Cotton *Research and Development Corporation*

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GENETIC ENGINEERING OF *HELICOVERPA* NUCLEAR POLYHEDROSIS VIRUS: PRE-COMMERCIAL RESEARCH

Project Number: CSE 29C

Commonwealth Scientific and Industrial Research Organisation
Division of Entomology

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A FINAL REPORT PREPARED FOR THE COTTON RESEARCH AND DEVELOPMENT CORPORATION

1. OBJECTIVES

The aims of the CRDC project CSE 29C were:

- to optimise the design of recombinant *Helicoverpa* viruses with increased insecticidal activity (Activities 1 and 2 of the overall program above),
- to develop suitable large-scale production systems (Activity 4) and
- to assess the potential impact recombinant viruses may have upon the environment (Activity 5).

Timescales for the overall program are shown in Figure 1. Objectives for each year of the CRDC project were as follows:

Year 1: Generation of recombinant HaNPVs with increased insecticidal activity
Development of small-scale *in vitro* production systems .

Year 2: Optimisation of recombinant HaNPV construction and selection of most suitable recombinants for commercialisation.
Optimisation of small-scale virus production and the commencement of field studies with non-recombinant HaNPVs.

Year 3: First field trials of recombinant HaNPVs.
Scale-up of small scale production systems.

2. INTRODUCTION

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 is 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, the entomopathogenic bacterium, *Bacillus thuringiensis* (Bt), a fair degree of commercial success has already been achieved. 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.

We have been working on another potential biological insecticide at CSIRO Division of Entomology since 1988, namely *Helicoverpa* Nuclear Polyhedrosis Virus (HaNPV). A closely related *Helicoverpa* virus was previously used under the commercial name of ELCAR™, but it suffered from a short half-life under field conditions, and a slow rate of kill. These factors made it unsuitable for use on many crops and led to its eventual removal as an option in all *Helicoverpa* control programs.

Therefore, to circumvent some of these previously experienced problems, our program aims to provide novel viral insecticides by inserting foreign genes, whose products have insecticidal activity, into the genome of *Helicoverpa* nuclear polyhedrosis viruses. The insertion of such genes will produce viral strains with increased insecticidal activity and field control potential, which will offer biological options to the cotton industry compatible with existing chemical control agents and practices.

To date the vast majority of Research and Development on baculovirus vectors world-wide has involved the NPV of *Autographa californica* (AcMNPV). The first phase of our program developed an efficient vector system based on a specific *Helicoverpa* NPV, which subsequently allowed us to begin the commercial development of a new generation of effective biological insecticides for the control of *Helicoverpa*.

Industry Significance

The benefits to the Australian cotton industry will accrue through the availability of alternatives to the currently used chemical insecticides. Such alternatives will not only have value in their own right but will also assist in the further refinement of *Helicoverpa* resistance management programs, thereby extending the useful life of chemicals such as pyrethroids.

1. SUMMARY

The Division of Entomology initiated its program "Genetic Engineering of *Helicoverpa* nuclear polyhedrosis viruses" in 1988. The program aims to modify the genome of *Heliothis* NPVs (HaNPVs) to generate more rapidly acting viruses for use as biological insecticides in *Helicoverpa* control strategies. Towards these ends the program can be divided into three distinct phases, with timescales shown in parentheses:

1)Research and development of recombinant viruses (1988-1992)
2)Pre-commercial development of recombinant viruses (1992-1995)
3)Commercialisation of recombinant viruses (1995-1999)

The overall program is carried out in collaboration with Zeneca Agrochemicals (UK) and Crop Care Australasia. The initial research and development phase of the program ended in March 1992. This CRDC funded project (CSE 29C) formed part of the second pre-commercial development phase of the program.

