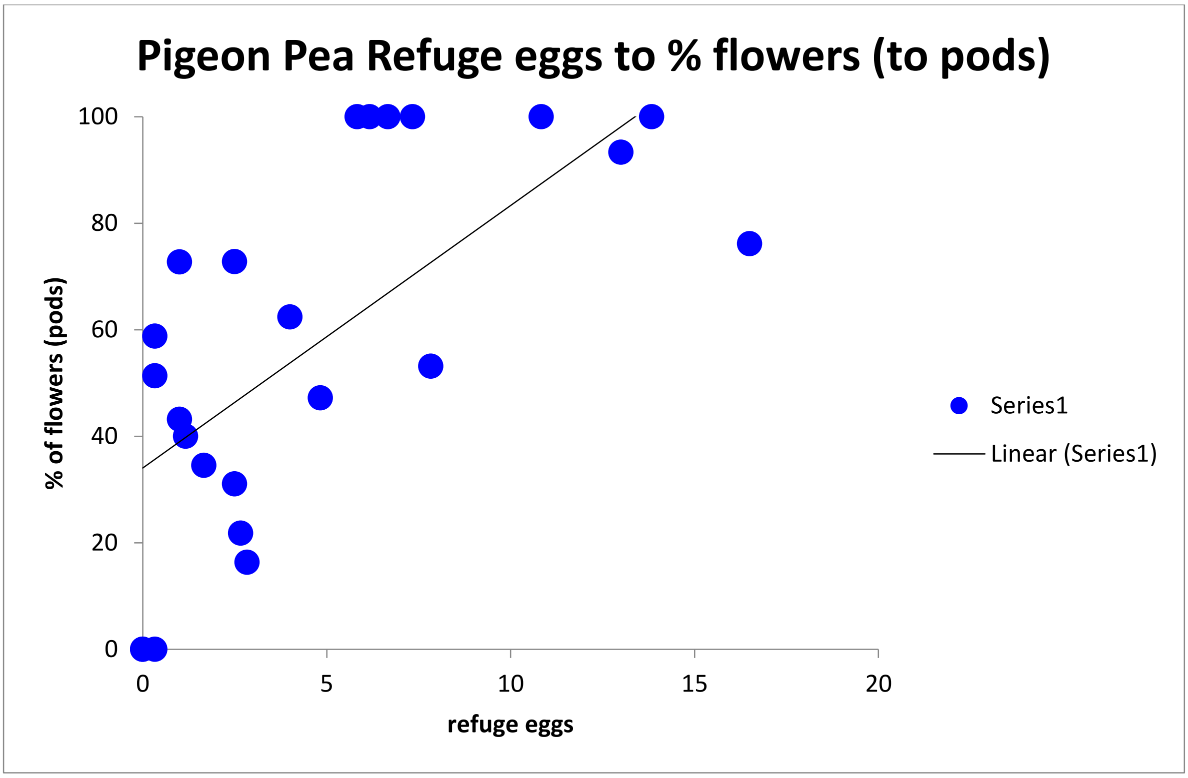

Figure 15. Amount of eggs found in relation to the percentage of pods which were also flowering. Spearmans rank correlation coefficient: Sample Size 21, d.f=19, P value=0.004. Due to low P value, there is a strong correlation between eggs being laid when there are a high number of flowers to pods percentage.

