Australian Government
Cotton Research and Development Corporation

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAQ116C**

Annual Report:	☐	Due 30-September
Progress Report:	☐	Due 31-January
Final Report:	X	Due 30-September (or within 3 months of completion of project)

Project Title: Assessment of the potential for resistance to Gemstar.

Project Commencement Date: 17/9/2001 **Project Completion Date:** 30/6/2004

Research Program: 3: crop protection

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Part 3.3 – Final Reports (due 3 months after completion of project)

(The points below are to be used as a guideline when completing your final report. Postgraduates please note the instructions outlined at the end of this Section.)

1. Outline the background to the project.

The emergence of multiple resistance to insecticides in *Helicoverpa* populations has had a significant impact on the production of most major field crops in Australia. This, coupled with industry's increasing awareness of the need to reduce environmental impacts from pesticide use, has led to the demand for effective alternatives to traditional pesticides. Current alternative control options for *Helicoverpa* available to cotton growers include the use of 'Gemstar', a nucleopolyhedrovirus (NPV) specific to Heliothine species. Gemstar has been widely used in cotton and grains crops throughout growing areas. It is often used in combination with other pesticides and, in Queensland, in repeated and low-rate applications.

This wide-scale and repeated use has led to industry concerns about the potential for emergence of resistance to Gemstar. There have been several examples in other insect species where resistance of between 5 and 800 times normal susceptibility to insect viruses has been generated in the laboratory, although there have been no reports of resistance to baculoviruses in the field. There is, therefore, a need to establish the baseline level of susceptibility against which future resistance can be assessed, to develop some understanding of the mechanisms of resistance to insect viruses, and to develop genetic markers that might lead to rapid identification of resistant populations.

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

To determine the base-line response of field-collected *H. armigera* larvae from across Australia, to identify resistant field populations

A base-line response was determined. 73 samples of eggs or larvae were received over the time of project, against a background of declining *Helicoverpa* incidence that has created difficulties for all resistance monitoring work. In the first season, only six samples were received, and these were in poor condition. A more widespread mailing of collection kits and publicising of the collection at grower and consultant meetings in subsequent years increased the number of samples returned in the last 2 seasons.

Many samples were in poor condition and the majority of egg samples collected did not, at first, hatch. Problems encountered included: Mite contamination of cultures, fungal and bacterial contamination of samples and cultures, damage of samples during transport (excessive heat and rough handling), not enough individuals in samples, too many individuals to handle at once (overcrowding), not enough individuals of one species in sample eg. a few *H. armigera* and a few *H. punctigera* only, occasional difficulties in speciation due to missing or damaged parts of pupae, poor yields of eggs / pupae after surface sterilisation, presence of viral contaminants in samples (NPV, Stunt virus), parasitiods in samples eg *Heteropelma*, *Tachinids*.

Improved protocols for obtaining viable insects from eggs were developed by modifying the protocol used by Dr Robin Gunning. This significantly improved the recovery of insects for testing from eggs. This protocol is attached.

Where possible, insects from the field were reared to pupation and identified to species before breeding the F1 generation for bioassay. Most samples received were *H. armigera*. A small number (6) were mixed *H. armigera* and *H. punctigera*, 2 were pure *H. punctigera*.

A total of 27 field populations, all but one of which were *H. armigera*, were subjected to a full bioassay using neonate larvae over a range of seven or five doses of virus plus a control, and compared to a ‘standard’ laboratory population treated with the same doses in every assay. In addition, 35 single-dose diet incorporation challenges were conducted, comparing mortality of the

field population to the laboratory colony at the same dose in each, to determine if a single discriminating dose could be used to detect resistant populations.

No resistant field populations were detected. There was very little variation in LC50 or LC90 in any of the populations assayed. The lack of significant difference in susceptibility from a laboratory standard was seen most clearly in comparison of the relative susceptibility with that of the laboratory colony. The field populations typically showed a variation of between one third and twice the susceptibility of a standard (i.e 3 times less susceptible and twice as susceptible), with only a single population being 8 times more susceptible (i.e. less resistant) to the virus. The single population of *H. punctigera* for which a full assay could be conducted was less susceptible to Gemstar than the laboratory colony (0.5 relative susceptibility), but was within the range of variation across populations. Overall, the differences in susceptibility are within the bounds to be expected in natural variation.

In summary, the results have established a baseline of susceptibility for the population with a mean LC_{50} of 2.8 x 10^5 occlusion bodies/ml in neonate larvae. While there is a small amount of variation in responses to viral challenge, there is no evidence of resistance in field populations.

To attempt to induce resistance in the laboratory.

Two attempts were made to establish resistant colonies in the laboratory by pooling populations collected from the field. However, in both years this was not successful as larvae ceased laying eggs in the autumn of both years, despite maintenance under a constant light and temperature regime.

To identify populations tested and any resistant populations using DNA microsatelite markers.

Moths emerging from all field collected samples were sent to the CID at UQ for microsatelite analysis. This was used to confirm species diagnosis, and to add to the microsatelite data analysis conducted independently by UQ.

To assess the stability of any resistance detected in the absence of selection pressure and after cross-breeding with susceptible insects. To determine if any resistance detected is at the level of gut infection or at a different stage in the infection process, and if it can be overcome.

Since no resistance was detected, and resistance was not successfully induced in the laboratory, this work could not be conducted.

3. Detail the methodology and justify the methodology used.

New and improved protocols developed during the project are attached.

Many samples were in poor condition and the majority of egg samples collected did not hatch. Protocols for obtaining viable insects from egg collections were developed by modifying the protocol used by Dr Robin Gunning, and this significantly improved the recovery of insects for testing from eggs.

