Cotton
RESEARCH & DEVELOPMENT

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

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

CRDC Project Number: **CSE95**

Annual Report: ☐ Due 30-September

Progress Report: ☐ Due 31-January

Final Report: ☒ Due 30-September
(or within 3 months of completion of project)

Project Title: Honey bee dissemination of Heliothis NPV onto cotton flowers.

Project Commencement Date: 1 July 2001 **Project Completion Date:** 30 June 2002

Research Program: 3 Crop Protection

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Part 3.3 – Final Report

1. **Introduction and background**

INGARD® Cotton varieties express Bt toxin in all plant parts except for the flowers. Because *Helicoverpa* moths frequently target flower buds and flowers as oviposition sites, a proportion of their neonate larvae may establish themselves on or in the flowers in a Bt-free location on the cotton plant. This may allow them to develop to a size at which they are more tolerant of Bt toxin on other plant parts. Such larvae may subsequently be capable of damaging fruiting structures and other plant parts before they are affected by the Bt toxin.

Honey bees are known to visit cotton flowers, and this project set out to determine whether they may provide an efficient means of depositing HNPV (*Heliothis* nuclear polyhedrosis virus) propagules directly into cotton flowers, and thereby help to eliminate an otherwise safe haven on INGARD® plants. Honey bee hives can be readily modified to disseminate virus or other microbial biocontrol agents, and the hives are easily transported to required areas. Honey bees from such hives have been shown to successfully act as vectors of HNPV against *Helicoverpa zea* on Crimson Clover Flowers (Gross *et al*. 1994), and as vectors of Bt for control of Banded Sunflower Moth on Sunflowers (Jyoti and Brewer 1999). Results from both these studies were impressive. For example mortality of *H.zea* larvae, when fed crimson clover flowers that had been visited by bees, increased from 12% in Control fields to over 80% in fields treated with HNPV (Gross *et al*. 1994).

The architecture of cotton plants and their flowers is substantially different from crimson clover or sunflowers. The temperature and UV radiation regimes within the cotton canopy are more severe. The feeding behaviour of *Helicoverpa* neonate larvae within flowers, and the foraging behaviour of honey bees visiting cotton flowers are also important factors influencing the incidence of NPV infection. This study was undertaken to determine the level of efficacy likely to be achieved using this technique in cotton crops in Australia.

If honey bee dissemination of NPV is efficacious it may provide a relatively cheap, target specific, non-disruptive bio-control option that growers implementing IPM may use to supplement their *Helicoverpa* control program. Furthermore, work undertaken by John Rhodes (NSW Agriculture, Tamworth, CRDC project DAN120C) suggests that honey bees have the potential to increase boll retention and yield in cotton by up to 15%. If this proves to be a consistent trend, it provides an additional incentive for cotton farmers to accommodate and utilize honey bees, and this in turn will increase reliance on non-disruptive IPM based pest control.

2. Project Objectives

The objectives of this study were to:

(i) Undertake pre-deployment sampling to determine background levels of NPV incidence in field collected *Helicoverpa* larvae.

Achieved: A total of 742 larvae were collected from chickpea, pigeonpea and cotton at 'Lowana' over 16 weekly sampling visits in the period 5 October 2001 to 17 January 2002. The larvae were reared on artificial diet in the laboratory to determine the natural incidence of NPV infection. Sampling continued after the deployment of NPV into the beehives and a further 67 larvae were collected from cotton and pigeonpea and reared on artificial diet.

(ii) Assess the number of wild honey bee visits to cotton flowers at a range of distances into cotton fields prior to deployment of bee hives.

Achieved: Observations and counts of honey bee visits to cotton flowers were made on the 4th and 10th of January 2002 prior to the deployment of commercial beehives.

(iii) Assess the number of honey bee visits to cotton flowers at a range of distances into cotton fields after the deployment of bee hives on the field edge.

Achieved: Observations and counts of honey bee visits to cotton flowers were made on 22nd January and 7th February 2002.

(iv) Deploy NPV powder formulation into pathogen applicator device trays in each bee hive beside treated fields and undertake a sampling regime to collect *Helicoverpa* spp larvae, bees, and flowers from the treated and untreated (control) fields.