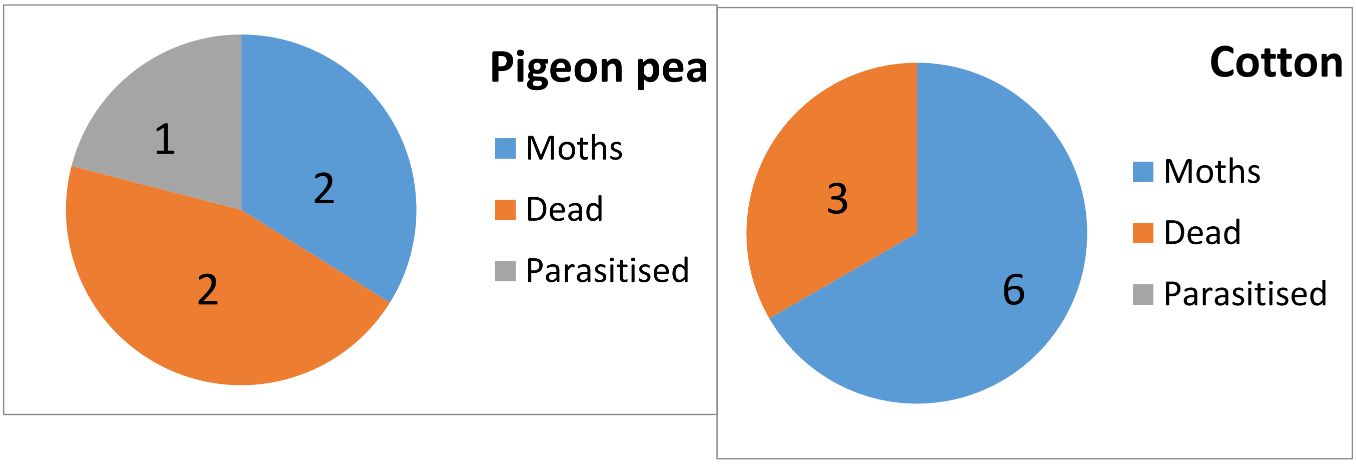

Figure 16. Relative proportions of emergences from pupae collected from pupae digs in pigeon pea and cotton refuges. The actual numbers of each category are indicated on the graphs.

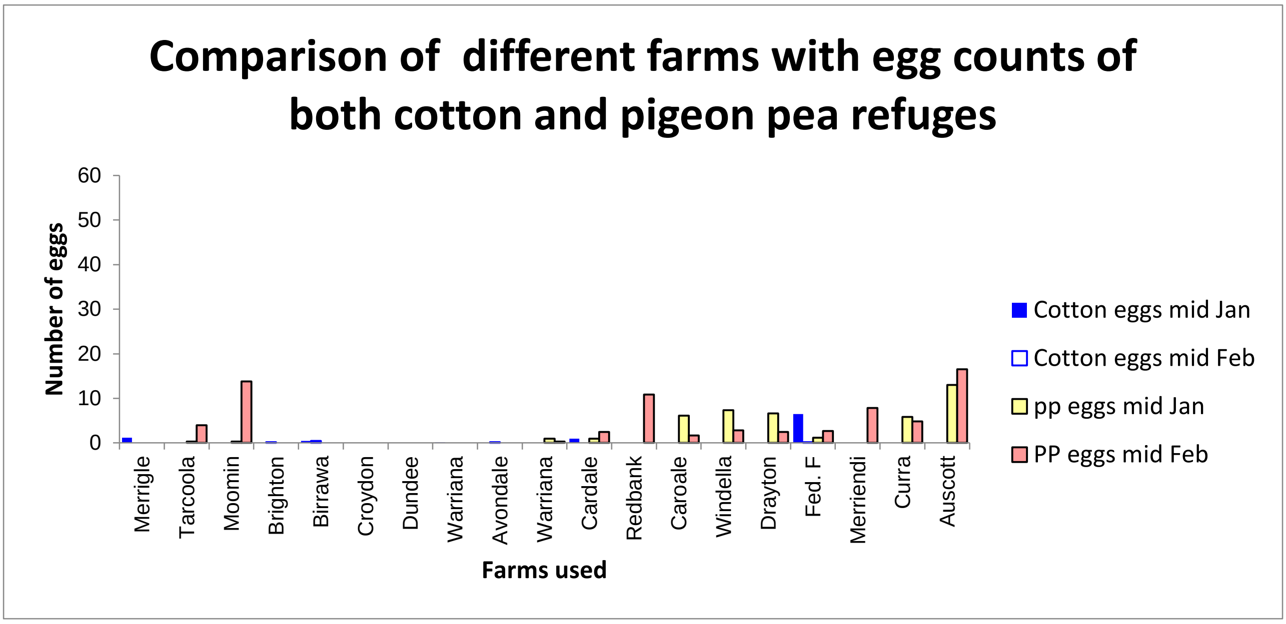

Figure 17. Shows the comparison made between the different farms and between cotton and pigeon pea egg counts. 2 analysis of variance were done here, one for the first egg counts at start of the season (Mid Jan) and one for the second egg counts in Sample 3. Kruskal-Wallis one-way analysis of variance (Sample 1): Chi-square=0.064, d.f=1, due to the P value being >0.05 we conclude that there is no significant difference between the pigeon pea and cotton in the initial egg count.