During this second phase of the program it was anticipated that there would be six major activities:

1) Optimisation of the design of recombinant HaNPVs to allow for the development of faster acting strains
2) Selection and optimisation of the genes to be inserted into a commercial product
3) Toxicological studies on the products of the genes selected for insertion into a commercial product
4) Development of suitable production systems for the recombinant viruses
5) Assessment of the potential environmental impact of these recombinant viruses and,
6) The development of suitable formulations for the delivery of these viruses.

All of the work to be undertaken in activities 3 and 6 was to be undertaken solely by Zeneca with CSIRO solely or jointly responsible for the four other activities.

Activity 1: Optimisation recombinant HaNPVs Design

We have made several important changes to the design of the vectors used to generate recombinant HaNPVs. The method for generation and selection of the recombinants has been refined so that a required recombinant can now be isolated in approximately 25% of the time that this took at the start of the project (a saving of over two months for each recombinant that we wish to generate). We have also developed the first generation of vectors that will deliver a virus in the format that is most compatible with existing application and formulation technologies i.e. in the occluded form.

Activity 2: Selection and Optimisation of Toxin Genes

Central to the commercial development of the overall program is the availability of a suitable gene for insertion into the HaNPV genome to improve its speed of action. However, these genes are in relatively short supply. A considerable proportion of the resources in the overall program have therefore been invested in the isolation of a suitable insert gene. Although the gene we are working on shows greater efficacy than other insects toxins, some delays have been experienced in its isolation. These delays have had ramifications through the rest of the program. For instance, toxicological studies (Activity 3) have yet to be commenced.

Despite the lack of a proprietary toxin, we have been able to demonstrate "proof-of-concept" with two (non-proprietary) toxins. Specifically, we have shown that the speed of action of HaNPVs can be significantly improved by the insertion of either of these two toxin genes. It is anticipated that further improvements will be possible through the correct selection of toxin-virus construct.

Activity 4: Development of Production Systems

Notwithstanding the delays caused by lack of a proprietary insert gene, considerable progress has been made in the development of suitable production systems for HaNPV insecticides. As viruses are intracellular parasites, they have to be produced in a suitable host cell system. This necessitates being able to produce the host-cell system on a large-scale. During the course of this project we have successfully adapted our *Helicoverpa* cell lines to static growth in the commercially available serum-free media Sf900-II, Insect Xpress and Excell-401 and then further adapted these lines to growth in suspension cultures. We have then used these lines to study the kinetics of virus production and have demonstrated that these systems give reproducible levels of virus production.

Activity 5: Environmental Impact Assessment

Another important aspect of the overall program is ensuring that a suitable registration/implementation strategy is in place. Given the lag period associated with many environmental impact studies that experiments it is important that such experiments are commenced well in advance. With respect to carrying out the necessary environmental impact assessment of recombinant viruses good progress has been made in laying down a baseline data on the field persistence and dispersal mechanism of wild-type (non-recombinant) HaNPVs and on the underlying genetic variability of HaNPV populations in the Australian environment. This work is continuing as part of a project funded by the CRC for Sustainable Cotton Production, with the aim of carrying out the first recombinant virus field trials in 1996/97.

Activity 6: Formulation Development

Over the last two years of the project some preliminary work has been carried out on the development of formulations that may be suitable for an eventual product. Formulations, developed by Zeneca and trialed in 1993/94 in collaboration with CSIRO, gave reasonable performance in terms of application, dispersal on the plant surface and ability to remain on the plant surface. Subsequent trials in 1994/95 utilised one of these formulations to gather more detailed information on the rate of virus decay on the plant surface and in soil. Future plans include the testing of new formulations that contain a U/V protectant and testing of *in vitro* derived virus in field situations.

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2. INTRODUCTION

Background

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OBJECTIVES

The aims of the CRDC project CSE 29C were:

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Timescales for the overall program are shown in Figure 1. Objectives for each year of the CRDC project were as follows:

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Optimisation of small-scale virus production and the commencement of field studies with non-recombinant HaNPVs.