Achieved: NPV powder was deployed into the pathogen disseminator trays in the bee hives on 10 occasions over the period 17 January to 11 March 2002. A total of 67 larvae, 511 flower samples, 95 bees, 20 pollen beetles and 10 honey samples were collected for bioassays and analysis. On two occasions 100 Sentinal larvae were placed into cotton flowers in treatment and control fields and retrieved 24 h after the virus dissemination for rearing and assessment of infection rates.

(v) Undertake laboratory bioassays of cotton flowers, bees, and honey from treated and untreated hives.

Achieved: Cotton flowers, honey bees, honey and pollen beetles were analysed using bioassays to quantify the incidence of NPV.

(vi) Undertake observations of neonate larvae placed onto flowers visited by NPV carrying bees marked with fluorescent powder.

Partially achieved: 8 captive larvae were successfully observed, and honey bees carrying NPV powder coloured by fluorescent dust were filmed visiting cotton flowers. However blacklight illuminated photographs of distribution of fluorescent NPV powder carried by bees into cotton flowers were not taken due to equipment failure.

3. **Methods**

(a) *Study site*.

The research was undertaken on the cotton property 'Lowana' near Pilliga NSW, in collaboration with the property owners Ken and Lou Platt, and a local apiarist Mr Gary Woolridge. Figure 1 shows a map of 'Lowana' and the location of each field used in the study. The cotton fields on 'Lowana' are located beside a branch of the Namoi River. The northern half of each field adjoins substantial areas of Coolabah and River redgum woodland. In good seasons substantial numbers of feral beehives are present in tree hollows in this woodland. The southern boundaries of each cotton field adjoin pasture and grassland.

Initial trials were conducted in Field 5 containing Siokra V3i INGARD® cotton. On 16 January 2002 an apiary of 10 hives was placed at the site marked by 'X1' beside Field 5 shown in Figure 1. A set of nil-treatment control hives was placed beside Field 2 at site 'X2'. Field 5 contained an early crop that matured prematurely and stopped flowering on 31 January 2002. The NPV treated beehives were subsequently moved on 4 February to site 'X3' beside Field 1, containing flowering "Delta Emerald" conventional cotton. As this crop matured, flowering began to decline in the northern end of Field 1, and on 20 February the NPV treated hives were moved to site 'X4' near the centre part of Field 5. On the 4 March the NPV treated hives were moved to site 'X5' beside Field 2 which contained two blocks of unsprayed Sicala 40 conventional cotton (5.16 Ha and 0.93 Ha respectively) that were separated by 16.5 Ha of pigeonpea (cultivar 'Quest').