Where possible, insects from the field were reared to pupation and identified to species before breeding the F1 generation for bioassay. Most samples received were *H. armigera*. A small number (6) were mixed *H. armigera* and *H. punctigera*; 2 were pure *H. punctigera*.

It has been suggested that all populations should be reared for a second generation in the laboratory to increase the proportion of homozygous resistant individuals that may be resistant in the population. An attempt was made to rear an F2 generation for some of the samples, however it was found that the additional work was beyond the resources of this project to routinely rear an F2 generation, and field collected populations often did not breed successfully under laboratory conditions. As most samples received were eggs, it is assumed that these are already the progeny of insects exposed to Gemstar, and it could be argued that these represent an effective F1 generation, with the progeny produced and assayed being F2. Assays were therefore conducted using the first generation of progeny from field collected insects.

A total of 27 field populations of both *H. armigera* and *H. puntigera* were subjected to a full bioassay using between 5 and seven virus doses and between 30 and 42 insects per dose. Neonate larvae were assayed using the modified droplet bioassay method of Hughes and Wood (1982). The DPI&F laboratory colony of *H. armigera* was used as a comparative 'standard' population in every assay using a full range of seven doses to establish the relative susceptibility of the field colonies and provide a point of reference between assays conducted on different dates. This bioassay method is used routinely in NPV bioassays by DPI&F and has proved the most reliable and consistent assay method.

The LC50 and LC90 of each population was calculated using a linear regression of logit transformed mortality against concentration of occlusion bodies per ml of test stock in Genstat. A relative susceptibility of each colony was calculated in Genstat by comparing the regression of the dose response in the field population to that of the laboratory standard.

In addition, 35 single-dose diet incorporation challenges were conducted to determine if a single discriminating dose could be used to detect resistant populations. Mortality of the field population was compared to the laboratory colony at a single dose of either 5×10^3 OB/ml of diet or 1×10^4 OB/ml of diet. This method might have practical advantages for future monitoring, as it can be done with relatively few larvae. However, results were disappointing and the method is not recommended.

Two attempts were made to induce resistance in a laboratory colony following variations of published methods (M.L. Milks, J. H. Myers & M. K. Leptich 2002, Evolutionary Ecology 16:369-385) . Field populations were mixed as adults to establish a 'pooled' colony over the season with discreet generations. The pooled colony was then closed and reared as a single colony for 5 generations. The colony was then divided into 2. In one half, larvae were reared on uncontaminated diet ('unexposed'), in the other larvae were reared on diet containing a concentration of virus sufficient to kill 90% of the larvae ('exposed'). In each sub-colony, adults were mated and the process repeated for their progeny. However, in both years it was found that adults in both the exposed and unexposed sub colonies would not lay eggs during the winter months, and both colonies were lost.

4. Detail and discuss the results including the statistical analysis of results.

No resistant field populations were detected. There was very little variation in LC50 (fig 1) or LC90. The LC50 of the field populations varied between 0.32 and 4.8 x 105 OB/ml. Only 5 populations showed an LC 50 that was statistically different from that of the laboratory standard: 2 were lower in LC 50 than the standard (ie more susceptible to virus) while 3 had a higher LC 50 (ie were less susceptible). However, the relative difference in LC50 to the standard was not great, ranging between 2.5 times higher (less susceptible) and 0.1 times lower (more susceptible) than that of the standard.

