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GENETIC ENGINEERING OF *HELIOTHIS* NUCLEAR POLYHEDROSIS VIRUS: ENABLING RESEARCH AND DEVELOPMENT FOR AUSTRALIAN CONDITIONS

Project Number CSE54C

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A Final Report to the *Cotton Research and Development Corporation*

PLAIN ENGLISH SUMMARY

The overall aim of the project was to carry out research to aid the safe and rapid adoption and implementation of genetically engineered viral insecticides into the Australian Cotton Industry. The project formed part of a larger program that aims to commercialise recombinant *Helicoverpa* NPVs (recHaNPV) with increased insecticidal potential for field control of *Helicoverpa/Heliothis* pests. The project had strong links with the CRC through the projects "Environmental Impact Assessment for Genetically Engineered Viruses" and "Identification and Predictive Classification of the Australian Heliothine Moths" and we carried out a number of field trials and collecting trips in collaboration with these projects.

The current project was focussed on two aspects of the overall program. One, to continue the development of effective recHaNPV insecticides and two, to ensure that commercial products arising from the program are well suited to Australian cotton cropping systems at to develop technologies that would allow for the rapid implementation of recHaNPV insecticides in these systems. Research in the first of the activities improved on our existing technology for developing recHaNPVs in a format suitable for delivery into cotton cropping systems. However, we were unable to isolate a gene suitable for insertion into the viral genome. Nevertheless, recent negotiations with another of our commercial collaborators indicate that a suitable recHaNPV, with improved insecticidal activity, will become available for commercialisation in Australia in the near future. Work on the second activity was centred around generating data that would allow for the first ever release of a recHaNPV into the Australian environment. This forms a critical part of the pathway towards regulatory approval for the general release of such viruses and for the eventual commercial release of a recHaNPV expressing an insecticidal-insert gene. This aim was achieved in the first quarter of 1998 when we carried out the release of a genetically "marked virus at the ACRI at Myall Vale. This trial was designed to test the field performance of containment structures and tio assess the effectiveness of various contingency measures for future releases. In addition to the above studies we have also identified a number of novel u/v protectants that can offer significant improvements to the field performance of recHaNPVs and thus improve their overall effectiveness in *Helicoverpa* control strategies in cotton.

The completion of the current project sees Australia well placed for the rapid integration of recHaNPV insecticides into the cotton industry. Effective recombinant viruses are becoming available and a regulatory pathway through to registration hads been identified and the first trials along this pathway have been successfully completed. It is anticipated that recHaNPV insecticides could become available in the early part of the next century.

BACKGROUND TO THE PROJECT

BACKGROUND

Helicoverpa armigera and *H. punctigera* are major pests of cotton crops in Australia. In the past, outbreaks of these pest species have been controlled by the conventional use of chemical insecticides. Over the last decade, however, resistance to a number of chemical insecticides has arisen in populations of *H. armigera* and there are currently no chemicals available which do not face a resistance problem. Therefore, it has been necessary to find new ways to augment and complement the existing methods of *Heliothis* control. One such form of control is based on biological insecticides.

Several biological options have been suggested for the development of insecticides. In one instance, with the entomopathogenic bacterium, *Bacillus thuringiensis* (Bt), a fair degree of commercial success has already been achieved and the development of transgenic plants carrying the Bt endotoxin has further extended the utility of this organism. However, recent demonstrations of field resistance to Bt in other species of lepidopterans has highlighted the fact that biological insecticides may engender resistance and that reliance on one particular agent is not a sustainable strategy.

CSIRO Entomology began development of novel insecticides based around *Helicoverpa* Nuclear Polyhedrosis Virus (HaNPV) in early 1988. The Program "Genetic Engineering of *Helicoverpa* NPVs" aims to modify the genome of HaNPVs to generate more rapidly acting and/or more virulent virus strains with the intention that these viruses will be available as biological insecticides against heliothine pest species to the Australian Cotton Industry. To achieve this aim CSIRO Entomology identified at an early stage that the Program would need to involve the close collaboration of a high profile Agrochemical company. Towards this end, CSIRO signed a collaborative agreement with Crop Care Australasia (then ICI Australia PLC) in 1989 and subsequently extended this agreement to include the parent company ICI Agrochemicals (later to become Zeneca Agrochemicals) in early 1992.

The research program to date can be divided into three distinct phases; 1) Research and development of recombinant viruses (1988-92), 2) Pre-commercial research and development of recombinant viruses (1992-95), and 3) Commercial Development of recombinant viruses (1995-99). The second phase of the Program was funded in part by the CRDC under grant CSE29C ("Genetic Engineering of Helicoverpa NPVs: Pre-commercial Research"). Components of the third phase of the program were funded *via* CSE54C "Genetic Engineering Of *Heliothis* Nuclear Polyhedrosis Virus: Implementation Research And Development For Australian Conditions".

The first phase of the Program aimed to develop a recombinant engineering technology for HaNPVs and achieved it's goals during the period 1988-92. The second phase of the project then aimed to demonstrate that recombinant HaNPVs (recHaNPVs) could be "armed" with genes that encode insect toxins to produce a virus with an improved rate of kill. The second phase achieved its aims during the period 1992-95. At the completion of the second phase of the project however, we were still restricted to the use of toxins i.e. a neurotoxin from the mite *Pyemotes tritici*, and one derived from the venom of the North African scorpion, *Androctonus australis*, for which the Intellectual Property (IP) resided outside of the Program Partners (CSIRO, Zeneca Agrochemicals and Crop Care Australasia). A major component of the third phase of the program was therefore to ensure that intellectual property around the toxin used to "arm" an HaNPV was as strong as possible. This was important because our commercial collaborators had indicated that their continuation after the third phase of the program was dependent on a strong proprietary position on the toxin.

From any early stage in the overall Program work involved with the engineering of HaNPVs had run in parallel with a project to isolate suitable insert genes for "arming" the virus. The insert of choice arising from

this project was a toxin from the parasitic wasp *Bracon hebetor* - chosen because of its very high specific activity against lepidopteran species. During the second phase of the project we had made good progress in identifying and isolating the toxin. However, from these studies we had discovered that the toxin contains four subunits (of between 32 and 17 kDa). This raised the possibility that all four subunits may have to be co-expressed in the recHaNPV product - making the development of a recHaNPV insecticide expressing the toxin a much greater technical challenge.

In addition to work on the development of a suitable insert gene (primarily *Bracon* toxin - BrTX) for a recHaNPVs, work in the CRDC funded part of the program also included generic studies aimed at enabling the rapid deliver of recHaNPVs insecticides to the Australian cotton industry. While we realised that it would be preferable to go forward into the final (Commercial Development) phase of the program with a recHaNPV containing an insert gene for which we held a controlling IP position, we also realised that in the interests of the Cotton industry bringing such products to the marketplace in a timely fashion was more important. During the second phase of the Program it became evident that a number of other companies had also become interested in the development of recHaNPV insecticides for use in Cotton and that two of these companies (American Cyanamid and Dupont) had managed to acquire a strong IP position on existing insert genes. With the possibility that either of these companies may be able to deliver a viable product ahead of the CSIRO-Zeneca project we considered that it would be sensible to invest in generic studies that could be used to "fast-track" recHaNPV insecticides to the Australian cotton industry. Work on such enabling technologies was developed through a close collaboration with the CRC for Sustainable Cotton Production funded project "Environmental Impact Assessment for Genetically Engineered Viruses".

By March of 1998, despite considerable effort from both CSIRO and Zeneca, all attempts to recover toxic activity from the recNPVs carrying the BrTX genes had failed. With no other toxins available to the CSIRO-Zeneca project for which a suitable proprietary position was available, the parties agreed to terminate their collaborative agreement.

Since this time, CSIRO has entered into an agreement with American-Cyanamid to collaboratively explore the potential of recHaNPVs generated by the latter for use in Australian cropping systems. This work is funded in part by American Cyanamid and in part through a collaborative agreement with the Grains Research and Development Corporation. While much of the work in these collaborations is initially directed towards the use of both wild-type (unmodified) and recHaNPVs in the Grains industry, CSIRO Entomology remains heavily committed to the delivery and integration of recHaNPV insecticides into the Australian Cotton Industry through its involvement in the CRC for Sustainable Cotton Production's project "Environmental Impact Assessment for Genetically Engineered Viruses" (see below)

CRC Links

The current project has had links with two CRC projects throughout it course. With one of these projects, "Environmental Impact Assessment for Genetically Engineered Viruses", the links were very strong. This project has the broad aims "to contribute to the implementation of viral insecticides for controlling insect pests of cotton by conducting environmental studies on genetically engineered viruses" This broad aim was complementary to the aims of the current project and in many instance field studies in the two projects were carried out co-operatively.

The current project also had links with the CRC project "Identification and Predictive Classification of the Australian Heliothine Moths". Links with this project helped greatly in formulating a conceptual framework around which to base our host range studies.

PROJECT OBJECTIVES AND ACHIEVEMENTS AGAINST THESE OBJECTIVES

AIMS

The overall aim of the project was to carry out research that will allow the rapid adoption of new genetically engineered viral insecticides into strategies for *Helicoverpa* control in Australian cotton cropping systems. Towards this aim there were two major components.

1) To develop a strong IP position and a viable product around HaSNPV and *Bracon* Toxin (BrTX).
2) To ensure that commercial products arising from the overall program would be well suited to Australian cotton cropping systems and to develop generic technologies and IP that would allow the rapid implementation of any recHaNPV insecticide into the Australian cotton cropping system.

Specific objectives were set for each year of the project as follows:

Year 1	Generation and testing of recombinants containing *Bracon* toxins Select virus isolate for recombinant construction
Year 2	Finalise toxin-virus construct Establish factors controlling virus efficacy
Year 3	Field-trial Australian formulations Begin assembly of registration package

PROGRESS AGAINST OBJECTIVES

1. Development of a viable product around HaSNPV and *Bracon* Toxin (BrTX).

Work against this objective progressed well during the first year of the project. At the commencement of the project we had identified the genes that encoded two of the toxin's four subunits and the genes for the remaining two genes were isolated fairly soon afterwards. At this stage of the project we began insertion of the genes into baculovirus vectors (singly at first) to ascertain if toxic activity could be recovered from a single subunit alone. This work was carried out in collaboration with our commercial partner Zeneca Agrochemicals - in the first instance using the *Autographa californica* (AcNPV) vector system.

In addition to testing each of the Br-TX subunits for toxic activity it was also necessary to further develop the HaSNPV system into a format more suitable for use in the field. In the context of baculoviruses this entails producing them in a polyhedrin+ (pol+) format. As all of the recHaNPVs generated up to this time have utilised the polyhedrin promoter for expression by *replacing* the polyhedrin coding sequence with the inserted gene, recombinants were pol-. We were able to generate pol+ recHaNPVs by inserting another copy of the polyhedrin gene and promoter into the transfer vector thereby ensuring that recHaNPVs carrying toxin genes could be generated in the correct format for use in the field.

The advances made in the first year strengthened our IP position surrounding both the HaNPV transfer vector system and the *Bracon* toxin. Provisional Patents for both of these areas of IP ("Recombinant helicoverpa baculoviruses expressing heterologous Australian" and "Novel Toxins") were submitted and completed respectively in this first year.

In the second year of the project little progress was made in attempts to recover toxic activity from recNPVs containing individual subunits of BrTX. Preliminary studies with a recombinant containing one of the individual subunits gave some improvement in activity when compared to wild-type controls, however, this improvement was not of a magnitude to be commercially viable. Subsequently, considerable effort was placed in the generation of recombinants containing more than one of the subunits.

Work on generation of these multi-subunit recombinant viruses carried on into the third year of the project. While a number of recombinants were generated that contained various numbers of the four subunits and one recombinant was generated containing all four of these subunits none of these recombinants were able to recover the toxic activity of BrTX.

2. Development of generic technologies for the rapid implementation of recHaNPV insecticide into the Australian cotton cropping system.

Activities in this .major objective were divided into two basic categories. One, generic studies involved with defining factors that could potentially limit the field efficacy of a recHaNPV insecticide and two, studies aimed at generating data that would assist in the development of a regulatory package for the eventual release and commercial development of recHaNPV insecticides.

Field Efficacy Studies

While field efficacy of a recHaNPV insecticide is in some part determined by the nature of the genetic modification made to the virus i.e. the speed-of-action of the virus is primarily determined by the inserted component of the recHaNPV, a number of other factors ultimately impinge upon the efficacy of the product. Many of these factors are more a feature of the virus' biology and the way in which the virus is formulated and delivered to the target pest. In many cases these factors are independent of the modification made to the virus and relate more to basic characteristics of baculoviruses *per se*.

A number of factors had previously been implicated in the apparently poor field performance of ELCAR™ - a *Helicoverpa zea* NPV developed for control of heliothine pests in the late 1970s. Most notable amongst these was inactivation of the virus by u/v irradiation. Prior to the commencement of the current project a number of significant advances had been made overseas in the development of effective sun-screens for biological insecticides. - in particular for baculoviruses.

While research in this activity was planned to commence in the second year of the project, the opportunity to commence work earlier than anticipated arose at the beginning of 1996 when CSIRO Entomology hosted Dr Marty Shapiro for a McMasters Fellowship. Dr Shapiro made the discovery that fluorescent brighteners (FlBrs) (compounds commonly used in the detergents and paper industries) could offer both u/v protection and enhance the activity of baculoviruses. In collaboration with Dr Shapiro we were able to develop a simple laboratory based assay for u/v irradiation studies (that uses natural daylight/sunlight in a solid phase system to mimic exposure on a leaf surface) and found that a number of FlBrs were able to offer good levels of protection against u/v.

In addition to their ability to protect HaNPVs from u/v inactivation, we also found that some FlBrs were able to enhance the intrinsic activity of the virus - effectively lowering the amount of virus that would need to be delivered to the target pest. While FlBrs showed some promise as u/v protectants we also carried out studies to elucidate the potential of other compounds as u/v protectants - most notably a range of food colourings and clay minerals (the choice of clays minerals came out of studies that were looking at the vertical dispersal of HaNPVs through the soil profile and the fact that virus dispersed less in high-clay soils but was more resistant to u/v inactivation in these soils). Laboratory studies showed that several of these compounds were able to offer some protection against u/v inactivation. Subsequent field studies supported these findings. Field studies carried out earlier this year (March 1998) were designed to look at the interaction between several of the compounds identified as potential u/v protectants in previous studies. Analyses of these samples are still underway - but it would appear that at least one combination of compounds offers considerable improvement over either of the compounds on their own.