Kruskal-Wallis one-way analysis of variance (Sample 3): Value of H=8.354, d.f=1, Chi-squared=0.001. Due to the low P value, we can conclude that the second egg count later in the season had significant difference between the cotton and pigeon pea refuges.

	Observed	Fitted	Residual	
C2	C3			
Alive	Cotton	6	3.57	1.62
	Pp	21	23.43	-1.81
Dead	Cotton	3	5.43	-1.94
	Pp	38	35.57	1.76

C3	Cotton	PP	Margin
C2			
Alive	1.366	0.26	1.626
Dead	1.297	0.162	1.459
Margin	2.662	0.422	3.085

Figure 18. Chi square test showing p=0.079, meaning the difference is not significant, however may be unreliable due to the p value not being that strong.

5. Discussion

This field survey showed refuges performing as expected for the 2012-2013 season. For example the egg counts in the cotton refuge were the same as in the Bt crop. Thus the cotton refuges showed no difference in the level of attractiveness as the corresponding Bt crop, suggesting that moths were not distinguishing between non-Bt cotton and Bt cotton. A study in the U.S also confirms this as they conducted an experiment comparing attractiveness of Bt cotton and other non-transgenic crops including cotton, and their results were such where both the Bt and non-transgenic cotton had no significant difference in their attractiveness (Jackson 2007). Egg counts in the pigeon pea refuge were on average twice those in the Bt-crop (Figure 14), with some pigeon pea refuges considerably out-performing the corresponding Bt crop. This fits in perfectly with our assumptions that a pigeon pea refuge is at least twice as attractive as a cotton refuge.

Attractiveness is important within a refuge crop. However not the whole plant may be attractive. During the experiment, vegetative data were also taken and correlated against egg lays. In cotton, there seemed to be a positive relationship between the number of eggs found per square metre and the number of squares. In pigeon pea, the number of eggs per square metre also increases with bud presence. This suggests that no matter the crop refuge type, the attractiveness of the crop depends on the amount of buds present. Further analysis of the vegetative attractiveness showed that the attractiveness in a pigeon pea plant related to the proportion of pods flowering (Figure 15). The data shows that *Helicoverpa* spp. were attracted to pigeon pea pods at the start of the flowering cycle, ie, when they had flowers. As the flowering cycle progressed, the flowers disappeared and pigeon pea lost attractiveness, becoming only pods and peas. In the few cases where the pigeon pea had not yet flowered, the crop was not attractive (Baker 2008). Therefore even though flowering pigeon pea attracts more than twice the number of eggs than cotton, the timing of the flowering could be problematic. Should there be a problem attracting enough *Helicoverpa* to a refuge, moth-attraction technology can be used. A study was done on a certain product called ‘Magnet’, this product was used in a cotton refuge and increased the *Helicoverpa* numbers in the applied area significantly (Addison 2010). However with pigeon pea already so attractive there is very little data to suggest applying magnet on this crop type will increase effectiveness.

When comparing the farms on a large scale, Figure 17, the results show that pigeon pea is most attractive when flowering starts, but then progressively decreases as the proportion of pods increases. The results from Figure 17, when compared to a previous study conducted by another summer scholarship student in 2011, shows a lower number of *Helicoverpa* moths, and therefore this season had lower pressure for this pest. That is there was no correlation between the attractiveness of eggs found and the number of flowers/metre but a strong correlation between attractiveness and the proportion of fruit that were flowers.

Temperature effects pigeon pea as well, (Silim, 2007) as higher temperature will reduce the time it takes to get to flowering, and lower temperatures lengthen it. An increasing temperature has other effects too, as the parasitoid *Trichogramma* is also affected by this. With optimum developing temperature at 27°C, the *Trichogramma* would also develop very fast, and still have a prolonged life, however once temperatures increase from there, both pigeon pea and the parasitoid will be adversely affected (Mawela 2013). Once temperatures decrease to 21°C, the parasitoid again will not emerge.