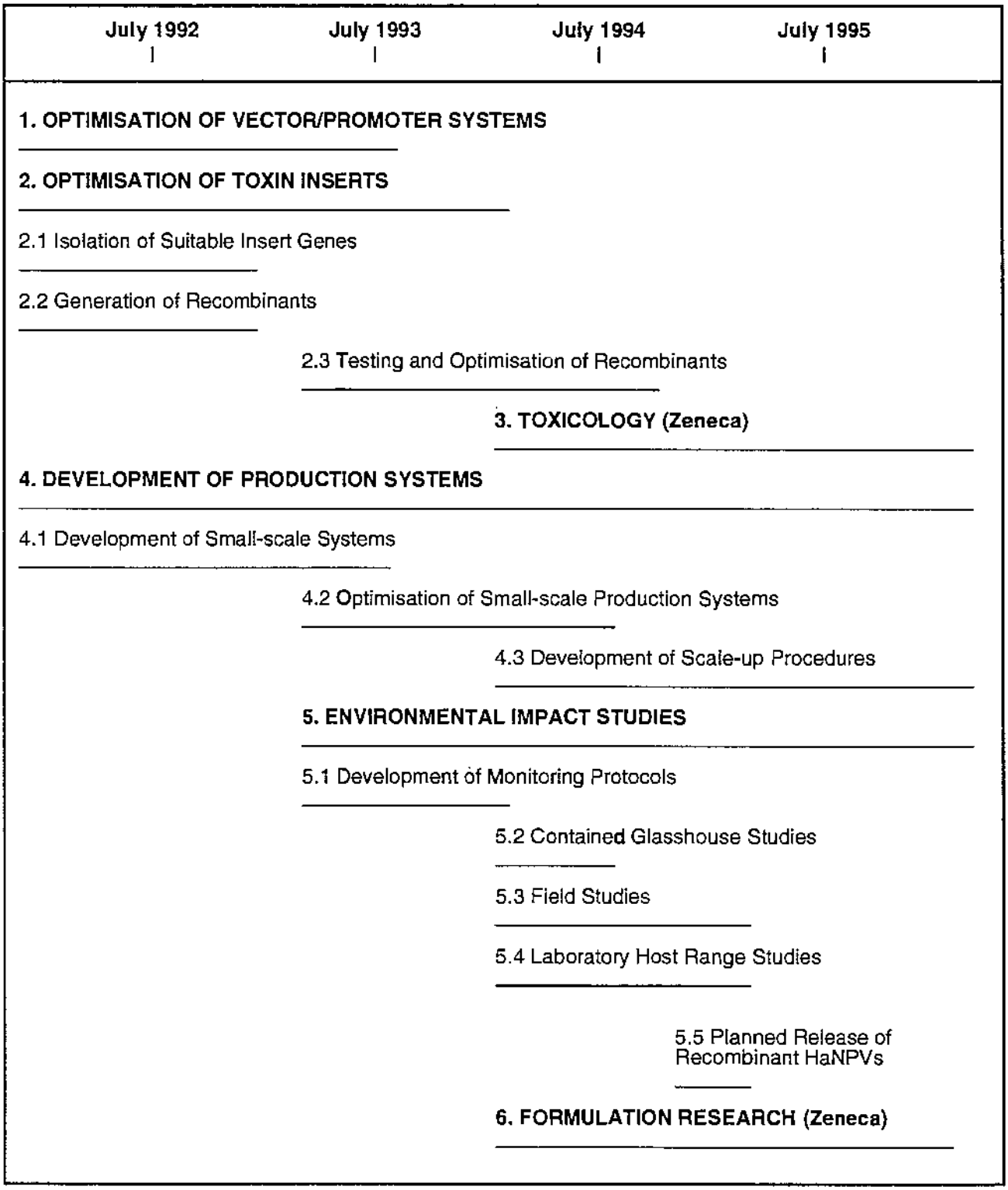
Year 3: First field trials of recombinant HaNPVs.
Scale-up of small scale production systems.