Development of a Regulatory Package

Work carried out in this activity involved extensive collaboration with the Sustainable Cotton CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses". This collaborative effort

was focussed on the objective of carrying out the first release of a recHaNPV in Australia. While the original project plan had anticipated that this release may occur during the 1996/97 field season for reasons outside of the control of this project the release occurred in early 1998 (see "Methodology and Justification of the Methodology").

The above trial is the first of any recombinant baculovirus carried out in Australia and as such marks an important milestone in the regulatory pathway through to the commercial introduction of recHaNPV insecticides. The trial comprised a series of field studies (using a benignly marked HaNPV) to establish the most practicable experimental approach for future field trials employing tox+ HaNPVs. The trial design aimed to elucidate approaches to minimise any risk to the environment of recNPVs without significantly compromising environmental realism. Specifically, the studies aimed to:

- assess the effectiveness of field containment approaches and define experimental procedure to minimise virus dispersal from release plots
- test and refine virus monitoring and contingency protocols
- establish experimental approaches for investigating the effect of spatial distribution of virus on translocation and transmission

The trial commenced in March of this year and lasted for a little over two months. The trial was successful in testing the containment offered by the experimental field structures and provided some important lessons for the future release of tox+ recHaNPVs. However, adverse weather conditions meant that the trial had to be terminated prior to completion of the studies on the effects of spatial distribution of virus on translocation and transmission of the virus. An application has since been made to carry out this part of the trial in the forthcoming field season (1998/99).

METHODOLOGY AND JUSTIFICATION OF THE METHODOLOGY

RESEARCH METHODOLOGY

Introduction

The current project (CSE54C "Genetic Engineering Of *Heliothis* Nuclear Polyhedrosis Virus: Implementation Research And Development For Australian Conditions") formed part of a larger overall program "Genetic Engineering of Heliothis NPVs". The overall Program was a collaboration between CSIRO, Zeneca Agrochemicals (UK) and Crop Care Australasia and at the beginning of the current project was entering its Third Phase "Commercialisation of recombinant viruses (1995-99)". With the overall aim of bringing a commercial recHaNPV product into the marketplace soon after the turn of the century, the Program had four major goals to address during the time-frame of the current project (1995-98):

1) Selection of Commercially Viable Recombinant HaNPVs.
2) Generation of a registration package.
3) Development of appropriate formulation and delivery systems.
4) Development of suitable production system(s).

Clearly for the overall program to progress there was a need to meet the first of the above goals within the time-frame of the current project. However, while some of the above objectives are inter-dependent there were nevertheless activities that could be carried out in parallel to ensure the most rapid route to commercial release of a product. Research in the current project was aimed to address issues relating to three of the above goals namely, 1, 2 and 3; to ensure that maximum advantage would ensue to the Australian Cotton Industry even in the event that the CSIRO-Zeneca could not deliver a suitable recHaNPV product.

The proposed research was also designed to run in parallel to the Project "Environmental Impact Assessment of Genetically Engineered Viruses" that is funded by the CRC for Sustainable Cotton Production. In the case of this project the aims are to "contribute to the implementation of viral insecticides for controlling insect pests of cotton by conducting environmental studies on genetically engineered viruses".

The research plan and methodology detailed below are very much as presented in the original application. However, because of the relative progress that was made in specific activities during the course of the project and the necessity to modify some of the objectives as the research progressed (for both scientific and regulatory reasons) objectives were added and amended. A summary of the research undertaken is presented in Figure M1.

1. Selection of Commercially Viable Recombinant HaNPVs

As previously outlined, at the termination of the previously funded Project "Genetic Engineering of Helicoverpa NPVs: Pre-commercial Research" (CSE29C) good progress had been made in demonstrating that recombinant HaNPVs could be generated with improved speed-of-action by the insertion of genes encoding insect specific toxins. However, the intellectual property surrounding the toxin genes utilised , was not owned by either CSIRO or it's collaborators. Nevertheless, good progress has been made identifying and isolating the genes that encode a toxin from the parasitic wasp *Bracon hebetor*. Because of the progress that had been made on the isolation of this toxin and because it represented a significant improvement (in terms of its specific activity) over all other toxins that had been utilised to "arm" recNPVs it was decided to focus research on this toxin as an insert for recHaNPV insecticides.

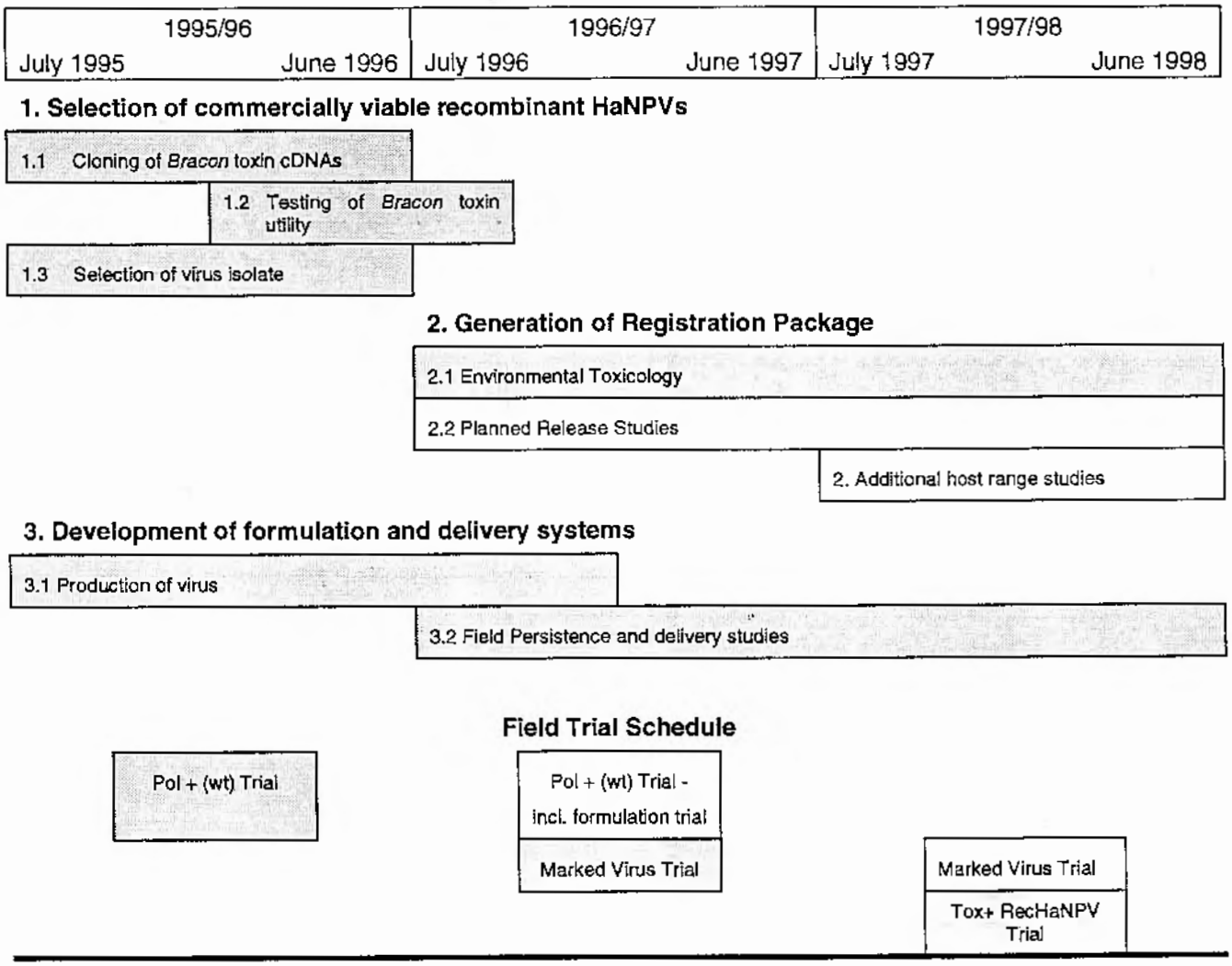
1.1 Cloning of Bracon *toxin cDNAs*

Prior to the commencement of the current project the toxin from *Bracon hebetor* (BrTX) had been found to comprise four subunits of between 32 and 17 kDa. Using a combination of "reverse engineering" technologies the genes encoding two of these subunits had been isolated and characterised. As we had no information on which or how many of the subunits would be necessary to recover toxic activity of BrTX the research plan involved:

a) Generation of additional protein sequence data from all four subunits - most importantly for the two for which we didn't have good data,
b) Generation of primers for use in the polymerase chain reaction (PCR) to identify additional cDNA clones and,
b) Characterisation of the putative cDNA clones isolated by PCR/hybridisation analysis

It was expected that this work would form the major commitment of resources available to the Program and would continue through 1995.

Figure M1. Gantt chart showing proposed and modified activities carried out in the current project and the schedule for field trials carried out in the course of the project.



1.2 Testing of Bracon *toxin utility*

Once the genes encoding the subunits of BrTX became available through Activity 1.1 the research plan involved inserting each of these genes into the genome of an NPV to ascertain if any toxic activity could be recovered. While the likelihood existed that all four genes would need to be inserted into the recNPV to recover the full spectrum of toxic activity, earlier studies had indicated that a subset of the subunits (and possibly only a single subunit) was required for some toxic activity. In the first instance it was therefore decided that it would be prudent to test each of the subunits in recNPVs but to also build into the research plan the facility for generating recombinant viruses containing all of the subunits (or some subset(s) thereof). While it would also have been desirable to carry out this research using the HaNPV vector system, it was also decided that there was greater utility at that time in using the *Autographa californica* NPV vector system for the initial demonstrations and to then transfer the knowledge gained from that system to HaNPV.

1.3 Selection of Virus Isolate

Twelve virus isolates had previously been cloned from the original HaNPV isolate (A44EB1) by an *in vitro* process (See progress Report submitted for CSE 29C). Several of these isolates had showed good levels of activity against both *H. armigera* and *punctigera* after repeated passage through cell culture. In many respects the virus "backbone" upon which a recHaNPV is based is as important as the gene that is inserted into the viral genome to generate the recombinant. For this reason, we considered that the choice of virus backbone was a very important component in the development of a recHaNPV insecticide.

The initial plans for work in this activity involved:

1) Ascertaining that these viruses can be used for the generation of recombinant viruses
2) To carry out comparative studies at the DNA level to check their identity against the reference A44EB1 isolate
3) Check the stability of the virus after passaging in both cell culture and insects

Data generated from the above studies would allow us to ascertain whether the "backbone" of any particular isolate was intrinsically better than any other, and that it was suitable for the generation of recHaNPVs.

2. Generation of a registration package

It was anticipated at the commencement of the current project that the eventual registration package required for a recHaNPV would involve a large body of data on all aspects of the toxicology and biology of the recombinant virus and/or it's individual components and products. Work in this activity in the current project was planned to focus on aspects of the registration package that we considered would be critical and specific to registration in Australia i.e. the potential effects of the virus on various components of the cotton cropping system and the potential impact that recHaNPVs may have upon the Australian environment. A number of environmental toxicology studies were planned (see Activity 2.1) which would complement collaborative studies underway in the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses".

However, due to the slow progress that was made on the generation of a commercially viable recHaNPV expressing *Bracon* toxin, it was not possible to commence studies on the environmental toxicology aspects of the work in this activity i.e. these studies were dependent upon having decided upon a toxin-expressing recombinant that would form the basis for a commercial product.

Despite the problems that were experienced in the generation of a suitable toxin-expressing recHaNPV, collaborative studies with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses" had progressed very well through the first year of the current project (1995/96). Using protocols developed for detection of HaNPVs in soil samples, an Australia-wide survey of the viruses present in both cropping and non-cropping systems was successfully undertaken. Results from these studies indicated that the natural distribution of wild-type HaNPVs is much wider than originally anticipated.

As a consequence of these findings it was realised that the range of potential sites into which a recombinant virus may disperse was much broader than originally envisaged. For instance, wild-type HaNPVs were found to be widely distributed through both cropping and non-cropping systems wherever *Helicoverpa* species occur - including uncultivated sites that contain native heliothine species e.g. *Heliocheilus* species.

In its own right, the above observations may not have had a significant impact upon the research required to support a planned release proposal for a recHaNPV. However, as a result of the "escape" of rabbit calicivirus from a "contained" field trial in 1996 the regulatory authorities changed their attitude towards "contained releases". Informal discussions with GMAC revealed that planned release proposals would be treated as **potential open releases -** irrespective of the containment conditions employed in the trial.

As a result of this change in the regulatory climate the schedule for field trials had to be modified to allow for a more cautious regulatory attitude to the release of a recHaNPV. This change in field-trial schedules had the result of effectively moving marked virus trials back a year (see Figure M1) and eventuating in tox+ recHaNPV trials being moved back two years (with an anticipated release date of early 2000). In addition to the need to move the trial schedule, as a result of studies carried out at Myall Vale during the period 1994-96 it was also recognised that to carry-out the marked virus trials we would first need to construct a suitable recombinant virus (see Activity 2.2a).

2.1 Environmental Toxicology

Work in this activity was planned to focus on the fate of toxins produced by recombinant viruses in insects, rather than the fate of the virus *per se*. However, due to the failure of the Program to generate a suitable toxin-expressing recombinant, it was not possible to commence the work in this activity.

2.2 Planned Release Studies

2.2a Generation of Marked-Virus Recombinant

A "marked virus" was designed to be constructed using standard molecular technique. The virus was intended to carry several silent site mutations. Using the introduced mutations, a number of primers were also designed to allow specific identification (using the polymerase chain reaction: PCR) of the released virus from the endogenous virus "background" at the release site(s).