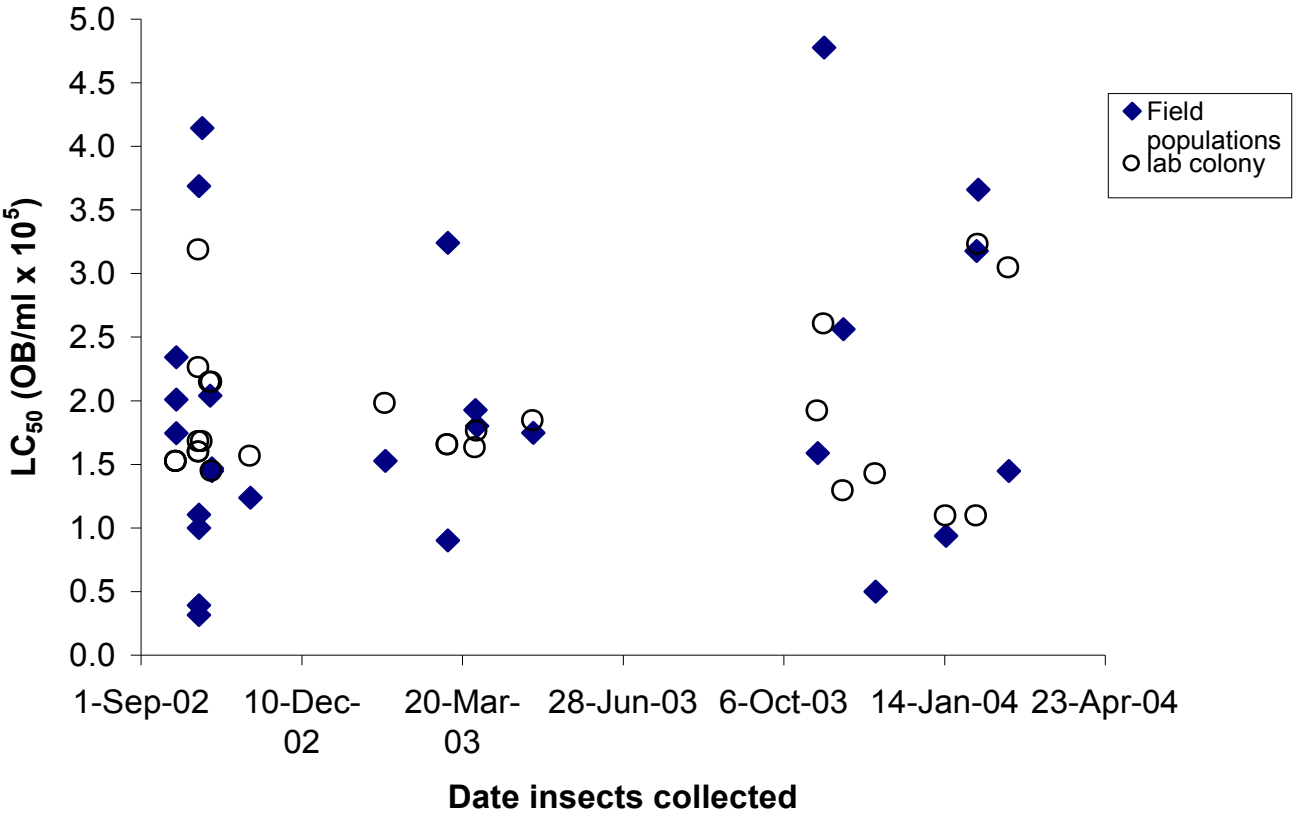

Figure 1: LC50 (OB/ml x 10^5) of field populations and laboratory colony.

The lack of significant difference in susceptibility from a laboratory standard was seen most clearly in comparison of the relative susceptibility with that of the laboratory colony (Figure 2). 11 of the 27 populations subjected to full bioassay had a relative susceptibility that was statistically significantly different from that of the laboratory colony. Of these, 7 showed susceptibility between only 0.65 and 0.34 that of the laboratory standard (i.e between half and one third less susceptible than the standard), while 3 had a relative susceptibility between 1.7 and 2 times that of the standard (i.e. less 'resistant'). One sample was 8 times more susceptible than the laboratory standard.

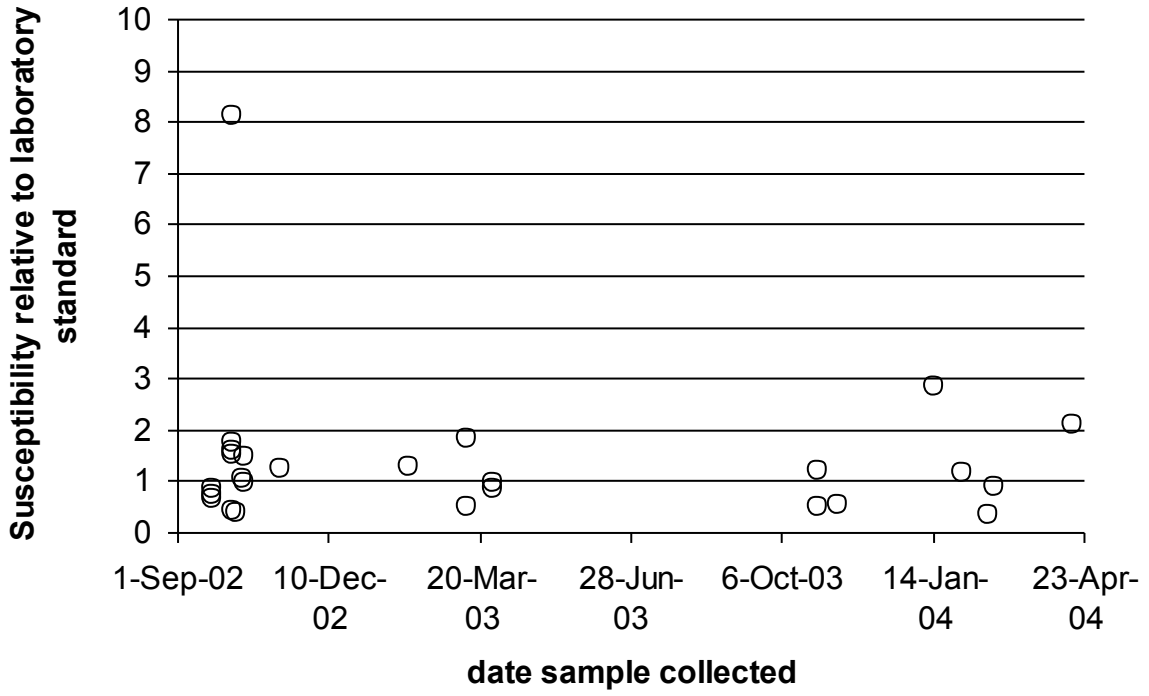

Figure 2: Susceptibility of field populations relative to laboratory population standard.

In summary, the field populations typically showed a variation in susceptibility of between one third and twice the susceptibility of a standard, with only a single population being 8 times more susceptible (i.e. less 'resistant') to the virus than the standard. These differences in susceptibility are within the bounds to be expected in natural variation.

The use of diet incorporation allows small numbers of larvae to be tested on each day of hatching. 35 single-dose diet incorporation challenges were conducted, comparing mortality of the field population to the laboratory colony at the same dose in each, to determine if a single discriminating dose could be used to detect resistant populations. Typically, 2 or 3 doses between 5 x 10^3 and 2x 10^5 OB/ ml were been used to determine an suitable dose.

Of the populations tested, none were significantly less susceptible than the laboratory colony or each other. The greatest difference detected was in a single population (sent from Byee) which had 57 and 65% of the mortality of the laboratory standard and other populations at 2 doses. However, the lack of replication in these assays prevents staistical comparison.

A single dose of either 5 x 103 OB/ml of diet or 1x 104 OB/ml of diet was found to give a mean of 82% and 74% kill respectively and could be used as a baseline in future monitoring. However, these results proved highly variable even in the laboratory colony, and are difficult to analyse statistically when only one dose is used. This method is not recommended for use when a bioassay is possible.