MATERIALS AND METHODS

1. OPTIMISATION OF VECTOR/PROMOTER SYSTEMS

The vector system developed in the first phase of the overall program was based around the polyhedrin promoter. As this is a very late promoter (not expressed until 18-24 post-infection) there are possibilities for increasing the control potential of recombinant viruses further by using earlier promoters. This activity aimed to identify and clone such promoters from HaNPV in order to provide alternatives for optimisation of the control potential of HaNPV recombinants (see activity 2.2).

FIGURE 1 **GANNT chart showing the critical research path and major milestones in the program , "Genetic Engineering of Heliothis Nuclear Polyhedrosis Virus: Pre-commercial Research". Activities that are the sole responsibility of ICI/Zeneca are indicated (ICI).**



2. OPTIMISATION OF TOXIN INSERTS

2.1 Isolation of Suitable Insert Genes

Several on-going projects within the Division and at Zeneca Agrochemicals in the U.K. were running in parallel with this project and aimed to isolate genes that may be suitable for insertion into the HaNPV genome. The most promising of these aims to isolate the toxin gene from the parasitic wasp *Bracon hebetor*. In addition, Zeneca were able to provide another two non proprietary toxin genes to the project that were inserted into HaNPV and their insecticidal potential assessed (see activities 2.2 and 2.3).

2.2 Generation of Recombinants

This activity was to involve the construction of transfer vectors and the generation and isolation of recombinant viruses containing suitable toxin-genes, as and when they became available.

2.3 Testing and Optimisation of Recombinants

Recombinants generated in activity 2.2 were to be assessed for increased rate of kill of *H. armigera* larvae, using standard bioassay procedures. It was intended that initial constructs would use a transfer vector in which the foreign (toxin) gene was linked to a second copy of the polyhedrin promoter and then recombinants would be optimised for reduced speed of kill using the alternative promoters isolated in activity 1. The most promising recombinants would then be passed on to Zeneca for further assessment and development.

The construct/recombinant showing the greatest *Helicoverpa* control potential would then be selected for further detailed biological and toxicological studies (activity 3). It was anticipated that a decision about the most suitable recombinant with which to proceed would be made in late 1993/early 1994.

3. TOXICOLOGY (ICI)

Toxicological data necessary to apply for the first planned release (activity 5.6) of the recombinant virus selected in activity 2.3 will be generated by Zeneca Agrochemicals. Toxicology will be performed on both the recombinant virus and the toxin that it is capable of generating. Toxicological data generated in this activity will also form the baseline data necessary for eventual registration of the selected recombinant as an insecticide.

4. DEVELOPMENT OF VIRUS PRODUCTION SYSTEMS

4.1 Development of Small-scale Systems

The parental *Heliothis* cell line used in our research is able to grow in two commercially available serum-free media. However, these media are very expensive (approx $40 per litre) and the recovery of virus is very low (see activity 4.2). This activity aimed to formulate our own serum-free media and to adapt available cell lines to growth in suspension culture.

4.2 Optimisation of Small-scale Production Systems

Several factors are known to attenuate the *in vivo* infectivity of baculoviruses that are produced *in vitro*. The most important of those factors identified to date are caused by genetic instability of the virus (Kool *et al.*, 1991) and the lipid content of the cell culture media (Tomkins *et al.*, 1991). This activity aimed to assess the *in vivo* infectivity of HaNPVs produced in systems developed in activity 4.1, thereby allowing us to optimise the recovery of infectious virus in small-scale serum-free production systems (up to 100ml).

4.3 Development of Scale-up Procedures

Two major systems are currently used for the large-scale production of cells in suspension culture, stirring and air-lift fermentation. The latter is used only when the cells to be produced are particularly sensitive to shearing.