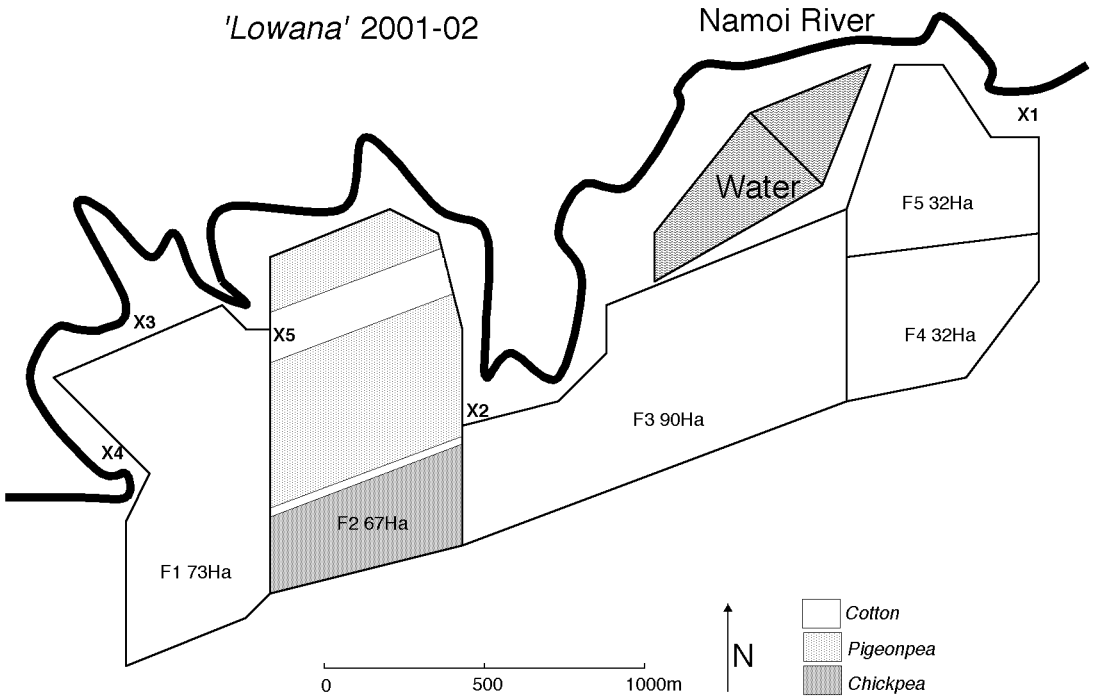

Figure 1. Map of 'Lowana' showing the location and size of each crop field. The locations of apiaries during the project are marked by the numbered crosses X1 to X5.

(b) *Honey bee hives*

A total of 22 commercial honey bee (*Apis mellifera*) hives belonging to Mr Woolridge were used during the study, however a maximum of 10 hives were deployed with NPV formulation at any one time. The hives were placed 10 to 22 metres from the edge of the cotton crops. Figure 2 shows a typical placement of the hives in an apiary beside Field 2.

The activity rate of each hive was quantified by counting the number of bees exiting and returning to each hive over 5 minute intervals. Three of these counts were completed prior-to and after each deployment of NPV powder formulation on 31 January, 7 February, and 15 February 2002. The PreNPV counts were made between 8.40 am and 9.05 am. The postNPV counts were made between 10.20 am and 11.30 am.

On 16 January 2002 one or more insecticide applications on neighbouring farms appear to have caused high levels of mortality in the bee hives. In particular, an application of fipronil (Regent) to a cotton field on a farm approximately 2km north of Lowana on 16th January 2002 corresponded with mass mortality of worker bees in most of the hives. Figure 3 shows dead bees that have been ejected from one of the hives. It is presumed that the bees were foraging in crops or vegetation and came in contact with the insecticide soon after it's application. Fipronil is highly toxic to the order Hymenoptera (wasps, ants and bees), and poisoned individuals that return to the hive (or nest) subsequently poison other individuals in the hive. Fipronil also has a very long residual period for bees – up to 28 days.

To counter the problems with insecticide mortality, Mr Gary Woolridge (apiarist) either added new worker bees from reserve hives to the 'sick' hives to bolster their numbers, or replaced sick hives with reserve healthy hives. When neighbouring properties subsequently notified Mr Woolridge that they intended to spray insecticides, all the hives were moved off-farm for 1 or 2 days before being returned.

(c) *Virus formulation*

The NPV powder formulation was derived from Gemstar® liquid concentrate. Gemstar® is an American biopesticide registered for use on cotton in Australia. It contains infective polyhedral inclusion bodies (PIBs) of nuclear polyhedrosis virus cultured from mass reared *Heliothis zea*.

A powder formulation of NPV was made by freeze drying 1 litre aliquots of liquid Gemstar® concentrate for 30 hours to produce approximately 25 g of dry material. The dry material was manually crushed and ground using a morter and pestle under liquid nitrogen.

The resulting powder was then mixed with 9 parts industrial talc and a trace of pink or orange fluorescent powder, to produce 250 g of NPV powder per litre of Gemstar®. Four batches were made during the course of the project. During each experiment, 5g of NPV powder formulation was placed into trays in each hive (figure 4).

Each 5g measure of the powder formulation applied to each bee hive was equivalent to 20 ml aqueous Gemstar. The recommended rate for applying Gemstar to cotton crops is 500ml/ha. Therefore, assuming it could be spread uniformly, each 5g of our final powder formulation contained sufficient active ingredient to treat 0.04 Ha (400m^2) of cotton crop canopy. However the formulation was spread in powder form by bees directly into cotton flowers and deposited as a highly concentrated sprinkling of particles onto surfaces that the bees landed on, predominantly in and around the flowers.

Figure 2. The apiary beside Field 2. Note the Pathogen Applicator Devices containing pink coloured NPV at the entrance to each hive.