2.2b Marked Virus Field Trials

The marked virus trial was planned for the first quarter of 1998. The trial design aimed to ascertain the most practicable experimental approach for future trials with fully active i.e. toxin-expressing (tox+) recombinant viruses. Specifically, the field studies aimed to:

- assess the effectiveness of field containment approaches and define experimental procedures to minimise virus dispersal from release plots
- test and refine virus monitoring and contingency protocols
- establish experimental approaches for investigating the effect of spatial distribution of virus on translocation and transmission

The overall trial was planned to comprise two separate trials involving three releases of the recHaNPV at 10 discrete sites. The two trials were planned as follows:

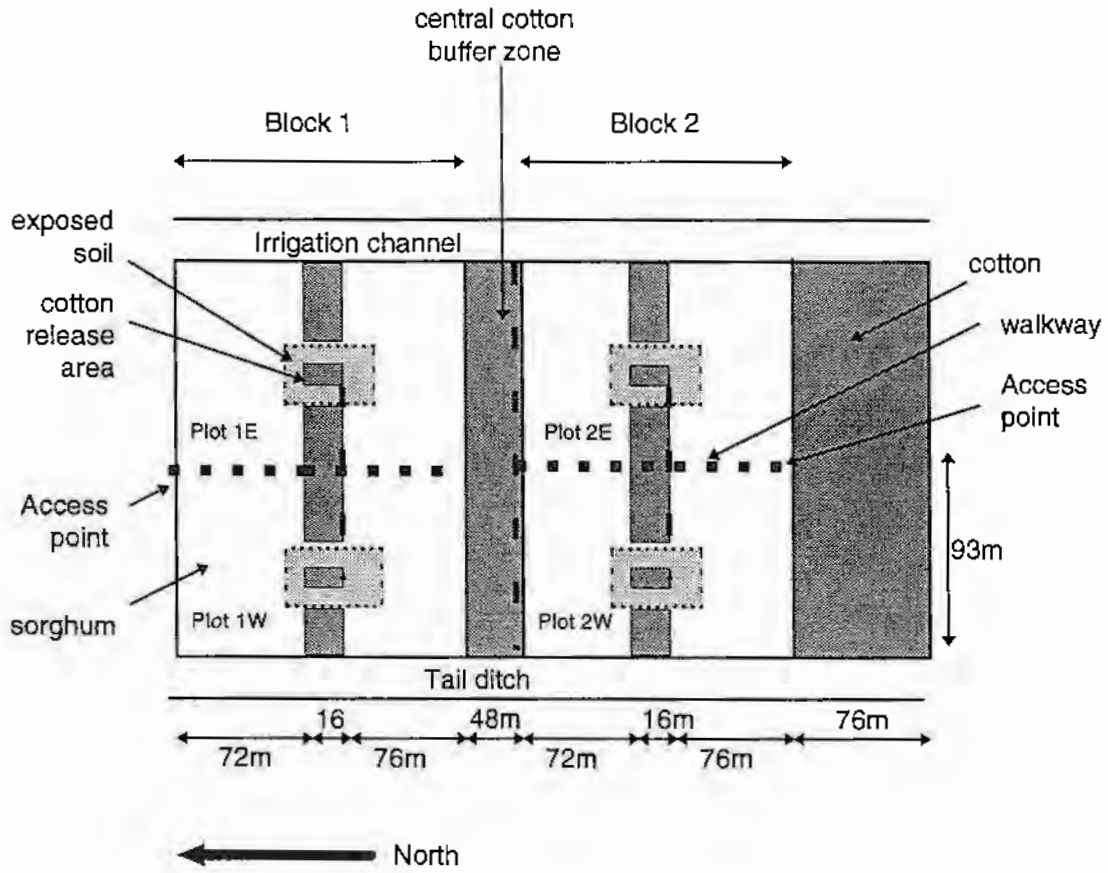
Trial 1 - Validation of the Effects of Physical Containment on HaSNPV Dispersal

Trial 2 - The Effect of Spatial Distribution of Inocula on Redistribution and Transmission of HaSNPV

A full outline of the trial protocols and procedures are provided by way of Appendix 1 and a plan of the trial layout is shown in Figure M2. The first trial (comprising two parts) was planned to proceed first, with the aim of investigating the ability of the trial structure to contain the released recHaNPV. The second part of the first trial aimed to assess the dispersal of the marked virus in the absence of the containment structure.

The second trial was to commence after the first trial had completely finished and aimed to test the effect of spatial distribution of inocula on redistribution and transmission of HaSNPV. The second trial was to be located in the central cotton buffer zone (see Figure M2).

Figure M2. Plan view of the plot layout for marked HaSNPV trials, field A2, Myall Vale



Because the probability of dispersal from the partially contained plot (the second part of Trial 1) was assessed to be greater than from the fully contained plot (the first part of Trial 1), the two treatments were separated in time. This allowed a greater likelihood of associating an identified virus dispersal event with a specific treatment. There were no suitable negative containment controls that could be included in the trial.

2.2c Additional Host range Studies

With the discovery that wtHaNPVs were much more widely distributed in the environment than we had originally envisaged came the realisation that the potential impact sites of a recHaNPV were much broader than previously anticipated. As a consequence, the range of heliothine species that may be impacted upon by a recHaNPV ie. those species that could encounter the virus in natural habitats is also broader than originally anticipated. Through collaboration with the CRC funded project ""Identification and Predictive Classification of the Australian Heliothine Moths". we were able to collect and rear several heliothine species in the laboratory in early 1997 e.g. *Heliocheilus pallida, Heliocheilus moribunda, Australothis rubrescens.* However, although we were able to rear these species for 2-3 generations, resources were insufficient to carry out extensive bioassays on these species at that time. Preliminary bioassays with wild-type viruses did however indicate that the *Heliocheilus* species are only mildly susceptible to HaNPV. Species at the "edge" of a viruses host range have been cited as species of particular interest in the analysis of host range for recombinant viruses as this is where one is most likely to pick up effective expansions in host range. For the above reasons we carried out additional field work in the final year of the program in the Darwin area with the intention of collecting, rearing and bioassaying several non-target heliothine species (including *Heliocheilus spp.* and *Adisura spp.*)

3. Development of appropriate formulation and delivery systems

While we anticipated that overall responsibility for the development of formulation(s) for a commercial product would ultimately lie with Zeneca Agrochemicals, we felt that there were a number of aspects relating to the development of the formulation that could best be undertaken by CSIRO to ensure that formulations would suit the Australian environment and heliothine pest complex.

3.1 Production of Virus

To undertake the field trials that formed part of the research plan it was necessary to generate a relatively large amount of virus. In addition to generation of the virus, in the case of the marked recombinant virus in 1998 it was also necessary to carry out a reasonably large amount of quality and assurance work (Q&A) to ensure that the virus being released was the intended virus.

3.2 Field Persistence and Delivery Studies

A number of factors have previously been implicated in the apparently poor field performance of ELCAR^{TM} - a *Helicoverpa zea* NPV developed for control of heliothine pests in the late 1970s. Most notable amongst these was inactivation by u/v irradiation. Recently there have been several advances in the development of sun-screens (u/v protectants) for biological insecticides and the research plan included work to assess whether any of these compounds showed potential for the development of formulations appropriate for Australian conditions (where it is generally recognised that u/v levels can be much higher than in other cotton growing regions of the world).

While research in this activity was planned to commence in the second year of the project, the opportunity to commence work earlier than anticipated arose at the beginning of 1996 when the Division of Entomology hosted Dr Marty Shapiro for a McMasters Fellowship. Dr Shapiro was responsible for the discovery that fluorescent brighteners (FlBrs) (compounds commonly used in the detergents and paper industries) could offer both u/v protection and enhance the activity of baculoviruses. In collaboration with Dr Shapiro we were able to develop a simple laboratory based assay for u/v irradiation studies (that uses natural daylight/sunlight in a solid phase system to mimic exposure on a leaf surface) and found that a number of FlBrs were able to offer good levels of protection against u/v and also give good levels of activity enhancement.

Much of the initial work with FlBrs was carried out in the laboratory but we were able to take several formulations into the field in early 1996. However, virus application problems with trial meant that no reliable data were generated form this trial. In early 1997, with a modified protocol, we therefore took several of our research compounds into the field to them test for their u/v protection properties.

RESULTS

1. SELECTION OF COMMERCIALLY VIABLE RECOMBINANT HaNPVS

1.1 Cloning of Bracon Toxin cDNAs

Work carried out during the second phase of the program had demonstrated that BrTX is comprised of 4 subunits. Protocols had been developed for the purification of the toxin and isolation of individual subunits. The four subunits range in size from 17kDa through 18kDa and 21kDa to 32 kDa. The subunits are generally referred to on the basis of these sizes. The procedure used for the isolation of subunit encoding genes involved a series of "reverse engineering" technologies. To summarise, these involved:

- Isolation of BrTX subunits
- Generation of protein sequence data from the isolated proteins (both N-terminus and internal/tryptic fragments
- Synthesis of primers for amplification of gene sequences from a cDNA library by polymerase chain reaction (PCR)
- Validation of the sequence from the amplified cDNA
- Synthesis of probes from the amplified cDNA fragment
- Probing of a phage cDNA library
- Isolation of the phage clone
- Sequence analysis of the phage clone

Using N-terminal sequence analysis of isolated BrTX subunits we had been able to successfully isolate the genes encoding the 17kDa, 18kDa and 32kDa subunits. However, N-terminal analysis had provided insufficient information to isolate the 21kDa encoding gene. To aid in isolation of this genes and to validate the identity of the putative 17kDa, 18kDa and 32kDa encoding cDNAs we subsequently isolated more material of each of the subunits for isolation of tryptic fragments. The basic protocol for the isolation of tryptic fragments from the subunits was as follows:

1) Separation of subunits from purified toxin by SDS-PAGE
2) In-gel trypsin digestion of the isolated bands
3) Elution of fragments from the gel and separation using the SMART system
4) Mass-spectroscopy of recovered peaks to ascertain amount and purity
5) Sequencing of suitable fragments

One millilitre of the purified toxin was used for each tryptic digestion run (approximately 3,500 venom gland equivalents). Material from the SDS-page gels for the 32 KDa and 17/18Kda subunits was saved in later runs for subsequent mass spectroscopy to further validate the identity of the putative subunit cDNAs. Sequence data obtained from tryptic digests are shown in Table R1.

Using the data generated from the tryptic digests it subsequently proved possible to isolate several putative cDNAs encoding the 21kDa subunit. Sequence analysis in conjunction with the above data and mass spectroscopy studies subsequently verified the identity of these clones.

1.2 Testing of *Bracon* toxin utility

From work carried out in Activity 1.1 clones were isolated that encoded each of the four toxin subunits. Each of these clones were then inserted into transfer vectors which were in turn used to generate recombinant AcMNPVs carrying each of the toxin subunits. Several recAcMNPVs carrying a single toxin subunit were then assayed for improved activity against *H. virescens* (all of this work was carried out by our commercial partner Zeneca Agrochemicals at their laboratories in the U.K.). None of the recombinants showed any significant improvement in their speed-of-action when compared to a wild-type virus control. However, two of

the clones encoding the 21kDa protein did show some slight improvement in activity - but not enough to make a commercially viable product.

Table R1. Summary of tryptic fragment sequence obtained from *Bracon* toxin subunits.

	Total number fragments sent for sequencing	Sequences obtained	Match to available nucleotide sequence
32 kDa	5	QIVTYYLDS(I/H)K	Yes
		INI(Q/R)VA	Yes
21 kDa	6	GIAQDVGHAAHSFTK	N/A
		(H/G)VHNPGNFR	N/A
18 kDa	11	PHTVYDKHESLQ	Yes
		WVHDNAGTLLPR	Yes
		DVHDNAGTLLPR	Yes
		MIKPGETYGDVTNK	Yes
17 kDa	6	FNPETHR	Yes
		EAYIQNHGA	

Previous studies with purified BrTX had indicated that a sub-set of the sub-units (probably the 32kDa and 21kDa subunits - which we had good evidence were covalently linked) may be sufficient to produce toxic activity. Recombinant viruses were then constructed containing all possible combinations of two and three subunits and all four subunits. This work constituted a major technical challenge and took several months to complete. Each of the recombinant viruses were then subjected to bioassay against *H. virescens*. As with the single subunit-containing viruses, no significant activity in speed-of-action was observed for any of the recombinants when compared to a wild-type control.

A number of technical problems restricted the subsequent analysis of the above recombinants - most notably the availability of suitable anti-bodies for testing that the various subunits were being expressed from the recombinant viruses. Two attempts had been made to generate anti-bodies against the toxin subunit. The first had involved the use of synthetic peptides derived form the N-terminus of each of the subunits. Three rabbits were use for immunisation of each of the subunits but no antisera with sufficient specific activity was generated against any of the subunits. The second attempt involved generation of fusion proteins in a bacterial expression system for each of the subunits. These fusion proteins were then used for immunisation of three rabbits for each subunit. Again, no suitable anti-bodies were generated that would allow the identification/discrimination of the subunits in the recNPV expression systems.