This activity aimed to assess the relative performance of selected cell lines in scale-up fermentation procedures up to the 1 litre stage. Initial data suggested that *Heliothis* cells are not particularly sensitive to shear stress and will perform well in stirred suspension systems. We therefore aimed to look at the performance of our selected *Heliothis* lines in a continuous stirring system and assess them primarily for cell growth . Only in the event that growth is severely impaired in this system, will an air-lift fermentation system be investigated.

Further optimisation of the systems developed in this activity will be undertaken by Zeneca Agrochemicals in conjunction with CSIRO.

5. ENVIRONMENTAL IMPACT STUDIES

Activities in this area of the program were planned to increase throughout the second half of the program and aimed to generate sufficient data for the application and subsequent release of a toxin-producing HaNPV in the growing season of 1994/95.

The primary aim of the proposed field trips was to identify natural HaNPVs that are present in *Heliothis* populations, both within cotton crops and in nearby crops; to assess the distribution of baculoviruses in species closely related to *H.armigera/punctigera* in these areas, and to determine the ability of these viruses to replicate in *H.armigera/punctigera*.

Experimental priorities and time-frames would alter within this area of the program depending on the requirements of the relevant regulatory bodies.

5.1 Development of Monitoring Protocols

As a pre-requisite to the planned field trials it was anticipated that it would be necessary to develop sensitive and convenient assays for monitoring the distribution and dispersal of viruses during the trials. The assay was expected to involve the polymerase chain reaction (PCR), which can be used to amplify specific fragments of DNA from very small amounts of starting material.

5.2 Contained Glasshouse Studies

Once a suitable recombinant was decided upon, small-scale glasshouse trials would be carried out to assess the control potential under more natural conditions and the rate at which the virus persists in insect cadavers, on the plant and in the soil.

5.3 Field Studies

The field study component of the project was planned for 1993-95 and will include surveys of NPVs in *Heliothis* and other lepidopterans in and around cotton growing areas before, during and after the normal growing season. The surveys were primarily aimed at identifying *Heliothis* overtly infected with NPVs but would also include random sampling of insects, vegetation and soil.

Once isolated from field samples NPVs were purified by an end-point dilution procedure and assessed for genetic homogeneity by restriction profile analysis of their genomic DNA. Homogenous genotypes isolated in this way were then compared by restriction fragment length polymorphism (RFLP) analysis against reference material already available to the project.

5.4 Laboratory Host Range Studies

In addition to the field studies detailed above, laboratory studies were planned to assess two parameters critical to subsequent GMAC applications for field trials of recombinant HaNPVs. The first parameter concerns the infectivity of both wild-type (primarily the strain around which the genetic manipulation work has been based) and engineered HaNPVs for species of insects that are found in cotton crops alongside *Heliothis*. The second parameter concerns the ability of our HaNPV to undergo recombination either with other strains of HaNPVs or other NPVs found in insects susceptible to infection with our HaNPV.

5.5 Planned Release of Recombinant HaNPVs

The data gathered in activities 5.2, 5.3 and 5.4, were to be used to make an application to GMAC (Genetic Manipulation Advisory Committee) for a field trial with the engineered toxin-containing recombinant in the 1994/95 field season.

6. FORMULATION RESEARCH (Zeneca)

Once a decision had been made as to which recombinant to develop as a commercial product, it was intended that Zeneca would begin formulation research to ensure that the viral insecticide will be stable both to u/v exposure and to the conditions on the cotton leaf surface.

5.RESULTS

1. OPTIMISATION OF VECTOR/PROMOTER SYSTEMS

In the original project design it was anticipated that this activity would include work to isolate a variety of alternative promoters that could be used to drive the expression of foreign genes. However, because of the delays experienced in the isolation of a proprietary insert gene and because of some concerns expressed over the use of earlier promoters (the earliest promoters to function in the virus' replicative cycle have been found, thus far, to function constitutively), this work has been delayed until there is a perceived necessity for it. However, before this work could have began there were other goals that had to be met to improve the existing transfer systems and to develop systems that would optimise the biological activity of recombinant virus(es) to form the basis of a commercial product. Towards this end, enabling work in this activity contained two major components:

1) Isolation of a number of viral clones, by *in vitro* methods, from the reference isolate A44EB1 to ascertain whether any intrinsic improvements in the biological activity of the virus could be made by more traditional selection methods and,
2) Generation of improved transfer vectors systems.