Figure 3. Hundreds of dead bees ejected from a bee hive following the aerial spray application of fipronil (Regent) insecticide on a neighbouring cotton farm approximately 2km to the north of Lowana on the evening of 16 January 2002

(d) *Virus dissemination*

Hive-mounted 'pathogen applicator devices' based on those described by Gross *et al.* (1994) were constructed by Mr Gary Woolridge at "Lowana", near Pilliga NSW (Figures 4, 5, 6). The devices consist of a removable wooden drawer with clear perspex panels that replaces the normal entry/exit panel at the base of each bee hive. Bees exiting the hive are forced to crawl through a removable tray in which the NPV powder formulation was placed, towards the clear Perspex window, before dropping down a gap and onto the take-off/landing pad. As they crawled through the tray they became contaminated with the virus dust which could be clearly seen as fluorescent pink or orange powder on their wings, legs, abdomen, thorax and head (Figure 5). Bees entered the hive by walking directly in from the landing pad below the pathogen tray and did not come in contact with the NPV powder.

For each treatment, 5g of NPV powder formulation was placed into the applicator trays in each hive and the time noted. A smoker was used to calm the bees, and a full apiarist suit was worn when attending the hives. At regular intervals the amount of powder left in the tray was assessed.

Figure 4. Filling tray with 5g of fluorescent pink dyed NPV +Talc powder formulation.

Figure 5. Inserting the NPV tray of the 'Pathogen Applicator Device'.

Figure 6. A honey bee worker exiting a bee hive covered in NPV powder.

(e) *Honey bees*

On two dates prior to the deployment of the bee hives, and on 3 dates after their deployment, counts of bee visits to 2 metre sections of cotton row were made at a range of distances (0, 10, 50, 60, 75, 100, 200 and 250 metres) into the cotton field (figure 7). On each observation date the counts of bee visits to cotton were completed between 0900 and 1100 hours. The behaviour of each bee observed was recorded and classified into 4 categories: Forage in flower; Forage on plant; Hovering; and Fly-by.

On three treatment dates, counts of bees exiting and returning to each hive were made both prior to, and after, the deployment of NPV powder into the pathogen applicator device in the treated hives. Counts of the number of bees per hive that were dying over a 2.5 hour period (presumably in response to contact with residual insecticide on neighbouring cotton farms) were made on 31 January 2002.

Samples of bees were collected at the exit and entrance to the hives to quantify their NPV load and net deposition.

Samples of bees visiting flowers were captured with a hand-net and stored alive in plastic containers. These were subsequently frozen at the laboratory for later bioassay to determine whether they were carrying NPV.

Figure 7. Observing behaviour and counting bee visits to 2 metre sections of cotton row in Field 5. Note the woodland bordering the cotton field.

(f) *Cotton flower samples*

On each trial date, sampling was undertaken both before and after the deployment of NPV powder formulation into the bee hives. A rectangular grid of 40 sampling points was established that extended 160 metres along the field adjacent to the bee hives, and 190 metres into the field. During each experiment 40 pre-NPV samples of 3 open white flowers were collected from cotton plants at each sampling point in the grid, and 4 hours after release of the NPV, another 40 samples of 3 white flowers were collected from the sampling grid. Each trio of flowers were placed into individual paper bags with labels indicating their point of origin in the sampling grid. The timing of the 4 hour post-NPV flower collection was based on observations that showed that the average time taken for a hive to disseminate all the virus powder formulation was about 3 hours.

Flowers from which sentinel larvae were retrieved were also collected and stored for bioassay. Separate samples of cotton flowers which were observed to have been visited by bees were collected and stored for later bioassay to determine whether they contained NPV. On 9 occasions the bees that visited flowers were captured and the labels on the bee and flower specimens were cross-referenced.

A range of drying times was trialled in order to evaluate the most effective protocol for storing, transporting and preparing the flowers for bioassay. These treatments were: (i) fresh flowers; (ii) 24 hours oven drying at 32^{o}; (iii) 48 hours oven drying at 32^{o}; and (iv) 4 days oven drying at 25^{o}. The procedure was standardised to dry flowers at 32^{o} for 48 hours before being sent to Canberra for bioassay to determine their NPV efficacy against captive *H.armigera* larvae.