The project also supplied a number of adults from field populations to the microsatelite marker analysis at UQ. Insects collected from the field were reared and mated in the laboratory. The progeny were used in resistance assays, while the adults were stored in alcohol and sent to UQ for microsatelite analysis. This is a useful model to make maximum use of valuable field collections in two projects and should be considered for future resistance monitoring projects.

5. Provide a conclusion as to research outcomes compared with objectives. What are the "take home messages"?

The results have established a baseline of susceptibility for the population by two assay methods: an estimation of LC50 by droplet bioassay, and a single dose diet incorporation assay. The results indicate that while there is a narrow range of variation in susceptibility within field populations, there is no significant resistance to Gemstar in field populations of *H. armigera*.

These results are significant because they provide a platform for future monitoring of resistance, should any concerns arise. However, no detailed studies of resistance were possible due to the difficulties in establishing a stable laboratory colony from the field populations.

While no detailed studies of the nature of resistance were possible, the results in general support the claim that resistance in the field is not likely to occur because of the complex biological and ecological relationship between baculoviruses and their insect hosts (see attached article from Heliothis Stateline for discussion). NPVs such as those found in Gemstar and Vivus are based on a complex 'swarm' of isolates. Over evolutionary time these variants have developed subtly different life history strategies, and complex interactions with their insect hosts. This suggests that, unlike for chemical insecticides, the development of resistance would not be a simple process for the insect populations. This is especially true now that new products based on different virus variants (such as Vivus Gold) are available to industry.

Where laboratory tests have shown some selection for resistance, these have sometimes incurred a penalty in fitness for the insect, although this was not the case in a recent study of loopers (Milks *et al* 2002). In laboratory studies, resistance has been rapidly selected against where outbreeding occurs with non-resistant populations, and in most studies once exposure to the virus stops (Fuxa & Richter 1989, 1998, though see Milks *et al* 2002). Conditions in the field are unlikely to lead to the generation of resistance, since, unlike with the use of chemical insecticides, natural enemies are

unaffected by the NPV. *Helicoverpa* larvae that survive virus challenge and that might carry a resistance to infection are likely to die from predation or parasitism.

NPVs cause an infection in the larva that can spread to other, uninfected larvae after death and persist and spread in the environment. *Helicoverpa* populations in Australia have a long history of natural exposure to these viruses, at both low levels and during wide scale epizootics, yet our results show that there is no resistance in the field populations even compared to a laboratory colony that has been reared without exposure to virus for over 30 years.

This has implications for the use of NPVs as insecticides, which have the potential to control *Helicoverpa* without disrupting natural enemy populations, and without the threat of resistance. This suggests that the widespread and repeated use of NPVs should be the first response to viable egg lay by *Helicoverpa*, rather than the use of disruptive chemical insecticides. Recommendations that restrict the repeated application of biopesticides based on NPVs should be reconsidered or removed.

The outputs of this project provide the techniques with which to monitor for resistance in the future, and a better understanding of risks of resistance on which to base current management decisions.

6. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?

The development of resistance to biopesticides like Gemstar and Vivus would have significant negative impacts on the industry and threaten strategies to reduce the use of conventional chemicals through "soft options" and Area Wide Management. By supporting the continued use of biopesticides, the work contributes to economic (maintaining viable insect control), environmental (maintaining viable 'soft' options) and social (reducing chemical inputs around rural communities) benefits.

7. Provide a summary of the project ensuring the following areas are addressed:

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.)

c) are changes to the Intellectual Property register required?

The outputs of this project provide the techniques with which to monitor for resistance in the future, and a better understanding of risks of resistance on which to base current management decisions. Recommendations that restrict the repeated application of biopesticides based on NPVs should be reconsidered or removed.

Protocols for rearing of insects from field collected material, especially from field collected eggs, have been improved and have been attached.

No changes in the intellectual property register are required.

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

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

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

(c) for future research.

Recommendations that restrict the repeated application of biopesticides based on NPVs should be reconsidered or removed.

A greater awareness of the likely lack of resistance will be disseminated to industry through the cotton IPM short course and in a concluding paper to be submitted to Cotton Grower.

The baseline susceptibility can be used in future follow-up monitoring. It is unfortunate that the laboratory population used as a standard is unlikely to be maintained now that the project has finished, due to lack of demand and funding for it's upkeep. However, future surveys at periodic intervals (such as every 4 years) should be conducted and use of the droplet assay at the dose range used here.

Induction of resistance in a population and subsequent studies on the nature of resistance is still required. We would like to investigate this further, and will discuss the possibility of student research in this area if a suitable pooled field population can be established.

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

Results were distributed through Heliothis Stateline and Hotline, through Area Wide management group meetings, through presentations to industry, and through the CRC extension team. Results have been included in the teaching materials and used in the Cotton IPM Short Course. An article published in Heliothis Hotline is attached.

Results were included in a paper at the International Congress of Entomology in 2004.

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

No.

11. **Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry or the Australian community.**

The outputs of this project provide the techniques with which to monitor for resistance in the future, and a better understanding of risks of resistance on which to base current management decisions.

Insecticides based on NPVs have the potential to control *Helicoverpa* without disrupting natural enemy populations, and without the immediate threat of resistance. This suggests that the widespread and repeated use of NPVs should be the first response to viable egg lay by *Helicoverpa*, rather than the use of disruptive chemical insecticides.

Recommendations that restrict the repeated application of biopesticides based on NPVs should be reconsidered and removed, as these uses would have significant benefits to industry.

Is resistance to virus a problem?