2.2 Planned Release Studies

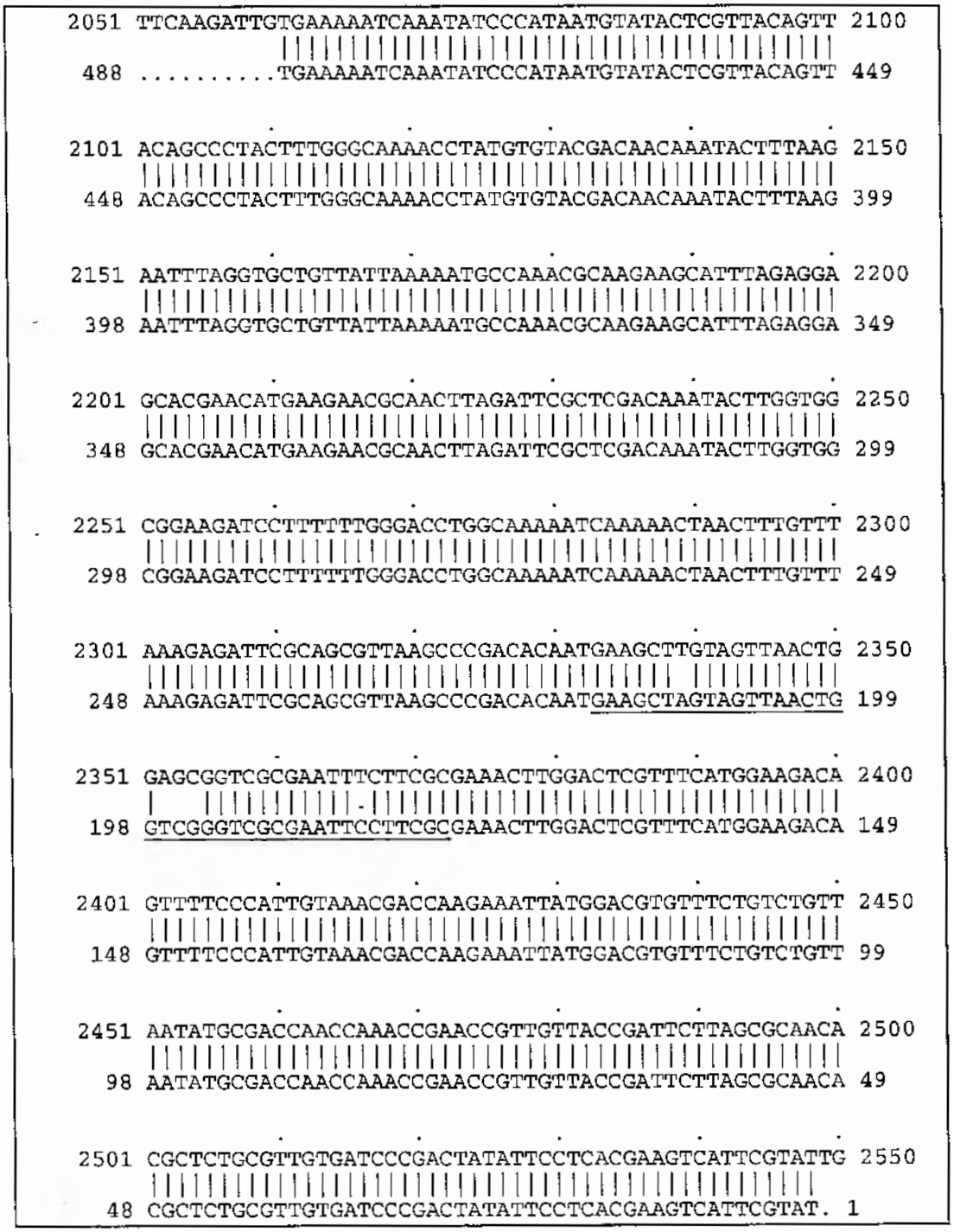
2.2a Generation of Marked-Virus Recombinant

Work carried out in collaboration with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses during the period 1994-97 had involved the use of wtHaNPVs and had aimed to elucidate some of the basic ecology of HaSNPV in cotton agro-ecosystems i.e. stability and dispersal of the virus and natural distribution both within and outside of cropping systems. Although these studies gave have given us a good basic understanding of the ecology of HaNPVs as a pre-requisite to trials involving a fully-active tox+ recombinant it was first necessary to carry out trials using a genetically defined virus that could be readily "tracked" through the course of the studies.

To carry out this trial we therefore generated a recHaNPV that contained a number of silent site mutations that would allow confirmation of identity by several methods. First, amplification of the virus from environmental samples would be achieved by bioassay using first instar *Helicoverpa armigera* larvae. DNA

could then be recovered from infected cadavers and PCR carried out across the region where the modifications have been introduced. Recombinant virus is identified by the ability of the virus to generate a product in PCR reactions and/or by the presence/absence of a number of restriction endonuclease sites.

Figure R1. Alignment of the wt viral sequence from HaSNPV-A44EB1 (top) with the sequence from the mutagenised transfer vector pA44POLMARK1. The region where mutagenesis primers anneal is shown underlined



The recHaNPV (A44POLMARK1) itself was generated using a variety of standard molecular/recombinant NPV technologies in early 1997. A summary of the mutations introduced into the viral genome is shown in Figure R1. The molecular and biological characteristics of the virus were determined in mid 1997 (for

inclusion of the data in the planned release application made to GMAC in September of 1997) and permission for the trial was granted in early 1998.

2.2b Marked Virus Field Trials

The planned release comprised two separate trials involving three releases of the GMO at 10 discrete sites. The two trials were planned as follows:

Trial 1 Validation of the Effects of Physical Containment on HaSNPV Dispersal (comprising two parts Trial 1a and Trial 1b)

Trial 2 The Effect of Spatial Distribution of Inocula on Redistribution and Transmission of HaSNPV

An outline of the overall protocol for the trials is provided in Appendix 1 and the plan of the trial-site shown in Figure M1. After a delay of over a week due to heavy rainfall, Trial 1a commenced on 17/02/98 (virus application conducted on 18/02/98) and terminated on 01/03/98. Virus release areas were decontaminated on 02/03/98 using 20% bleach solution and the containment structures dismantled following treatment. The effect of the decontamination treatment was monitored for several days after the completion of Trial 1a. Trial 1b was initiated on 09/03/98 (virus application 10/03/98) and terminated on 19/03/98. Virus release areas for Trial 1b were decontaminated on 20/03/98 and monitored as for Trial 1a.

After the completion of Trial 1b it had been intended to commence Trial 2. However, because of the delays experienced at the start of Trial 1 due to the weather and the very advanced stage of the crop resulting from particularly favourable early season conditions, it was decided to abort this trial and to make an application to carry it out in the following 1998/99 field season.

Analysis of the material collected from the field trial are still underway, however, preliminary analysis of the material generated from within the virus release plots, and samples form the decontamination studies have been analysed. A summary of the results obtained thus far from the trial are attached as Appendix 2. In brief, the results obtained to date have shown:

1) That high levels of virus inocula were generated within the contained virus release plots that coincided well with the "amplification" of the virus arising from the *H. armigera* larvae released into those plots.
2) All virus isolated from the virus release plots by bioassay analysis thus far, have been the marked virus recombinant
3) The marked virus has not dispersed from the containment structure into the surrounding bare-earth of sorghum/cotton crops.
4) u/v inactivation of the virus was reduced within the containment structure
5) Decontamination procedures were effective at removing up to 98% of the virus present in the soil at the completion of the trial(s).

2.2c Additional Host Range Studies

Results obtained prior to the commencement of the current project had shown that the two major species of heliothine pest in Australia (*Helicoverpa armigera* and *H. punctigera*) along with the North American pest species (*Helicoverpa zea* and *Heliothis virescens*) were all highly susceptible to the wt virus (A44EB1) around which the construction of recHaNPVs was based. In the current project further host range studies centred upon additional non-target Australian heliothine species which have the potential to be impacted upon by a recHaNPV.

In early 1997, through collaboration with Dr Marcus Matthews and the project ""Identification and Predictive Classification of the Australian Heliothine Moths" we were able to collect and rear several heliothine species in the laboratory. These species included *Heliocheilus pallida, Heliocheilus eodora, Heliocheilus moribunda* and *Australothis rubrescens*. However, although we were able to rear these species for 2-3 generations, resources were insufficient to carry out extensive bioassays on most of these species at that time with quantitative data being generated only for *Heliocheilus pallida* (see Table

R2). Preliminary (single dose) bioassays with the species *Heliocheilus eodora* and *Heliocheilus moribunda* were in agreement with the data for *H. pallida*, suggesting that *Heliocheilus* species were only partially susceptible to HaNPVs.

Species at the "edge" of a viruses host range have been cited as species of particular interest in the analysis of host range for recombinant viruses because this is where one is most likely to pick up effective expansions in host range. For this reasons we carried out additional field work in the final year of the program in the Darwin area with the intention of collecting, rearing and bioassaying several heliothine species (including *Heliocheilus spp.* and *Adisura spp.*).

TABLE R2. Relative Susceptibility (LC_{50}) of larvae of *Helicoverpa armigera*, *H. punctigera*, *H. zea*, *Heliothis virescens*, *H. punctifera*, *H. subflexa*, *Heliocheilus pallida*, *Trichoplusia ni*, *Spodoptera frugiperda* and *Chrysodiexis argentifera* to HaSNPV-A44EB1 and HzSNPV.

	A44EB1			HzSNPV		
	[1] LC_{50}	[2] 95% C.L.	[3] b (+/- S.E.)	LC_{50}	95% C.L.	b(+/-S.E.)
H. armigera	0.191 b	0.142-0.256	1.269 (0.083)	0.157 bc	0.077-0.273	1.299 (0.139)
H. punctigera	0.250 b	0.171-0.356	1.455 (0.105)	0.328 c	0.215-0.473	1.572 (0.141)
H. zea	0.022 a	0.011-0.042	1.136 (0.142)	0.032 a	0.016-0.057	1.389 (0.172)
H. virescens	0.072 a	0.039-0.132	1.687 (0.185)	0.062 ab	0.031-0.120	1.223 (0.185)
H. punctifera	0.206 b	0.139-0.299	1.430 (0.148)	0.299 c	0.200-0.462	1.514 (0.150)
H. subflexa	21.649 c	9.488-40.175	1.299 (0.161)	[5] ND		
H. pallida	5.994 c	2.589-11.312	0.877 (0.093)	[5] ND	-	--
T.ni	[4] $>10^2$ d	-	-	$>10^2$ d	-	-
S. frugiperda	$>10^2$ d	-	-	$>10^2$ d	-	-
C. argentifera	$>10^2$ d	-	-	$>10^2$ d	-	-

[1] LC_{50} values expressed in PIBs/mm^2 based upon 4 rates/isolate, 25 larvae/rate and a minimum of three replicates for each rate. LC_{50} values with overlapping confidence intervals are indicated by the same letter.

[2] 95% confidence intervals.

[3] Slope (+/- standard error) of probit regression.

[4] No infection recorded at diet contamination rates of 100 PIBs/mm^2

[5] ND = Not Determined

Females of several heliothine species including *Heliocheilus cramboides*, *H. ferruginosa*, *H. flavitincta*, and *Adisura* spp. were successfully collected and ggs were produced by all of the species collected. However, we were unable to establish any of these in culture using the techniques that had previously proven successful for (particularly) *H. pallida* and insuffcient insects were generated for bioassays.

3) Development of appropriate formulation and delivery systems

Studies carried out in early 1995 in collaboration with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses" established a baseline for u/v inactivation of HaNPVs in cotton cropping systems (see Table R3). Under the most severe conditions tested (exposed positions in the leaf canopy under high u/v intensity) half-lives for virus activity were found to be as little as 1.6 hours.

Table R3. Virus activity half-life estimates and associated statistics from virus inactivation studies conducted at Myall Vale, mid January-mid March, 1995

Crop Growth Stage	Canopy Shading	Position in Canopy	UV Intensity Through Trial	Activity half-life (hours)
Final emergence (January)	None	Exposed leaf	High	3.7
	None	Soil surface	High	459
Flowering (February)	Intermediate	Exposed leaf	Very high	1.6
	Intermediate	Shaded leaf	Very high	12.9
	Intermediate	Soil surface	very high	Months/ years
Boll set (March)	Maximum	Exposed leaf	Moderate	5.65
	Maximum	Shaded leaf	Moderate	18.1
	Maximum	Soil surface	Moderate	Months/ years

With u/v inactivation having the potential to be so damaging to HaNPV activity in the field it was felt that studies in this activity should focus on developing virus formulations that have the ability to offer u/v protection to HaNPVs under field conditions. Preliminary studies were carried out in Canberra using a solid-phase sunlight-exposure system to estimate the u/v protecting potential of a number of compounds.

The compounds initially chosen are a group of chemicals commonly termed fluorescent brighteners (FlBr). These stilbene-derived compounds have been used extensively in the paper and detergents industries and were recently found to offer a relatively high degree of u/v protection to a number of entomopathogens - including baculoviruses. In addition to their u/v protection capabilities, several FlBrs have also been shown to have the ability to enhance the intrinsic biological activity of several baculoviruses. Through a collaboration with Dr Marty Shapiro (USDA, Beltsville, Md) we were able to obtain access to several FlBrs and test their u/v protection capabilities with HaNPVs.

The results from these initial trials are summarised in Figure R2. In addition to the u/v protection studies, the ability of the available FlBrs to enhance intrinsic HaNPV activity was also tested. The results of these tests are shown in Table R4. With the promising results obtained from the laboratory based trials we decided to proceed to the field with our FlBr-containing formulations in early 1997. Several formulations were applied using a hand-held boom-spray to small plots of cotton at the ACRI at Myall Vale. However, subsequent analysis of the samples recovered from the trials showed a much lower level of activity than had been anticipated from studies carried out in previous years. Subsequent analysis revealed that there were no problems with the intrinsic activity of the formulations (even after several weeks storage at 4^{o}C), which indicated that the problem was with the application of the virus to the cotton crop.

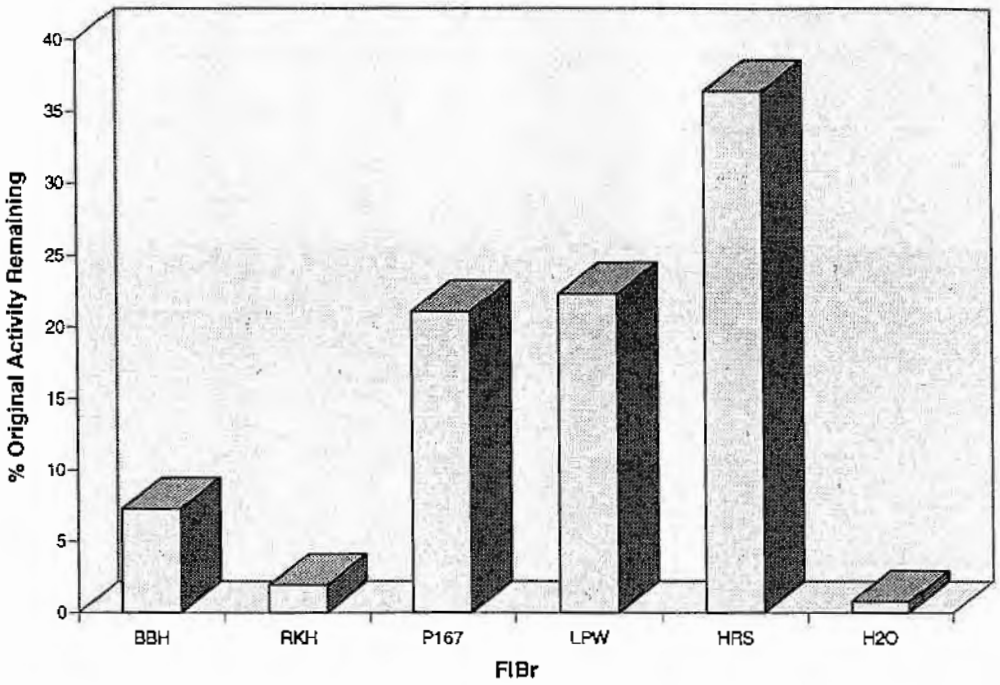


Figure R2. Percentage of original HaNPV activity remaining after 6 hours exposure to sunlight (Canberra, February 1996) on a solid matrix. FlBrs were used at a concentration of 0.1%.