Due to this flux of weather affecting the pigeon pea, over time, it is possible that the pigeon peas themselves have changed. The pigeon pea variety originally used as the refuge crop in the 1990s was Quest, but pigeon pea has not been attempted as a commercial crop in Australia since the 1980s, so there has been no incentive to maintain this variety's characteristics. In addition, the pigeon pea seed used to plant refuges for Bt cotton generally is produced from past refuges; therefore the seed used is that which has survived *Helicoverpa* attack over the years. Thus pigeon pea refuges used today are extremely variable, and may not be as productive (in terms of moths) as those in the past (Whitehouse 2012). Pigeon peas are day length sensitive, requiring day lengths under 12.5 hours for flowering and seed production (Silim 2007). However over time the species complex may have changed and therefore requiring different day lengths or prolonged the cycle to account of *Helicoverpa* attack. Pigeon pea usually flowers more than once a season and therefore can hold more than one generation of moths. However given our results the timing is now essential with planting the pigeon pea at such a time, depending on the variety, where the plant flowers with pods, at the same time that *Helicoverpa* spp. are laying in the adjacent Bt cotton field (Fitt 2005).

The productivity of these crops depends on the amount of moths emerging. It is believed that pigeon pea is twice as productive and attractive than normal cotton refuges. While there was a trend for pupae collected under cotton to have a better survival rate in comparison to those collected under pigeon pea, pigeon peas overall produced over three times the number of moths as cotton refuges (although the numbers are very low). Thus in terms of productivity, pigeon pea refuges sampled in the survey behaved as expected, and were at least twice as attractive as cotton refuges. A study done recently, showed that although pigeon pea was producing more pupae, the refuge's respective Bt crop, produced more live pupae (Baker 2008). If 'live pupae abundance' was taken into consideration instead of pupae abundance, this finding suggests that the effectiveness of susceptible moth production, from pigeon pea refuge crops in countering emergence of potentially resistant moths from Bt crops, might be compromised (Baker 2008).

During this period of time of collection, a lot of factors come into play in terms of accuracy of the data. Counting egg for this experiment was difficult as there is another pest insect with similar looking eggs known as the *Earias huegeliana* (rough boll worm). This insect is not included in this study, and to limit the experimental error we made certain before taking any notes, that we knew the differences between a rough boll worm egg and a Helicoverpa egg. Another contributing factor to experimental error in this experiment, was the poor state in some farms, as some farms as mentioned earlier may not have irrigated as often as they should have, or had severe weed problems along with low germination rates. These poor management practices can all effect the attractiveness and productivity of a refuge crop. To limit the variability, not all farms were in the best shape and bias was taken out of the equation, keeping all the 1 metre plots random. Another point of concern is that there were very few pigeon pea refuges in this survey that were in the vegetative stage. That means that we could not gauge the attractiveness of non-flowering pigeon peas, even though they are common in the area. While cotton refuges were scarce and we used any that we could obtain access to, pigeon pea refuges were plenty. To obtain access to these refuges, we asked consultants and growers to direct us to pigeon pea refuges. We suspect that to help us they directed us to only the flowering refuges. During the time this experiment was conducted, there were some floods which may have lowered the numbers and affected the statistics, however we accounted for this by making sure all there was corresponding cotton and pigeon pea refuge farms close to each other.

6. Conclusion

Overall pigeon pea is more attractive and productive than cotton as a refuge. Our figures show it produces significantly more moths and attracts more eggs. This is extremely important when looking to reduce resistance in Bt fields and looking at pigeon pea more specifically, with figure 16 in conjunction with 18, we can conclude that depending on the stage of the life cycle pigeon pea is at, will depend on the attractiveness of the refuge. With pods while flowering, being the most attractive stage in its life cycle, it means nothing if the plant does not reach this stage. A combination of proper water, weed and fertiliser management, will allow for optimum growing conditions and full attractiveness throughout its life. Therefore with our results, pigeon pea performed as expected in theory and was twice as more attractive, productive and economically viable than cotton as a refuge. With pods while flowering being the stage of most attractiveness, there is no current literature or published work to support this theory.

7. References

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