By Dr Caroline Hauxwell, DPI AFFS, Indooroopilly, 07 3896 9362

The threat of Heliothis developing resistance to the nuclear polyhedrosis virus (NPV) in Gemstar® and Vivus® still concerns and confuses many people. The concern is that use of virus over a wide area, at frequent intervals and at below-label rates may cause NPV to follow conventional chemicals into the resistance trap. Yet the possibility of resistance to NPV is thought to be of little concern by many experienced insect pathologists. The reasons for this are, like the biology of these viruses, complex.

Most conventional insecticides work in a relatively simple way - they inhibit a biochemical step, such as one involved in transmitting nerve signals. When chemicals are applied at high rates, those Heliothis with the relatively minor mutations required to 'get around' the chemical are rapidly selected and the population becomes resistant.

So why aren't the experts worried about viruses? The virus in Gemstar® and Vivus® causes an infection in the insect, a much more complex process than blocking a nerve signal. What's more, the insects have been exposed to similar viruses for many millennia, usually at low levels. During the evolutionary tug of war between insects and viruses, the insects have evolved resistance mechanisms, but the viruses have also evolved ways to overcome them. One result is that the wild Australian viruses (and the commercial products) are a mix of many strains, each with a slightly different infection 'strategy'. It would seem logical that Heliothis would find it difficult to become resistant to them all.

Could heavy reliance on virus in Australia increase resistance above natural levels? A comparable example is the use of a similar virus against the velvetbean caterpillar, *Anticarsia gemmatalis*, a pest of soybean in Brazil. The Brazilians have been using the virus on a large scale for almost 20 years, and currently treat around 1 million hectares a year. Some concern was raised when studies showed that *Anticarsia* could develop 1000-fold resistance to the virus in the laboratory, but further studies showed that maintaining resistance is 'expensive' for an insect, and tends to be rapidly lost when selection pressure is removed. In the field, insects from areas that had been sprayed with virus for longest were very slightly less susceptible than unsprayed populations, yet the *Anticarsia* virus is still effective as an insecticide. Even so, the Brazilians are monitoring the field situation, just to be sure.

The move towards 'soft options' and Area Wide Management involves the use of viral biopesticides on an unprecedented scale. CRDC have funded a 3 year monitoring study by QDPI to survey Heliothis populations so that we can spot resistance if it occurs. QDPI, with University of Queensland, are also conducting laboratory studies to see if resistance can be artificially generated in the laboratory and to understand the genetics and mechanisms of that resistance. If resistance is detected in the field or created in the lab, it will be very different from resistance to chemicals, and understanding how it works will be fundamental to finding a solution.

The good news is that there are already a few options for overcoming resistance if it ever occurs. These include the commercial production of different virus strains (such as native Australian strains), or by adding optical brighteners to the spray mix, which was shown to break the laboratory-generated resistance in Brazilian *Anticarsia*.

Aussie farmers are world leaders in the wide-scale use of viral biopesticides, and are rightly concerned about their loss to resistance. However, the probability of resistance emerging is small. As for below label rates, Heliothis in the field are always exposed to low-level virus from natural sources or from previous applications. Low rates may not be very effective for pest control, but they are even less likely to cause resistance than the high selection pressure from full-rate sprays. More work – and time – is needed before we can draw a firm conclusion. Sensibly, monitoring has started. For now, resistance to virus shouldn't cause any loss of sleep.

DAQ 116 – Protocol 3 Handling and Rearing Helicoverpa spp. Eggs from Plant Material

Introduction

Eggs collected in the field usually arrive still attached to the plant material they were deposited on. It is not always easy to find and separate the eggs from the plant material, especially if the sample consists of corn silks. This method was devised to reduce the amount of labour required to harvest individuals from difficult samples. It also reduces the number of samples that are lost due to fungal and bacterial growth when plant material is placed directly onto diet. The decreased amount of handling of individual eggs reduces losses arising from mechanical damage.

Documents Required
Running Record (Attachment 1)

Materials
Rectangular plastic 3.5L food storage box with modified lid (Rectangular hole 110mmX170mm centrally located on the lid) (Décor)
Plain white Nappy liners, (Generic brand), White only for ease of identifying neonates only.
Forceps
Paintbrush
Clinical waste disposal bag
Absorbent Paper
5% Sodium Hypochlorite solution (5L sodium hypochlorite solution [12.5g/L available chlorine] in 100L H_2O)
Freshly prepared Heliothis diet cups (Solo P100 plastic Soufflés containing approximately 6.5 g of Heliothis diet)
Tissue squares (Kleenex – 1ply, 50mm X 50mm square)
Solo PL1 Plastic Lids with 9 holes for air exchange
Trays to hold completed diet cups
Adhesive labels and marker pen

Method

1. Process and assess the condition and quarantine status of samples on arrival. (Outlined in Protocol 2 – Steps 1 to 5)

2. Cut absorbent paper to size so it fits into the base of the rectangular plastic box easily. Cut five (5) to six (6) per box depending on sample size.

3. Place one absorbent paper sheet on the bottom of the box.

4. Evenly spread the leaf material over the absorbent sheet and cover with another sheet. Evenly spread more leaf material over this sheet and cover. Continue this procedure for several more layers. Avoid packing the leaf material in too densely or adding too many layers into the box. Large leaves should be spread in a single layer and dense material should be gently teased apart and spread as thinly as possible.

5. Place two (2) white nappy liners creating a double thickness over the top of the box, overlapping the edges.

6. Seal firmly with the modified lid.

7. Label with the sample's unique number and date set up (arrival date), place in a controlled temperature / humidity room.

8. Check daily. Change the absorbent paper as required, if excessive moisture in the sample, absorbent paper may need to be changed daily until paper remains relatively dry. As neonates hatch – remove them and place them into diet cups with a layer tissue, seal with holed lids and place them into an appropriately labelled tray. Neonates tend to move upwards in the box and are often found on the Nappy liners under the lid.