While the methodology used in the above trial had been similar to that used in previous trials, we had lowered the nozzle pressure to reduce the total volume of formulation required for each treatment. We concluded that this change in application protocol resulted in the virus being applied primarily to the upper surface of the leaf surface whereas in previous trials where we had used higher nozzle pressures the virus was forced down into the canopy creating turbulence and leading to deposition of virus on the undersides of the leaf as well as the upper side. Clearly, virus deposited on the underside of the leaf will be more resistant to u/v inactivation than virus on the upper surface.

Table R4. Relative activity of HaNPV-A44EB1 in the presence of several FlBrs. FlBrs were used at a concentration of 0.1%

FlBr	LC_{50}	Lower-Upper Confidence Limits	Slope	Error of slope
None	0.104	0.057 - 0.172	1.328	0.127
RKH	0.027	0.010 - 0.055	1.363	0.144
HRS	0.020	0.009 - 0.034	1.175	0.140
P167	0.048	0.029 - 0.080	1.129	0.126
BBH	0.018	0.011 - 0.027	1.206	0.140
LPW	0.017	0.008 - 0.028	1.414	0.152

As a result of this trial we decided to modify trial protocols for future u/v protection trials so that the formulation would be applied directly to the upper surface of exposed leaves with a shortened sampling period to reflect the much higher u/v exposure in this canopy position (see Table R3).

While FlBrs appeared to have the potential to offer some level of u/v protection we were concerned that the concentrations needed were still relatively high (around the 0.1% level). To circumvent this potential problem we begun looking at other compounds (that may have the potential to be used in lower concentrations) and at the possibility of bringing the u/v protectant and the active ingredient (HaNPV) into juxtaposition on the crop surface - rather than relying on the protectant working in an aqueous film around the virus. Throughout the remainder of 1996 we tested a number of other compounds for the u/v protection capabilities - in particular a range of food dyes and clay minerals.

The lead for looking at clay minerals came from some studies that we carried out in collaboration with the CRC Environmental Impact Assessment project where we had looked at relative inactivation rates of virus on the surface of different soil types. A summary of the results from these studies are shown in Table R5.

Table R5. Virus activity half-life estimates for 5 soils selected for variable colour & sand content

Soil description	Activity half-life under test conditions (hours)
non-cultivated fine red clay	24
non-cultivated red soil	13
cultivated grey clay (Myall Vale soil)	10
cultivated pale soil with sand	6
non-cultivated fine red sand	5

These results seemed to suggest that the red soils (containing a large amount of ferric oxide) may have some intrinsic u/v protection capability. We therefore decided to test a number of common soil-forming minerals for their ability to afford u/v protection to HaNPVs. Included in these trials were a number of FlBrs and some artificial food colourings (selected because it is well known that a number of dyes e.g. congo red, brilliant yellow, afford some degree of u/v protection to NPVs). The results from these studies are shown in Figure R3.

Table R6. Relative activity of HaNPV-A44EB1 in the presence of several soil forming minerals. Kaolin (1) and (2) were obtained from different sources.

Mineral	LC_{50}	Lower-Upper Confidence Limits	Slope	Error of slope
None	0.462	0.287-0.711	1.306	0.103
Aluminium hydroxide (Gibbsite)	0.803	0.353-1.920	1.276	0.125
Kaolin (1)	0.602	0.428-0.823	1.656	0.204
Kaolin (2)	0.699	0.473-1.078	1.644	0.245
Atttapulgite	1.191	0.423-5.509	1.394	0.172
Illite	3.641	1.778-18.777	1.138	0.291
Ferric oxide (Haematite)	2.207	1.051-7.067	1.099	0.158
Bentonite	0.183	0.089-0.301	1.636	0.349
Talc	0.839	0.436-1.773	1.554	0.155

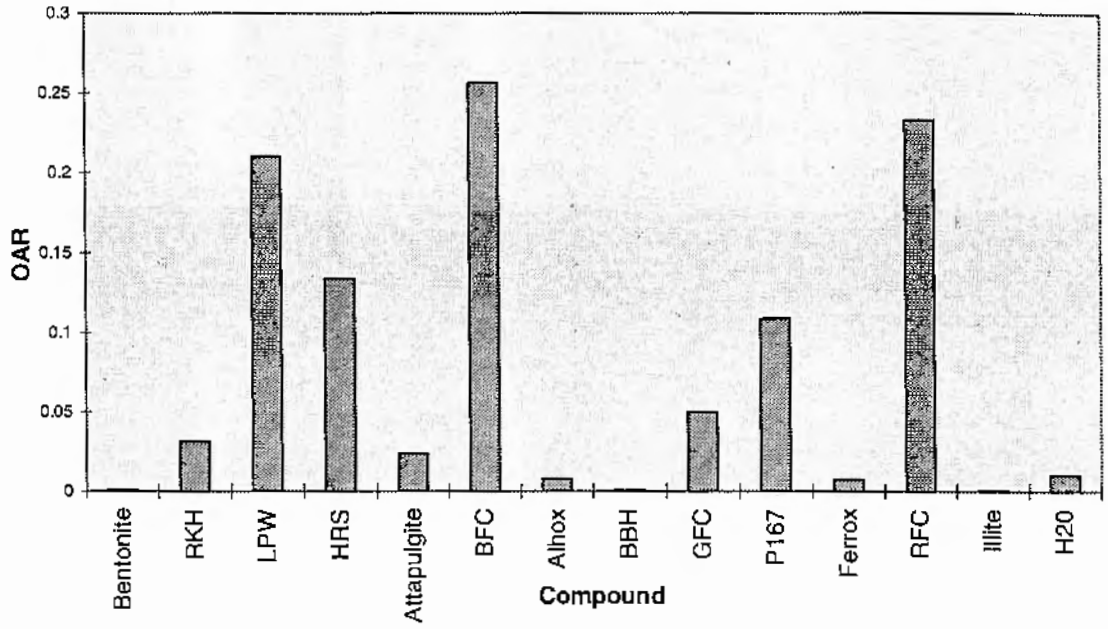


Figure R3. Percentage of original activity remaining (OAR) after 6 houris exposure to sunlight on a solid matrix. Control (H2O) contained no other compound. Compounds are as follows: RKH, LPW, HRS, P167 and BBH are FIBrs; BFC, GFC and RFC are blue, green and red food colourings respectively. The remaining compounds are soil-forming minerals; Alhox is aluminium hydroxide (gibbsite), Ferrox is ferric oxide (haematite).

Surprisingly we found the most startling u/v protection capability was offered by the food colourings (Aeroplane Pty Ltd) - which performed much better than the FIBrs. The clay minerals did not appear to offer any intrinsic protection capability in their own right. However, it is well known that clay minerals are good absorbers of a number of organic compounds - including proteins and viruses. We therefore hypothesised that it may be possible to absorb both virus and u/v protectant onto clay particles thereby bringing virus and protectant into juxtaposition in the formulation and on the leaf surface.

Initial studies were carried out to assess the relative activity of HaNPV in the presence on a number of soil forming minerals (to ensure that the mineral itself didn't reduce virus activity). The results form these studies are shown in Table R6. With the exception of two minerals (illite and ferric oxide) the mineral appeared not to interfere with viral activity - and in one case there was some indication that the mineral may have actually had an enhancing effect on the virus (bentonite).

In parallel with these studies we also carried out experiments to determine if, and how well HaNPV could bind to these soil-forming minerals. Results of these studies are summarised in Table R7. An analysis of variance revealed significant heterogeneity amongst the means of unbound material ($F = 3.394$; $df = 6$; $P = 0.0144$) but the overriding pattern was that all of the minerals tested bound virtually all of the available HaNPV.

Table R7. Percentage of unbound HaNPV after incubation with a range of soil-forming minerals. $1x10^7$ polyhedral inclusion bodies were incubated for 1 hour at room temperature in the presence of 10mg/ml of the mineral in a total volume of 10ml. Clay-PIB complexes were removed from solution by flocculation with the polyacrylamide based flocculant LT25 and the unbound concentration of PIBs estimated by bioassay against *H. armigera* larvae.

	Illite	Alhox	Ferrox	Kaolin (2)	Attapulgite	Kaolin(1)	Talc
Mean	1.169	0.678	0.055	0.317	0.510	0.264	1.815
Standard Error	0.214	0.119	0.013	0.142	0.115	0.040	0.837

In the first quarter of 1998 a number of trial formulations containing different combinations of clays and compounds with good u/v protection capabilities e.g. FlBrs and food colourings were tested under field conditions for their u/v protection capabilities. The materials from these trials are still being analysed although there are indications that some of the combinations may be performing better than predicted from the u/v protection capabilities of their individual components.

DISCUSSION OF THE RESULTS

Analysis of research outcomes compared with objectives

Two major objectives were set for the project at its commencement:

1) To develop a strong IP position and a viable product around HaSNPV and *Bracon* Toxin (BrTX).
2) To ensure that commercial products arising from the overall program would be well suited to Australian cotton cropping systems and to develop generic technologies and IP that would allow the rapid implementation of any recHaNPV insecticide into the Australian cotton cropping system.

Progress against most of the components of these objectives was good throughout the course of the project with the only major difficulty encountered being the inability to recover toxic activity from cDNA clones encoding BrTX and hence, the inability to generate a viable product around HaSNPV and BrTX. However, despite this problem significant advances were made in the development of systems for the generation of HaNPV recombinants and in developing generic technologies and IP that will aid in the progress of recombinant HaNPV insecticides through the regulatory framework and into the hands of the cotton industry. A fuller discussion of the research outcomes against the other major activities in the project (as shown in Figure M1) is presented below.

Generation of a registration package

The passage of any insecticidal agent from the laboratory, into field trials and ultimately to delivery as a commercial product involves a wide range of studies and a broad range of interactions with the relevant regulatory authorities. For a recombinant product the process is probably even more involved as they represent a new class of agents with little accumulated experience of how such products should be regulated and what studies are necessary to carry out the appropriate risk assessment. In addition, recent experience with chemical insecticides - that has seen the rapid development of field resistance amongst target insects - has led regulatory authorities to become more cautious about the way in which it will ultimately allow a product to be used. A point in case is the restriction placed upon the newly introduced transgenic Bt toxin-expressing cottons. These restrictions have resulted from a broader concern about the development of field resistance to Bt toxin and the consequent loss of this pest control option in cropping systems outside of cotton.

The regulatory pathway for recombinant baculovirus (BV) insecticides presents all of the above challenges for the relevant authorities. Unlike chemical insecticides however, BVs are replicating agents that can remain viable in the environment for many years (Thompson *et al.* 1981). Therefore, once a recombinant BV (recBV) is established in the environment there is a finite probability that it cannot be removed if this is desired. As a consequence regulatory authorities are likely to be much more cautious in their approach to such agents and will undoubtedly demand greater analysis of the potential impacts that these agents may have upon the environment prior to their release (Richards *et al.*, 1988).

With the above in mind, the broad aim of this activity during the course of the current project was to obtain permission for and carry out the release of a recHaNPV into the Australian environment. In collaboration with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses" we achieved this objective in the first quarter of this year, when Australia's first ever release of a genetically engineered insect virus was carried out at the ACRI in Myall Vale (Richards and Christian, 1998).

While the virus released was only modified to include a number of silent site mutations i.e. did not contain any additional genetic material, it was an important stage in the overall process of obtaining regulatory approval for a fully active tox+ recHaNPV. The trial also validated a number of basic protocols and mitigation strategies that will form an integral part of future releases with a tox+ recHaNPV and submission has been made for a trial in the forthcoming (1998/99) season that aims use another marked HaNPV as a 'tracer' to

determine how differences in the spatial location of infected insect cadavers in the crop affect secondary disease cycling in susceptible host populations. This approach is intended to model the behaviour of tox+ recHaNPVs which are known to differ from wt viruses in both the amount of secondary inocula they generate and the way that this inocula is distributed in the cropping systems. Successful completion of this trial should lead to an application for the release of a tox+ recHaNPV for the 1999/2000 field season.

While the overall objective of this activity was to obtain permission for and carry out the release of a recHaNPV into the Australian environment it should be noted that to achieve this aim in collaboration with the CRC project 'Environmental Impact Assessment for Genetically Engineered Viruses", we have had to generate a considerable body of data on the basic ecology of HaSNPV in the Australian ecosystem. In particular:

- Baseline data on the u/v stability of HaNPVs in the Australian cotton agro-ecosystem and how this component of the virus' biology impacts upon its long term persistence in the environment and within different environmental niches
- Data on the distribution and of HaNPVs both within and outside cropping systems where heliothine species are known to occur. These data have shown that HaNPVs are very widely distributed throughout Australia and occur at a very much higher population density than previously thought. In addition, we have gathered considerable data within a range of cropping systems that indicates that crop type can have a profound effect on the amount of virus generated by the infesting heliothine population.
- Data on the abiotic dispersal mechanisms acting within the cotton agroecosystem - in particular the effects of irrigation on the dispersal of HaNPVs.