(g) *Helicoverpa larvae*

(i) Sampling of wild *Helicoverpa* larvae

To quantify the level of natural incidence of NPV infection prior to the commencement of the project, larvae were collected from all fields of chickpea, pigeonpea and cotton on Lowana before the deployment of bee-disseminated NPV. Larval collections continued after the project commenced to monitor for any changes in NPV incidence. Sampling was conducted visually on cotton and pigeonpea, and by sweepnet in chickpea. Pre-NPV sampling was undertaken once per week during the period 5 October 2001 to 16 January 2002. Post-NPV sampling was undertaken each week during the period 17 January 2002 to 21 March 2002. To determine species (*H.armigera* or *H.punctigera*) and NPV status, collected larvae were placed on artificial diet in the laboratory and reared at 25^{o}C until they either died, or survived to emerge as moths.

(ii) Sentinel larvae

Two experiments were undertaken in which sentinal larvae were placed into marked cotton flowers at a range of distances from the beehives and left for 24 hours during the deployment of NPV powder. The experiments were carried out on 4 March 2002 and again on 11 March 2002. On each occasion 100 3^{rd} instar *H.armigera* larvae were individually placed into marked newly opened white cotton flowers prior to the deployment of NPV powder into the treatment hives. 80 larvae were placed at set distances along two transects extending out into the cotton field immediately in front of the beehives, and 20 larvae were placed into flowers in a separate block of untreated cotton located 720 metres away as a control (Figure 1). Immediately after all the sentinel larvae had been placed into the flowers, NPV dust formulation was deployed into the applicator trays in the beehives. The marked flowers and surrounding plant parts were searched 24 hours later to retrieve the sentinel larvae. The

flowers in the nil-treatment control area were checked first to minimise the risk of cross contamination from the treated area. In both the treatment and control blocks all the marked flowers were collected, and labels noted whether resident larvae had also been retrieved from each flower so that in the event the larvae was positively infected with NPV the flower could also be cross-checked by bioassay.

(h) *Laboratory bioassays.*

Samples of cotton flowers, bees, honey and pollen beetles (*Carpophilus* spp) were bioassayed to determine whether they had been contaminated with the NPV dust formulation. The laboratory bioassays were undertaken in Canberra by Dr Andy Richards and Ms Janelle Scown (CSIRO Entomology, Black Mountain laboratories). Samples were prepared as follows: Cotton flowers were homogenised in 100ml of distilled water. Honey bees were homogenised in 3ml distilled water. Honey was mixed with distilled water in the ratio of 500ul honey: 4500ul water. Pollen beetles were ground under liquid nitrogen and then re-suspended in 10ml distilled water.

The methodology of Richards and Christian (1999) was used for the bioassay procedure. An agar based Soyflour artificial diet was placed into trays containing 25 separate wells. A 100ul drop of the liquid suspension from each sample to be tested was placed onto the centre of the diet in each well. A glass rod was used to smear the sample solution evenly over the surface of the diet. The glass rod was flame sterilised over an ethanol burner between each sample. During each bioassay, three control treatments with known levels of NPV were also included. The controls consisted of Distilled water (No NPV), a solution containing NPV at $5x10^3$ PIBs/ml, and a solution containing NPV at 1×10^4 PIBs/ml. The diet and sample solution were set aside until the surface had fully dried. Captive reared mid-first instar (neonate) *H.armigera* larvae (24 hours old) were placed onto the diet in each well and the tray maintained at a constant 28^oC. Infection rates were scored 10 days afterwards. Larvae with a positive NPV infection were clearly indicated by presenting a flattened non-rigid cadaver (‘flat-splat’).

4. **Results**

Incidence of NPV in wild Helicoverpa larvae

A total of 742 wild *Helicoverpa* larvae were collected from all fields of chickpea, pigeonpea and cotton at ‘Lowana’ prior to the first deployment of NPV dust into the bee hives (Table 1). The incidence of NPV infection in these larvae was 2.3% in chickpea, 8.8% in pigeonpea and 0 in cotton.

A total of 67 wild *Helicoverpa* larvae were collected from fields of pigeonpea and cotton at ‘Lowana’ at weekly intervals after 17 January 2002 following the deployment of NPV dust into the bee hives (Table 1). The incidence of NPV infection in these larvae was much higher than the incidence of NPV in the pre-treatment samples. 23.3% of larvae collected from pigeonpea and 14.3% of larvae collected from cotton became infected with NPV. It is not possible to determine the extent to which the NPV treated honeybees may be responsible for this increase.