9. Record daily the number of neonates harvested onto running record sheet and retain with the sample.

10. This process will continue for a few days. Once neonates have ceased to emerge, dispose of plant material and absorbent paper. Sanitise rectangular plastic boxes in a 5% sodium hypochlorite, rinse and dry well before next use.

Attachment 1 – Sample Copy – Running Record

Batch No: **Species:** ***Helicoverpa*** **Generation:**

Date	**No. set up in tray**	**No. left in tray**	***Sample Health***		*Comments*
			Bacterial		
			Chemical		
			Deformed		
			Fungal		
			Mechanical		
			NPV		
			Other		
			Parasitised		
			Stunted		

DAQ 116 Protocol 4: Handling and Rearing Helicoverpa spp. Eggs not from plant material, Larvae and Pupae.

Introduction

The condition of field samples is influenced greatly by the way they are transported to the Laboratory. Summer heat can destroy the samples before they reach the laboratory if they have not been packed appropriately. Even if they survive the transport they are difficult to rear. On arrival it is important to assess their condition and attempt to establish them as quickly as possible in the controlled temperature / humidity room (CT room). It is difficult when working with small numbers to establish successful individual colonies. Initial handling of samples on arrival can influence the rearing success of the samples but often factors occurring prior to receival have a greater influence. This protocol introduces techniques used to improve the success of establishing field samples as viable colonies.

Documentation Required

Receipt Information (Attachment 1)
Running Record (Attachment 2)

Materials

- For field samples of larvae

Freshly prepared Heliothis diet cups (Solo P100 plastic Soufflés containing approximately 6.5 g of Heliothis diet)
Tissue squares (Kleenex – 1ply, 50mm X 50mm square).
Solo PL1 Plastic Lids with 9 holes for air exchange.
Trays to hold completed diet cups

- For field samples of pupae

1L Sodium Hypochlorite solution 2.5% (25mL sodium hypochlorite solution [12.5g/L available chlorine] in 975mL H_2O)
1.5 L Plastic Bowl and Sieve

- In general

Forceps
Paintbrush
Clinical waste disposal bag
Absorbent Paper
Field Sample ring binder
5% Sodium Hypochlorite solution (5L sodium hypochlorite solution [12.5g/L available chlorine] in 100L H_2O)
Adhesive labels, white paper, pencil and marker pen
70mL plastic screw-top storage container.
100% Ethanol
Disposable plastic weigh boats
5 L plastic bucket
Square Perspex lid with cloth, (nappy liners) slits
Blue and white Nappy Liners, (Johnson & Johnson)
70mL plastic screw-top storage container modified to hold a centrally located cotton wick with a
10% Sucrose / Ascorbic acid / Streptomycin Solution

Methods

For Field Samples of Larvae

1. Process and assess the condition and quarantine status of samples on arrival. (Outlined in Protocol 2 – Steps 1 to 5)
2. Transfer each surviving larva from the plant material or diet they were transported on using clean sterilised (either with sodium hypochlorite solution or by steam sterilisation) forceps or paintbrush into a clean diet cup containing freshly (less than 1 week old) prepared Heliothis diet. Be careful to avoid contamination by decontaminating forceps or paintbrush frequently with a sodium hypochlorite solution (2.5%),.
3. Place a square of 1 ply tissue on the diet cup and secure with a holed lid, lay diet cup on its side in a labelled tray accompanied by the running record for the sample.
4. Place tray in CT room set at 16-hour day length and 60% humidity.
5. Check twice a week, record any deaths, their cause and the date on the running record sheet. Also record any relevant observations, for example the presence of a parasite. Retain all dead insects and parasites storing them in 100% Ethanol, empty and replace with fresh 100% Ethanol after twenty-four (24) hours. Write the details including unique number, date, generation and species on a small white piece of paper with a pencil and place inside the 70mL plastic specimen storage container. Label the outside with the same information and store in a cool place out of direct sunlight.
6. Speciate using visual identification guides and separate the larvae into *Helicoverpa armigera* and *Helicoverpa punctigera* into separate trays. Clearly label with a Ha for *Helicoverpa armigera* and Hp for *Helicoverpa punctigera*. The unique number will remain the same but –Ha or – Hp may be attached to the end, for example 04030Ha or 04030Hp and 04030AHa or 04030AHp.
7. Allow larvae to pupate unhindered.
8. Once pupated and hardened remove from diet cup and surface sterilise with a 2.5% Sodium Hypochlorite solution in a bowl for between five (5) to ten (10) minutes, rinse well in the sieve with clean water and dry on absorbent paper. Place in a plastic weigh boat.
9. Place in a labelled 5L bucket with perspex lid and hanging nappy liner.
10. Check daily for emerging moths.
11. Remove moths, confirming species and sex, to a separate labelled bucket with nappy liner. Place a 70mL plastic screw-top storage container modified to hold a centrally located cotton wick with a 10% Sucrose / Ascorbic acid / Streptomycin Solution into the bucket. (Ensure the wick does not touch the nappy liner).
12. These moths are the F_0 generation. If your sample is mixed you will have a bucket for *H. armigera* and for *H. punctigera* remember to label the buckets carefully.
13. Each day remove and store nappy liner in a labelled and slightly inflated plastic bag sealed firmly with a rubber band. The eggs in this bag are the next generation.
14. Once neonates emerge repeat the process. REMEMBER: At this point the samples should have been speciated but it is advisable to check. Also remember that this is a new generation and the correct generation number should follow through onto all labels and documents associated with these individuals.