All of these data formed an integral part of the application for the first release of a recHaNPV and will continue to form the basis of our understanding of the ecology of HaNPVs in the Australian environment. Such data will also be crucial for future Environmental Impact Evaluations for recHaNPV insecticides. In addition, as well as the immediate practical application of the data to the release application, it has also played a pivotal role in developing a theoretical framework for designing strategies to evaluate the environmental impact of recombinant baculovirus insecticides (Richards *et al.*, 1988)

Development of appropriate formulation and delivery systems

The major objective of this activity was to ensure that any products and formulation(s) developed during the course of the project would suit Australian conditions and the Australian cotton industry. Foremost amongst the factors that needed to be taken into account when developing a product with the above specifications are:

1) the Australian heliothine pest complex is unique - with any product needing to have good activity against both *H. armigera* and *H. punctigera* (our major pest species)
2) that u/v levels across many of the cotton cropping regions of Australia are much higher than in other regions of the world. As u/v inactivation has been cited as one of the most important factors in the performance of baculovirus insecticides it was important to ensure that formulations would have maximum u/v protection potential to make any product as effective as possible for use in Australian cotton cropping systems.

To a great extent, the first of the above issues had been addressed from the beginning of the program by choosing to work with an HaNPV isolate that had been chosen for its good intrinsic activity against both *H. armigera* and *H. punctigera* (see also Final Report for the project CSE 29C). For this reason the major focus in the current project was placed upon developing formulations and formulation components that could combat Australia's high u/v levels.

Overall, the research program in this activity achieved its objectives in identifying a number of compounds that are able to afford some degree of u/v protection to HaNPVs under Australian field conditions. Some of the compounds tested i.e. fluorescent brighteners (FlBrs) had previously been shown to act as both u/v

protectants (Shapiro, 1992) and as enhancers of NPV activity (Shapiro and Vaughn, 1995). Some of these compounds however, have not been investigated before and offer some significant potential as u/v protection agents. Perhaps more significant though are the interactions that we have identified between a number of soil forming minerals and HaNPVs and the potential that these minerals have in the development of novel formulations. With the best of the compounds identified to date, the potential exists for significantly extending the half-life of HaNPV insecticides and thereby increasing their overall effectiveness in the field.

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IMPACT OF THE RESULTS

The major impact of the research outcomes from the current project comes from the trials carried out in the early part of this year which comprise the first ever release of a recHaNPV into the Australian environment. Through the course of the current project the regulatory framework in which a recHaNPV insecticide will be assessed has changed quite dramatically. This change has come about as a result of both specific events i.e. the unplanned release of rabbit calicivirus onto the Australian mainland from a "contained release" on an offshore island, and from a more general climate in which the release of genetically engineered products are being treated with more caution. As a result of this change in the regulatory climate the overall approach and pathway to the release of a tox+ recombinant baculovirus insecticide has by necessity been more gradual than originally anticipated. As a result the first release of a recHaNPV into the Australian environment and the anticipated release of a tox+ recHaNPV in the 1999/2000 field season has been slower than originally thought.

While CSIRO and Zeneca decided to terminate their collaborative agreement on the development of recHaNPV insecticides at the end of the current project it still looks likely that similar products will reach the Australian marketplace over the next few years. Two American companies have been running parallel programs to our own over the last several years (American Cyanamid and Dupont) and both have recently completed field trials with recombinant heliothine NPVs in the last northern summer.

CSIRO has recently entered into a heads-of-agreement with American-Cyanamid to collaboratively explore the potential of recHaNPVs generated by the latter for use in Australian cropping systems. This work is currently funded in part by American Cyanamid and in part through a collaborative agreement with the Grains Research and Development Corporation. While much of the work will be focussed upon the use of HaNPVs (both wt and recombinant) in grains cropping systems it is anticipated that the focus of the research will be broadened in time to include cotton cropping systems.

While the major impact of the research outcomes has been in the regulatory area relating to the release of recHaNPV insecticides (and more broadly, of genetically engineered insect viruses in general), there have been some important discoveries throughout the course of the project that may well impact upon the cotton industry more broadly. The most important of these relate to our findings around the widespread distribution of HaNPVs in the Australian agro-ecosystem and the management of transgenic insect-resistant crop eg. Ingard cotton. From our studies we have identified a number of crops e.g. sorghum and lucerne, where HaNPVs can dramatically regulate the numbers of insects in the crop and consequently the numbers of pupae produced. If these crops are to be used as refugia in the Bt resistance management program then the effects of NPV upon *H. armigera* populations needs to be taken into account.

Transgenic crops form an increasingly important component in the management of insects pests, particularly *Helicoverpa* species, in the Australian cotton industry. An essential component of the management program for these crops is the provision of refugia. Refugia are designed to generate pupae and adults of (primarily) *H. armigera* that are susceptible to the insecticidal traits introduced into the plants. Through the ecological research that we have carried out in collaboration with the CRC funded "Environmental Impact Assessment for Genetically Engineered Viruses" Project we have found that HaNPVs present in the environment, primarily in the soil, have the potential to initiate viral epizootics in a number of crops e.g. lucerne and sorghum. While these epizootics can have value in such crops, when they are used as refugia in transgenic crop management the reduction in the *Helicoverpa* population that results from the effects of HaNPVs could reduce their effectiveness as refugia. Understanding the populations dynamics of the virus-host system and the factors that control those dynamics will allow strategies to be developed that reduce the effect of virus on pupal productivity from these refugia. Methods to increase the

number of pupae produced per unit area of refugia crop could allow refugia areas to be reduced and overall profitability to be increased.

DESCRIPTION OF THE PROJECT TECHNOLOGY

Throughout the course of the research program we have attempted to ensure we have as strong an IP position as possible around the technology developed. Only in this way did we feel that it would be possible to have a significant impact upon the eventual application of the technology and some assurance that its implementation would provide maximum benefit to the Australian agribusiness in general and the Australian Cotton Industry specifically.

To achieve the above objectives we have submitted patent applications on two aspects of the overall program. The first patent application was based around the identification and isolation of the cDNAs encoding the subunits of BrTX (Novel Toxins) and was continued in-part in the application Biological insect control agent. (1997).

This patent application was originally submitted in the names of both CSIRO and Zeneca. However, since the termination of the collaborative agreement with Zeneca they have decided to relinquish their interest in this application. CSIRO has taken the option on prosecution of the application for US and Australia for its sole use. While the program was unable to demonstrate the recovery of toxic activity from the cDNAs encoding the subunits of BrTX there may ultimately be some value in the developed technology for although BrTX is obviously a complex toxin it is still the most active insect neurotoxin isolated to date (with a specific activity against lepidopterans 2-3 orders of magnitude greater than any other published toxin.). It is for this reason and because we feel we have a dominant position with the patent that we have decided to continue with its prosecution.

The second area of project IP for which we have submitted a patent application is around the technology for the generation of recHaNPVs. While there is other technology in the area (with the overall area being dominated by the original patent granted for the generic process for generating recombinant baculoviruses held bby Texas A&M University) we still feel that we have a strong position with respect to the introduction of the technology into Australia for use against heliothine pests. This patent application is currently in the national examination phase and is being prosecuted in both Australia and the USA.

In addition to the above, some of the work that we have carried out on u/v protectants may have generated patentable IP. We are currently assessing the prior art in the area and may decide to proceed with a patent application in the near future.

TECHNICAL SUMMARY OF RESEARCH PROJECT DISCOVERIES

In addition to the patentable IP generated in the current project, in collaboration with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses" we have made a number of methodological advances relevant to future research activities involving the development and application of recHaNPV insecticides. A brief summary of these methodological advances in presented below.

A Quantitative Bioassay for HaNPV in Environmental Samples

An outline of this methodology is presented as part of Appendix 2. While a very simple technique, that relies on incorporation of soil samples (after drying and crushing) directly into synthetic diet, it has proven remarkably robust in its application to a wide range of soil types and has been modified to allow for the assay of other environmental material such as crop residues. A paper describing this methodology has recently been accepted for publication in the Journal of Virological Methods (see "Publications Arising from the Research").

The technique is very sensitive and allows estimation of virus concentrations to below 50 polyhedral inclusion bodies per gram of soil. However, in addition to providing sensitivity it also allows for an amplification step in the overall process for isolating and characterising HaNPVs from environmental samples, which has added advantages in the identification of the viruses present in the samples (see below).

Protocols for the Identification of HaNPVs In Environmental Samples

Direct isolation, quantification and identification of organisms from soil samples has consistently proven to be a technically challenging process. This is particularly true where the identification step involves molecular techniques and requires the isolation of DNA from the isolated samples, as humic acids and other organic compounds present in the soil interfere with many of these biochemical procedures.

However, in conjunction with the soil bioassay procedure outlined above we have found that identification of materials from environmental samples is a relatively straightforward process. DNA is extracted from cadavers generated in bioassays and subjected to the polymerase chain reaction (PCR). Amplified DNA generated in the PCR reaction can then be characterised by a variety of standard molecular biological techniques including restriction enzyme digestion and nucleotide sequencing.

In trials such as the marked recombinant trial carried out earlier this year, the presence of well defined "markers" in the genome of the released virus allows for rapid identification of the virus isolated in the bioassay step of the overall analysis (see above). The above technique in conjunction with the current protocol therefore, allows not only quantification of virus loading in the environmental samples but also identification of the viruses present in the sample.

Accurate Methodology for Field Testing of Trial Formulations

Field trials have comprised a major component of the current project and by and large have run relatively smoothly. However, in some trials - and in particular a formulation trial that we carried out in early 1996 - we have experienced problems with the application of virus to the crop. In the trial in question, nozzle pressure was reduced in applying the virus samples (to reduce the amount of virus needed for the trial) which we concluded resulted in reduced turbulence in the canopy and consequently reduced amounts of virus deposited on the underside of the leaf surface. Because the sampling strategy was based on the results from earlier trials little data was gathered from these trials as the majority of virus was inactivated by u/v radiation much quicker than anticipated.

To avoid such problems in future trials and to reduce the amount of virus needed for each formulation to be trialed, we designed a trial protocol that relies on the direct application of virus to the leaf surface. In this protocol, the virus is applied directly to pre-marked leaves on their upper surface using a micropipette. Because a well defined amount of virus is applied to each the protocol reduces significantly the variability between replicates (with the ability to trate each leaf as a replicate) and because the selection of application site can be carefully made the effects of factors such as canopy position can be much more readily controlled.

Post-trial processing of samples is significantly reduced as each leaf sample is homogenised in a given volume and then used directly in surface contamination bioassays. In trials in which the virus is applied with a boomer sprayer a large number of individual leaves need to be collected and subsamples taken from those leaves by collecting leaf-discs. Thus the number of formulations that can be processed in a given time is significantly greater for the direct application methodology described above.

For testing of multiple trial formulations we have found that the direct application methodology is much more time-efficient than more traditional spray-based procedures and less prone to variation created by the application procedure.

RECOMMENDATIONS FOR DEVELOPMENT AND DISSEMINATION OF PROJECT TECHNOLOGY

FURTHER DEVELOPMENT OF PROJECT TECHNOLOGY

Although CSIRO Entomology has terminated its collaborative agreement with Zeneca Agrochemicals for the development of novel recHaNPV insecticides it remains committed to the to the delivery and integration of recHaNPV insecticides into Australian agribusines and to the Australian Cotton Industry. IN the immediate future its commitment to the Australian Cotton Industry in this area continues through its involvement in the CRC project "Environmental Impact Assessment for Genetically Engineered Viruses". Work in this project will continue to generate data relating to the safe and effective application of the technology to the Cotton Industry in particular and to Australian agribusines in general. This work will continue to be carried out in the public arena as we feel strongly that work which relies upon a full and open regulatory process can only be carried out in this way.

To ensure the most efficient and timely route through to delivery of recHaNPV products to the Australian Cotton Industry and other agribusiness sectors CSIRO has recently entered into a heads-of-agreement with American-Cyanamid to collaboratively explore the potential of recHaNPVs generated by the latter for use in Australian. American Cyanamid are at the forefront in the commercial development of recombinant baculovirus insecticides and have already carried out several successful trials in the USA with recHaNPV insecticides. The combination of CSIRO's expertise in the Australian regulatory process and their knowledge of working with HaNPVs in cotton cropping systems and American Cyanamid's recHaNPVs provides the best available opportunity for a rapid delivery of the technology to Australian cotton growers.

DISSEMINATION OF RESULTS

Due to the commercially sensitive nature of much of the research that has been carried out in the current project it has not been possible to publish or disseminate many of the results of the research within normal time-spans. However, wherever possible summaries of the research results have been published in industry journals, and will eventually appear in scientific journals. Direct dissemination of results to the cotton industry remains a high priority for the overall program and over the last three years we have contributed:

- articles to the Proceedings of the Australian Cotton Growers Conference
- interviews to the video magazine "The Cotton Report" and,
- an article to the Australian Cotton Grower on the first recHaNPV trial

We will continue to disseminate and publish the results of our research in the above ways whenever the opportunity arises.

PUBLICATIONS ARISING FROM THE RESEARCH

PAPERS

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Appendix 1

OUTLINE OF TRIAL PROTOCOL FOR PLANNED RELEASE TRIAL CARRIED OUT AT NARRABRI, FEBRUARY 1988

The planned release comprises two separate trials involving three discrete releases of the GMO at 10 discrete sites. The two trials are as follows:
Trial 1 - Validation of the Effects of Physical Containment on HaSNPV Dispersal
Trial 2 - The Effect of Spatial Distribution of Inocula on Redistribution and Transmission of HaSNPV

Trial 1 will involve two releases of the GMO over a four week period. The first part of the trial (Trial 1 Part 1) will involve a single release that will be monitored for 12 days. After completion the virus release area will be decontaminated and the success of the decontamination procedure monitored. Two-three days following the first part of the first trial a second release will be made (Trial 1 Part 2). This release will be monitored for 12 days prior to virus release area decontamination and assessment of the decontamination procedures.
Trial 2 will involve release of virus into six trial plots. Trial 2 will take place after the completion of Trial 1 and will be for a duration of 3 weeks.

Trial 1 - Validation of the Effects of Physical Containment on HaSNPV Dispersal

Aim

To assess the effectiveness of physical containment in minimising the dispersal of recombinant HaSNPVs from trial release plots.

Rationale

Previous studies using wild-type HaNPVs have investigated abiotic routes of virus dispersal but an assessment of the importance of biotic mechanisms and the risk of dispersal during application procedures has not been possible. This difficulty arises from one, confirming that an observed infection is due to the virus released, and two that it is the result of a biotic dispersal event and not due to abiotic mechanisms eg spray drift or accidental dissemination by field workers.

Experimental Design

The experiment will comprise two treatments:
(a) *full containment* ie release plots physically enclosed for the duration of the experiment, and
(b) *partial containment* ie release plots physically enclosed for the virus application only with the containment structure then removed for the remaining experimental period
The containment structure will be 20m x 4m x 2m and will be manufactured in sections to allow easy transport, assembly and removal. The structure will comprise a frame covered with a light material with a mesh fine enough to allow the dissipation of heat while preventing the escape of spray droplets. Within the containment structure will be an area of cotton measuring 30m^2 which will be used as the virus release area. The containment structure will be surrounded by an area of bare earth bordered by larger areas of flowering sorghum and cotton.
The trial will be divided into two parts. The fully contained plot will be run first and will be sited in Block 1 as shown in Figure 3. After completion of the fully contained trial (Trial 1 Part 1) and clean-up of the virus release area the partially contained design (Block 2) will be tested. To standardise conditions as much as possible, the sowing date of the cotton and sorghum in each of the two blocks will be staggered by 14 days

Experimental Protocol:

Virus application procedure: An aqueous suspension of formulated marked *Helicoverpa* NPV will be applied to each cotton release plot at a rate of 1 x 10^{13} PIBs/ha @ 150 litres/ha, using an hydraulic 2m boom sprayer.

Monitoring and sampling:

(i) One day prior to the marked virus application 10,000 x 48 hour old laboratory reared *H. armigera* larvae will be distributed throughout each cotton plot; the first of five rounds of soil, foliage, and larval samples will be taken at this time.

(ii) immediately following marked NPV application soil and foliage samples will be taken from cotton, bare earth surround, and sorghum trap crop areas. Larval samples (100 individuals) will be taken from cotton release plots only.

(iii) leaf samples will be collected from an exposed canopy position from the release area at intervals of 0.5, 4, 8, 24, and 48 hours after treatment for construction of virus inactivation curves. These will be compared against virus inactivation curves for wt HaSNPV constructed for the parental virus (A44EB1) inoculated directly onto cotton leaf surfaces by pipette in cotton plots outside the containment structure, in the east-west buffer zones.

(iv) Pitfall traps will be sited along selected transects and emptied daily. A Malaise trap will be sited in one of the release plots for both fully contained and partially contained treatments. The presence of the GMO on body surfaces, in the gut, and in frass from collected insects will be assessed by bioassay.

(v) at 4, 8 and 12 days following the marked HaSNPV application, soil, foliage and larval samples will be taken from the cotton, bare earth surround, and sorghum components of each plot.

(vi) at the termination of each trial, all cotton plants in the virus release area will be removed and incinerated. Any virus present at the soil surface will be treated by saturation with 20% hypochlorite solution (1% available chlorine). The treated area will be covered with black plastic sheeting, and soil samples taken for bioassay after a suitable time and the containment structure removed. The sheeting will remain in position for the duration of the test bioassays and until confirmation that the marked HaSNPV has been removed.

Trial 2 - The Effect of Spatial Distribution of Inoculum on Redistribution and Transmission of HaSNPV

Aim

The aim of this trial is to investigate spatial redistribution and Transmission of HaSNPVs that originate from two positions in the cotton crop: (a) in the leaf canopy, and (b) at the soil surface beneath the leaf canopy. Each of these treatments will be replicated three times. Control samples will be taken from outside the trial plots within the central cotton buffer zone. A plan view of the general layout for this trial is shown in Figure 6.

Rationale

For neurotoxin-producing NPVs (tox+NPVs), the long-term impact of the soil virus reservoir on nontarget populations may be particularly important. One effect of neurotoxin producing recombinant NPVs is larval paralysis. Individuals infected with these viruses tend to fall from the host plant before death and therefore before progeny virus is released onto the foodplant for transmission to foliage feeding populations. Because these viruses are therefore, likely to move directly to the soil-NPV reservoir with little or no solar inactivation the question arises of how the difference in spatial origin of virus inocula (foliage versus soil) influences the redistribution and Transmission of virus to host feeding sites and the subsequent generation of secondary infections in susceptible populations ?

Experimental design

The Transmission study plots will be sited in the central cotton buffer zone.

Experimental Protocol.

Two hundred and fifty larvae infected as third instars will be positioned in a $1m^2$ area at the centre of each release ploteither in the leaf canopy (on the uppermost leaf whorl) or on the ground. To prevent larval movement and cuticle rupture prior to release, infected larvae will be frozen prior to death. A cohort of infected larvae will be retained in the laboratory and virus counts conducted to determine the loading of virus in the release plots.

Monitoring and sampling

(i) soil and leaf samples will be taken at 1m intervals along each transect at 1, 3, 6 and 9 days after the release of the infected cadavers. Leaf samples will be taken from both exposed and shaded leaf canopy positions. Control samples of foliage and soil will be taken from the control transect shown in Figure 6. One sample of upper canopy and one of lower canopy foliage and 4 soil samples will be taken from each of the five arms of the transect.

(ii) pitfall traps will be positioned along selected transects and material collected from these traps tested for the presence of the marked HaSNPV.

(iii) On day 10, up to 10,000 second instar larvae will be released at 8 points in each plot, collected on day 14, and reared on sterile insect diet to compare infection rates in the two treatments. After the collection of larvae on day 14, samples of foliage (upper and lower canopy) will be taken from plants in the virus release area along with soil samples.

(iv) at the termination of the trial, all cotton plants in $1m^2$ release area will be removed and incinerated. The surface of the $1m^2$ virus release areas will be treated with 20% hypochlorite solution, and soil samples removed for bioassay for confirmation of virus decontamination.

Appendix 2.

SUMMARY OF PRELIMINARY RESULTS FROM 1998 PLANNED RELEASE TRIAL

TRIAL DESIGN

The trial comprised two treatments of:

a) *full containment* i.e. release plots physically enclosed for the duration of the experiment, and

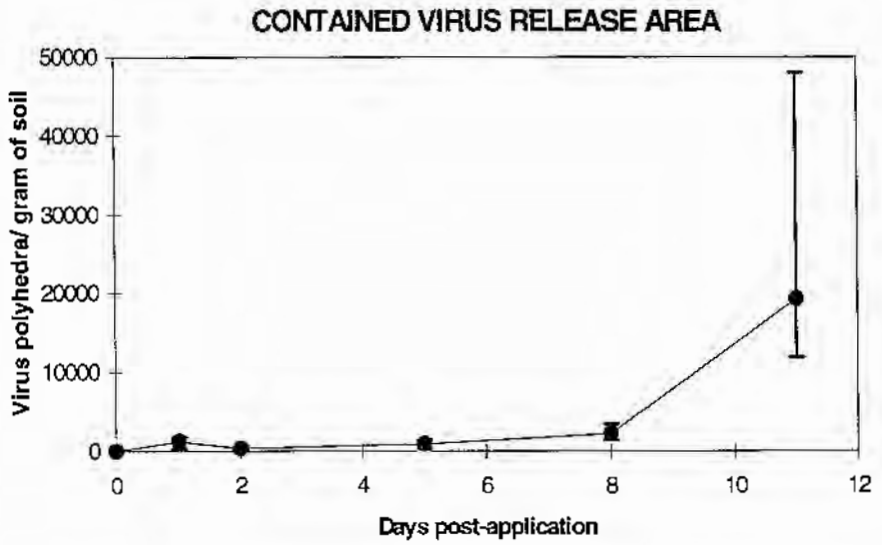
b) *partial containment* i.e. release plots physically enclosed for the virus application only with the containment structure then removed for the remaining experimental period

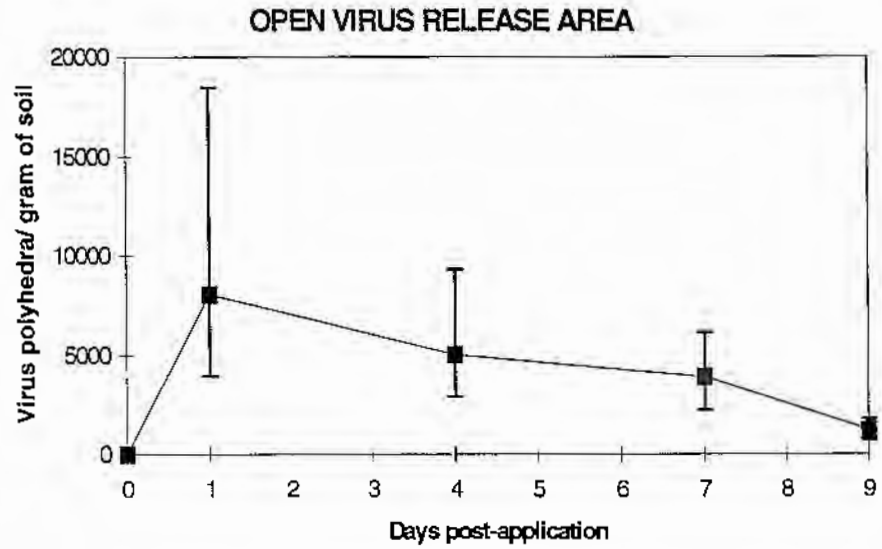
Each of the treatments comprised two replicate plots layed-out according to the plan shown Figure 2A-3. Further details of the trial protocols are provided in Appendix 1.

1. MONITORING MARKED VIRUS AT THE SOIL SURFACE

Method: Ten samples of soil were taken from each plot at each time point, dried, combined and bioassayed against a laboratory culture of *H. armigera.* Estimates of soil virus concentration obtained as detailed in the attached protocol "Procedures for the detection of HaSNPVs in soil".

Figure A2-1. Estimates of marked virus populations in soil inside contained and open virus release areas (data given as virus polyhedra per gram of soil with +/- 95% confidence limits)

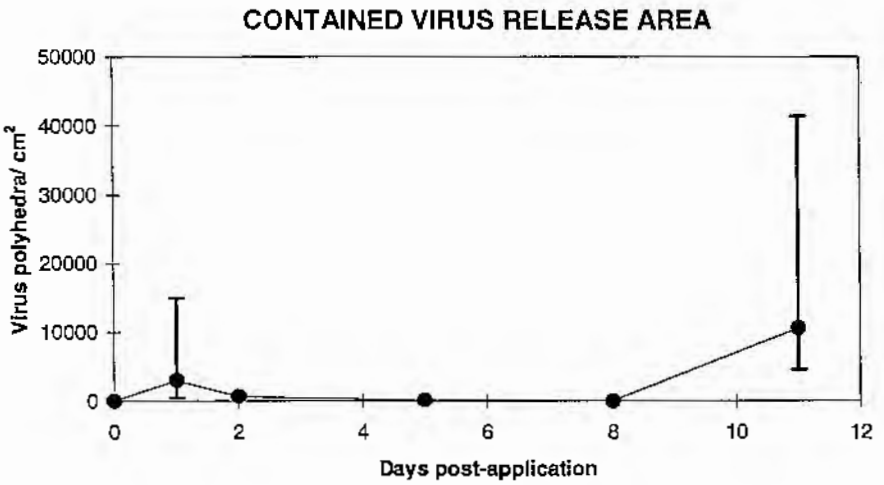


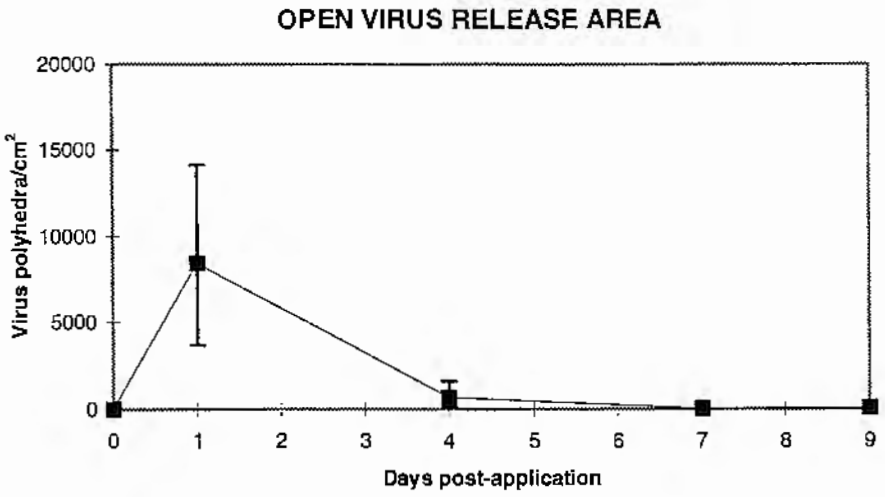


2. MONITORING MARKED VIRUS AT THE LEAF SURFACE

Method: Twenty whole leaves were taken from each virus release area at each time point and frozen prior to analysis. One hundred leaf discs (each 2cm^2) were removed from each batch and homogenised in 100ml of distilled water. From this, 100µl aliquots were applied to the surface of a sterile insect diet in each of 25 larval feeding cells and bioassayed against one day old *H. armigera* larvae. Percent mortality was recorded at 10 days. Estimates of virus concentration per ml of leaf homogenate were calculated from standard virus response curves of probit mortality against log virus concentration. These were constructed using wt HaSNPV. These data were then used to calculate virus concentration per cm^2 of leaf.

Figure A2-2. Marked virus populations at the cotton leaf surface in contained and open virus release areas (data given as virus polyhedra per cm^2 of leaf with +/- 95% confidence limits).





3. SITE DECONTAMINATION

Method: Five samples of soil were taken from each plot at each time point, dried, combined and bioassayed against a laboratory culture of *H. armigera*. Estimates of soil virus concentration were then obtained as detailed in the attached protocol "Procedures for the detection of HaSNPVs in soil".