For Field Samples of Eggs that Arrive or are on Non-plant Material

1. Process and assess the condition and quarantine status of samples on arrival. (Outlined in Protocol 2 – Steps 1 to 5)
2. Assess the media the eggs are on. If it is okay leave the eggs on it, if not gently remove them and place into a clean diet cup containing freshly (less than 1 week old) prepared Heliothis diet.
3. For eggs on egg cloths you can, if you choose to, carefully surface sterilise them when they are mature (older than 24 hours) using a 2.5 % sodium hypochlorite solution for 2 – 4 minutes.

Rinse well with water and dry on absorbent paper towel. Place in a plastic bag to hatch and then place the hatched neonate onto a clean diet cup containing freshly (less than 1 week old) prepared Heliothis diet. Beware surface sterilizing these eggs may retard hatching.

4. Place a square of 1 ply tissue on the diet cup and secure with a holed lid, lay diet cup on its side in a labelled tray accompanied by the running record for the sample.
5. Check 2 –3 times per week. Speciate when possible and record information on the running record sheet for the sample.
6. Follow the steps 4 through 14 from the section of this protocol, " For Field Samples of Larvae"

For Field Samples of Pupae

1. Process and assess the condition and quarantine status of samples on arrival. (Outlined in Protocol 2 – Steps 1 to 5)
2. Remove from their transport container and surface sterilise with a 2.5% Sodium Hypochlorite solution in a bowl for between five (5) to ten (10) minutes, rinse well in the sieve with clean water and dry on absorbent paper. Place in a plastic weigh boat and place in a labelled bucket.
3. Follow the steps 9 through 14 from the section of this protocol, " For Field Samples of Larvae"

Attachment 1 - Sample Copy – Receipt Information
Receipt Information

Accession Number	
Place of Origin	
Date Received	
Date Collected	
Collector	
Received From	
Property Name	
Property Owner	
Paddock Number	
Crop	

Sample Type	**Number Received**	**Condition**
Eggs		
Larvae		
H.armigera		
H.punctigera		
Dead on Arrival		
Pupae		
H.armigera		
H.punctigera		
Dead on Arrival		
TOTAL Number Received		
TOTAL Number Dead on Arrival		

Bioassay Date	
Generation Bioassayed	

Generation Culture Terminated	**Date**
F0	
F1	
F2	
F3	

COMMENTS:

Attachment 2 – Sample Copy- Running Record

Batch No: **Species:** ***Helicoverpa*** **Generation:**

Date	No. set up in tray	No. left in tray	***Sample Health***		***Comments***
			Bacterial		
			Chemical		
			Deformed		
			Fungal		
			Mechanical		
			NPV		
			Other		
			Parasitised		
			Stunted		

DAQ 116 Protocol 5: Maintaining *Helicoverpa* spp. as individual multigenerational colonies and pooled colonies.

Introduction

Field samples submitted are initially reared as individual colonies (F_0), if required, further generations are established, F_1, F_2, F_3. Progeny from these colonies contribute to resistance bioassays, susceptible colonies and the establishment of a resistance colony. Generally the F_0 samples are not in sufficient numbers or at an appropriate stage to supply individuals for bioassay work so an F_1 colony is established to increase numbers. This is difficult as F_0 colonies are fragile and limited numbers of adults may be produced. Samples submitted, in some cases, contain a mixture of species. It is not uncommon for a sample to contain both *Helicoverpa armigera* and *Helicoverpa punctigera* and it is necessary to set up two (2) distinct colonies. This also reduces your available numbers.

Documents Required

Receipt Information (Attachment 1)
Running Record (Attachment 2)
Bucket Record (Attachment 3)

Materials

Materials outlined in Protocol 4

Methods

Follow protocol 4 - All steps for Heliothis rearing.
Samples should all be speciated and separated accordingly.

Single population

15. Surface sterilise the selected nappy liners containing the egg lay you require 24 to 48 hours after collection with a 2.5% sodium hypochlorite solution for 2 - 4 minutes. This step may be omitted or a reduced strength sodium hypochlorite solution may be used. Rinse eggs with clean onto a fresh nappy liner. Allow eggs and liner to dry. Gently place between 2 layers of absorbent paper and seal into a slightly inflated, labelled plastic bag. Put aside and allow eggs to hatch.

16. Transfer each neonate, using clean sterilised (either with sodium hypochlorite solution or by steam sterilisation) forceps or paintbrush into a clean diet cup containing freshly (less than 1 week old) prepared Heliothis diet. Be careful to avoid contamination by decontaminating, with a sodium hypochlorite solution (2.5%), forceps or paintbrush frequently.

17. Place a square of 1 ply tissue on the diet cup and secure with a holed lid, lay diet cup on its side in a labelled tray accompanied by the running record for this generation of the sample. Ensure the generation is clearly stated on all labels and documentation.
18. Place tray in CT room set at 16-hour day length and 60% humidity.
19. Check twice a week, record any deaths, their cause and the date on the running record sheet. Also record any relevant observations, for example the presence of a parasite. Retain all dead insects storing them in 100% Ethanol, empty and replace with fresh 100% Ethanol after twenty-four (24) hours. Write the details including unique number, date, generation and species on a small white piece of paper with a pencil and place inside the 70mL plastic storage container. Label the outside with the same information and store in a cool place out of direct sunlight.
20. Allow larvae to pupate unhindered.

21. Once pupated and hardened remove from diet cup and surface sterilise with a 2.5% Sodium Hypochlorite solution in a bowl for between five (5) to ten (10) minutes, rinse well in the sieve with clean water and dry on absorbent paper. Place in a plastic weigh boat.
22. Place in a labelled 5L bucket with perspex lid and hanging nappy liner.
23. Check daily for emerging moths.
24. Remove moths, confirming species and sex, to a separate labelled bucket with nappy liner. Place a 70mL plastic screw-top storage container modified to hold a centrally located cotton wick with a 10% Sucrose / Ascorbic acid / Streptomycin Solution into the bucket. (Ensure the wick does not touch the nappy liner).
25. Each day remove and store nappy liner in a labelled and slightly inflated plastic bag sealed firmly with a rubber band. The eggs in this bag are the next generation.
26. Once neonates emerge repeat the process from step 1 – 12. REMEMBER: At this point the samples should have been speciated but it is advisable to check. Also remember that this is a new generation and the correct generation number should follow through onto all labels and documents associated with these individuals.