Table A2- 1. Effect on marked virus populations present in soil inside contained and open virus release areas following saturation with approx. 10% hypochlorite solution (data given as virus polyhedra per gram of dry soil with 95% confidence limits in parentheses).

1a. Contained virus release areas

	NPV concentration prior to treatment (PIBs/gram of soil)	Virus concentration after treatment (PIBs/gram of soil)	Percent reduction in [NPV]
Replicate 1	24075 (15908-35635)	176 (101-256)	99.3%
Replicate 2	19185 (11832-47965)	240 (150-336)	98.7%

1b. Open virus release areas

	Prior to treatment	24 hours post-treatment	48 hours post-treatment	72 hours post-treatment	9 days post-treatment	Percent reduction at 9 days
Replicate 1	660 (317-1017)	9 (2-22)	44 (17-80)	554 (406-731)	143 (78-215)	78%
Replicate 2	1801 (1068-2587)	187 (110-271)	226 (139-319)	187 (110-271)	49 (20-88)	97.3%

DISCUSSION

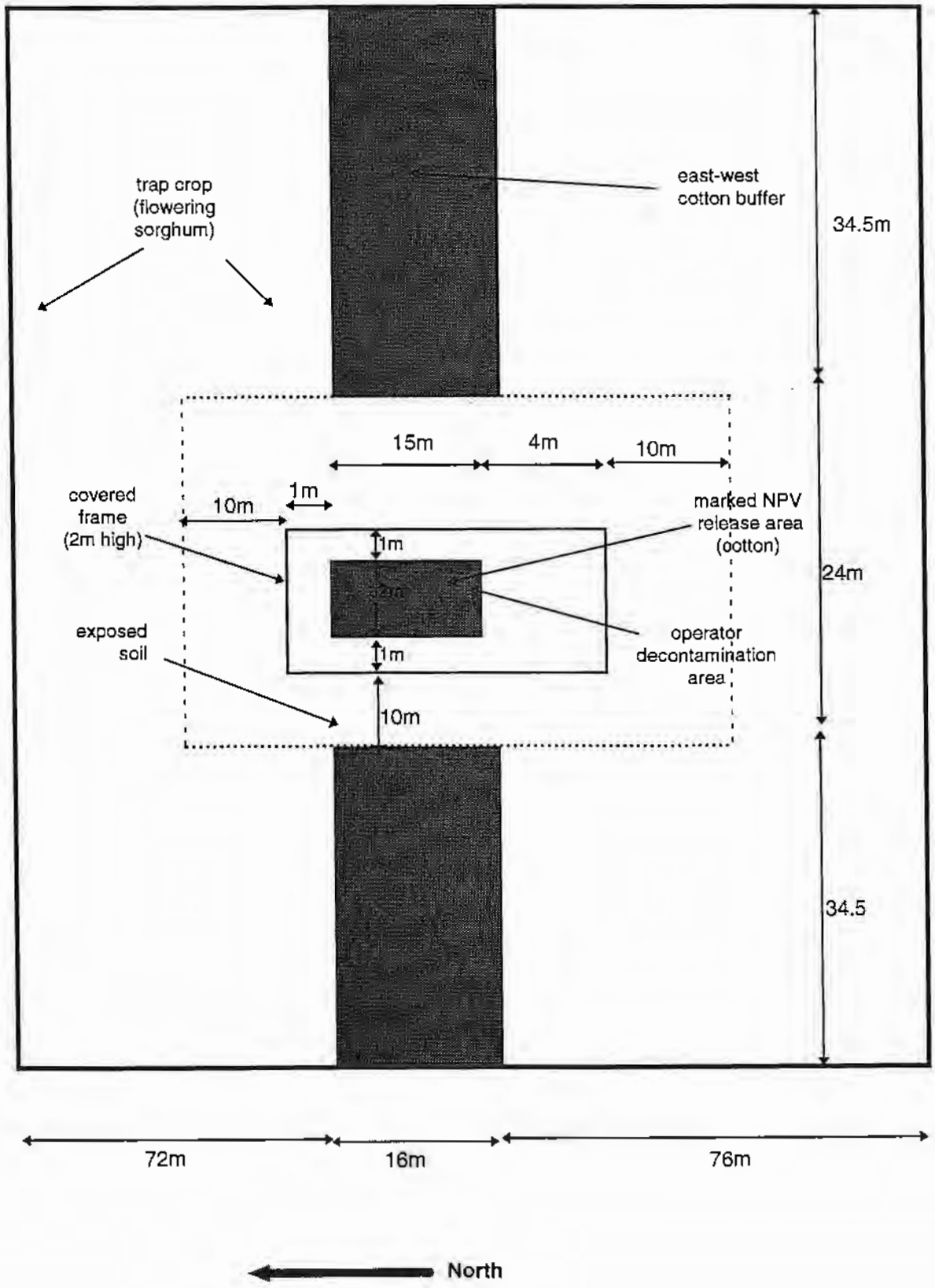
The preliminary results presented above suggest that the behaviour of the released virus under the plot two treatments was quite different. In the contained plots, the virus population in the soil increased through the course of the trial - with the rapid increase between days 8 and 10 post release being attributable to the amplification of the virus in the larvae that were released into the plot. The same basic pattern was also observed in the virus population present at the leaf surface - with the initial increase in population on day 1 being attributable to the virus that was applied to the plot.

In contrast, in the open plots (where the virus was applied under cover of the containment structure after which, the covering of the structure was removed), the population of virus at the soil surface decreased through the course of the trial. There was no obvious increase towards the end of the trial attributable to biological amplification of the virus from larvae released into the plots as was observed in the first part of the trial. The initially high population observed a day after application of the virus was probably due the slightly more open structure of the canopy in this trial and the fact that a greater proportion of the applied virus made it through the canopy to the soil surface. Like the contained plots, the same basic pattern in population change was observed at the leaf surface - although with much greater u/v exposure in this position the virus was inactivated much quicker than at the soil surface.

The decontamination trials produced fairly consistent results across the four treated plots irrespective of the containment treatment i.e. with greater than 95% inactivation being observed over a 7-9 day period . The apparently low reduction in the open trial Replicate 1 plot is due to the very low (relative) concentration of virus present in that plot at the start of the decontamination process. The low level of virus left at the end of the decontamination treatments in all of the plots may well represent the natural population of virus that is present at depths below that which the decontamination treatment can penetrate to. Studies are currently

underway to establish whether the viruses detected at the end of the decontamination process are the released virus or the natural background of wt virus.

Figure A2-3 Trial plot design for Trial 1 (parts 1 and 2). Not to scale.



PROCEDURES FOR THE DETECTION OF HaSNPVS IN SOIL

MATERIALS AND METHODS

Insect Source: *Helicoverpa armigera* were maintained from hatching on artificial diet (see Appendix 4) at 25°C at CSIRO Entomology (Canberra, Australia). The strain used had been continuously maintained in the laboratory for a period of more than 5 years.

Virus Source: Eight *Helicoverpa* isolates were used in the course of the study. HzSNPV was originally collected from larvae of *H. zea* (Ignoffo, 1965) and was used to produce the commercial formulation marketed under the tradename Elcar™. A44WT was originally isolated from a larva of a *Helicoverpa* species (probably *H. armigera*) from Brookstead, Qld, Australia. H25WT was isolated from larvae of *Helicoverpa armigera* from Biloela, Queensland, Australia and A35WT was isolated from larvae of *Helicoverpa punctigera* from Gleneagle, Qld. Australia. All Australian isolates were obtained from Dr Bob Teakle (Centre for Tropical Pest Management, Brisbane, Qld). The isolates HaChWT and HaInWT were obtained from Dr William McCarthy (Department of Plant Pathology, Pennsylvania State University, Pennsylvania) and are as described in Gettig and McCarthy (1982).

These isolates were passaged three times through late third instar *H. armigera* larvae at a rate of 100 polyhedral inclusion bodies (PIBs)/mm^2 and PIBs purified after each passage by differential centrifugation at 500xg and 5,000xg. They were then subjected to two rounds of limiting end-point dilution, *in vivo,* to isolate genotypic variants carried out at surface contamination rates that gave approximately 20% mortality (Smith and Crook, 1988). Polyhedra were isolated from single infected cadavers and DNA purified as described in Appendix 1. The DNA was digested with the restriction enzymes *Eco* RI, *Hind* III and *Bam* HI to assess homogeneity. Those isolates showing no submolar bands were subjected to a second round of limiting end-point dilution, viral DNA assessed for homogeneity as described above.

Construction of Soil NPV Concentration Response Models - *Preparation of soil*: The soil used in the construction of the standard response models was a cracking grey clay (60% clay; <1% organic matter; pH 8.1) collected from the Australian Cotton Research Institute, Myall Vale, Narrabri, NSW. The soil was crushed to a fine powder and passed through a 1.4mm guage sieve to remove woody debris and small stones prior to autoclaving at 120°C for 15 minutes. The soil was then dried in a fan-driven oven at 35°C for 24 hours. The final water content was 15% (w/w).

Soil-diet incorporation: The artificial diet into which soil was incorporated comprised 1.25% w/v agar, 4.2% w/v soyflour, 2.5% w/v stabilised wheatgerm, 0.3% w/v of ascorbic acid, 0.1% sorbic acid and 0.3% w/v methyl-p-hydroxybenzoate. After preparation the diet was maintained at 50°C -55°C. The soil sample was mixed into a fine paste with the addition of 25ml of distilled water. The soil paste was then mixed thoroughly into the diet using a hand-held electric blender. Soil was incorporated in this manner to final concentrations of 25%, 10%, 5%, 1% and 0% (w/v). One millilitre of wtA44HaSNPV suspension, containing a known concentration of polyhedra was inoculated into soil-diet mixtures and homogenised using a hand-held electric blender. The diet-soil-virus mixture was then dispensed into plastic jelly trays (J2 Cavities; Nu-trend Containers, Jacksonville, Fl.) and the diet allowed to cool. A single mid-first instar *H. armigera* larva was then placed into each well of the tray. Insects were maintained at 28°C +/- 1°C and mortality scored at X and Y days after transfer to the diet. Five NPV concentrations per soil rate were used: 5 x 10^4, 2.5 x 10^4, 1 x 10^4, 5 x 10^3 and 1 x 10^3 NPV polyhedra/ml soil diet. Three replicates were performed for each concentration for each soil incorporation rate.

Data analysis: The proportion of larvae killed by NPV infection was used in a maximum-likelihood computation of weighted linear logit mortality on log virus concentration, first as PIBs/ml of soil-diet then as PIBs/g soil. Non-NPV deaths (idiopathic mortality) represented less than 10% across all bioassays and was not included in the analysis. Estimates of soil virus loading in blind test samples and all subsequent estimates were computed using the Polo program (LeOra Software, California, 1987). Estimates of LC_{50} and 95% confidence intervals were computed for each soil incorporation rate. Mean percent mortality for six *H. armigera* SNPVs were compared and the effect of soil bioassay rate on larval survivorship and pupal

weights was assessed using the Generalised Linear Interactive Modelling programme (GLIM, version 3.77; R. Stat. Soc. 1985). The significance of any change in model deviance was assessed after calculation of the appropriate Chi-square value or F-ratio.

RESULTS

Effect of soil incorporation on *H. armigera* survivorship: The impact of diet quality on survivorship of test larvae was determined for all soil incorporation rates using control soil-diet treatments only ie no virus. The effect of rate of soil incorporation on survivorship was assayed after 10 days exposure (concurrent with the recording of the NPV mortality response) then again at 20 days to record survivorship to pupae. There was no significant effect of soil incorporation rate on larval survivorship at 10 days (χ^2=1.7; df=4; P=0.79) or survivorship to pupae (χ^2=2.75; df=4; P=0.6). However, there was a significant effect of soil incorporation rate on pupal weight ($F_{4,232}$ = 52.8, P<0.0001).

Generation of Soil NPV Concentration Response Models: The isolate HaSNPV-A44EB1 was used to construct all concentration response. The LC_{50} values and associated statistics for the logit response regression lines computed for the 5 rates of soil incorporation (0%-25%) are given in Table 1. All LC_{50}'s were characterised by narrow confidence limits which did not exceed the LC_{50} value by more than two-fold. Although there were highly significant differences in the intercepts of the regression lines for the different soil rates tested (χ^2=23.04; df=8; P=0.003) the slopes were not significantly different (χ^2=9.07; df=4; P=0.06) indicating a constant increase in NPV mortality for a given increase in NPV concentration across all rates. Table 2 shows the LC_{10} and LC_{50} values computed for each soil incorporation rate as polyhedra per gram of soil. These data indicate the high degree of sensitivity of soil HaSNPV detection obtained with incorporation soil incorporation rate.

Validation of Soil Concentration Response Models: A series of blind tests were conducted to validate the concentration-mortality regression models and test the validity of this method for estimating soil NPV concentration. Tests were conducted by one investigators using samples inoculated with a suspension of virus of known concentration by a second investigator. The inoculated soil was prepared as previously described and incorporated it into bioassay diet at a range of rates. The bioassay response for each soil rate was used to compute an estimate of NPV concentration and these were then used to give an overall mean estimate of PIBs/g soil for each sample. In each case, the estimated virus concentration was within the calculated 95% confidence intervals indicating that the regression models were reliable for the soils used in this study.

To assess whether or not the regression models generated for the isolate HaSNPV-A44EB1 were likely to be applicable to a broader range of HaSNPVs we compared the bioassay mortality response for six genotypic variants of *Helicoverpa armigera* SNPV isolates from Australia, namely A44EA17, A44EB1, A44EC7, A35EF2, H25EB9, and H25EA3. For each isolate, 1ml of virus suspension containing $1.5x10^4$ PIBs was inoculated into 100ml of diet mixture containing 25% autoclaved soil and assayed against 1 day old *H. armigera* larvae as described above. Three replicates of this concentration for each isolate were performed and the mean percentage NPV mortality for each isolate compared. Mean percent bioassay response (NPV mortality) for six Australian HaSNPV isolates was not significantly different (χ^2=1.48; df=5; P=0.92) implying that the quantal models designed upon the A44EB1 type isolate were likely to be applicable across a broader range of HaSNPV isolates

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