Pooled colony. A continuous colony consisting of multiple samples being added over a specified time then closed and treated as a single entity.

1. Follow steps 1 to 9 from the previous section, " For a single sample generation."
2. Once the adults begin to emerge, confirm and record sex, species and pupal deaths onto running record for that sample.
3. Add adults into a labelled bucket (Label = Pooled Colony, Commencement Date and Species) containing adults taken from other samples.
4. Record these additions documenting the number added, generation, sex and date on the bucket record for the pooled colony bucket.
5. Proceed with this method until colony is closed and no more moths are added. Once closed rename colony, Generation 0 (G_0), carefully archive colony records and begin recording this colonies history. Always make sure records about G_0 colony origins are easily traced.
6. Now treat this colony as a single sample colony.
7. Select the use for this colony's progeny: -
   - Continuation of discreet generations,
   - Exposure to NPV for bioassay or
   - Exposure to NPV for resistance colony formation.

Attachment 1 - Sample Copy –Receipt Information
Receipt Information

Accession Number	
Place of Origin	
Date Received	
Date Collected	
Collector	
Received From	
Property Name	
Property Owner	
Paddock Number	
Crop	

Sample Type	**Number Received**	**Condition**

Eggs		
Larvae		
H.armigera		
H.punctigera		
Dead on Arrival		
Pupae		
H.armigera		
H.punctigera		
Dead on Arrival		
TOTAL Number Received		
TOTAL Number Dead on Arrival		

Bioassay Date	
Generation Bioassayed	

Generation Culture Terminated	**Date**
F0	
F1	
F2	
F3	

COMMENTS:

Attachment 2 – Sample Copy – Running Record

Accession No: **Species:** ***Helicoverpa*** **Generation:**

Date	**No. set up in tray**	**No. left in tray**	*Sample Health*		*Comments*
			Bacterial		
			Chemical		
			Deformed		
			Fungal		
			Mechanical		
			NPV		
			Other		
			Parasitised		
			Stunted		

Attachment 3 - Sample only – Bucket Record

Bucket Record

Bucket Label:

Species:

Accession Number	Date of Addition	Generation added	Number & Sex added	Eggs Produced & Date

Culturing/Bioassay Artificial Diet for *Helicoverpa armigera*

Equipment

Biscuit dispensing guns
Solo P100 plastic soufflés
Trays to hold diet cups
Heat resistant gloves
Large plastic spatula
2L plastic beaker
Paper towel
10 ml measuring cylinder
5L plastic beaker
Balance
Spatula and weigh boats
Blender or stick mixer
Electric kettle (2L)
Microwave oven

Safety and Contamination Control

1. **Always wear an apron**
2. **Always handle hot materials with the heat resistant gloves provided**
3. **Keep the diet prep room very tidy and clean.**
4. **Do not bring soil or insecticides into room, EVER**

	Full Batch	**Half Batch**	**1/4 Batch**
Dry Ingredients			
Wheat germ	300g	150g	75g
Yeast	250g (550)	125g (275)	62.5g (137.5)
Soy Flour	340g (890)	170g (445)	85g (222.5)
Boiled Water	2400mL	1200mL	600mL
Agar	50g + 1200mL hot tap water (NB: **not boiled**)	25g + 600mL hot tap water	12.5g + 300mL hot tap water
Anti-bacterial			
Nipagin	15g (905)	7.5g (452.5)	3.75g (226.25)
Sorbic acid	5g (910)	2.5g (455)	1.25g (227.5)
1-ascorbic acid (Vitamin C)	15g (925)	7.5g (462.5)	3.75g (231.25)
Anti-fungal	5mL	2.5mL	1.25mL

Anti-fungal Solution

Prepare in a Fume Hood

Propionic acid	42ml
Phosphoric acid	4ml
Water	54ml

Add ingredients and store in a glass container

How to prepare Agar

Tare 2L beaker on balance and add Agar. Add hot tap water. Microwave for 3 mins on high, put on heat resistant gloves, take out and stir with spatula, then microwave on high for another 2 mins. Put gloves on again and take out of microwave. Add carefully (hot) to dry ingredients mixture. When finished, put beaker under hot water so it's easy to wash later.

Method

1. Put water in kettle and boil while you weigh out dry ingredients
2. Weigh out sorbic acid, 1-ascorbic acid and Nipagen while you microwave dry ingredients. Use plastic 1 oz portion cups to separately weigh out the sorbic acid, nipagen and 1-ascorbic acid. If time also weigh out the agar.
3. Measure out anti-fungal solution
4. Boil tap water in 2L kettle
5. Measure out wheat germ, yeast and soy flour on balance into a 5L beaker (See table)
6. Carefully add the required amount of boiled water to 5L beaker containing dry ingredients
7. Stir mixture with spatula
8. Microwave mixture on medium high for 5 mins. Using heat resistant gloves, remove mix and stir using spatula. Microwave on medium high for another 5 mins
9. Pour mixture into blender, or leave in beaker and use a bar mixer.
10. Blend mixture well and when the temperature is below 60°C, add sorbic acid, anti-fungal solution (see instructions below), Nipagen and 1-ascorbic acid (Vitamin C).
11. Add melted agar.
12. When all ingredients are well mixed, pour into another 5L beaker with handle and then pour into biscuit guns, ready for dispersion into plastic cups.