Generation and Characterisation of Defined Viral Clones

As an initial step in this part of the activity, twelve viral clones were generated from the reference (A44EB1) isolate by *in vitro* methods. These clones were then assessed for:

1) Non-occluded virus (NOV) and polyhedral inclusion bodies (PIBs) production in *Helicoverpa zea* cells
2) Biological activity against *H. armigera* and *H. punctigera*.

Experiments to ascertain virus productivity in *H. zea* cell lines were first carried out when the only production system available was a static culture based system (see Activity 4). We ascertained that there was a large level of intrinsic variability in production parameters in such systems (see Table 1). We had already observed such patterns of variation with other viruses in these static systems. The only variable that we could associate with the observed variability was the growth stage of the cells - a factor which can be more readily controlled in suspension culture systems. Work in this activity was therefore focussed on experiments to determine the biological activity of the viral clones, until appropriate suspension systems were available. Results from the studies on biological activity are shown in Table 2.

Improvement of Existing Transfer Vector Systems

The original transfer vector (pA44NS1) used to generate recombinants was based around a unique insertion site adjacent to the polyhedrin promoter of in the HaNPV genome. Subsequent modifications made during the course of this project were:

1) Introduction of a polylinker into the unique *Bam*HI site of pA44NS1 site to allow more ready insertion of a variety of genes (pA44NS31 series of vectors),
2) Deletion of the 2 kb at the very 3' end (relative to polyhedrin) of the A44EB1-NS31 series vectors to allow for the insertion of large genes (>1kb) more efficiently.

3) Generation of a transfer vector that contains a second copy of the polyhedrin promoter along with the intact polyhedrin gene. The second copy of the polyhedrin promoter is inserted upstream of the normal promoter in an *Eco*RV site originally generated in the construction of the A44EB1-NS1 transfer vector.

Table 1 PIB Production Experiments carried out with clonal variants of A44EB1.

	Expt 1(1)	Expt 2(1)	Expt 3(1)	Expt 4(2)	Expt 5(2)	Expt 6(2)
Clonal Isolate						
E7F10		62.5		248.0		110.57
E7C12			75.0	296.0	255.0	
E7B9		164.0			625.0	
H10B3	403.0			265.0		
H10B10		60.0		1350.0	88.8	
H10B12		25.0		578.0	246.2	
D12B4			50.0	275.0	212.5	
D12D8	169.0			272.4		
D12F12			95.0	142.4	215.0	105.77
C9B3		25.0		215.2		
C9A12		18.0		202.4	147.6	
C9H3	405.0			132.0	548.8	100.96

(1) - Experiments initiated from GS-P0 i.e the reference NOV stock for the clonal isolate
(2) - Experiments initiated from GS-P1 i.e. the first passage of the reference NOV stock for the clonal isolate

In addition to the changes made to the transfer vector design, we also managed to achieve some significant improvements in the speed and efficiency of generating recombinants. Most notable of these improvements has been the use of a linear (rather than the circular) form of HaNPV to carry out transfections. This change in protocol has led to over a ten-fold improvement in the frequency with which we can generate recombinants. This turn has led to a 3-4 fold improvement in the amount of time required to isolate a particular recombinant from a transfection mixture (a saving of several weeks from each recombinant that we generate).

2. OPTIMISATION OF TOXIN INSERTS

Work in this activity was focussed around the isolation of a suitable gene for insertion into an HaNPV recombinant to improve its speed of action. With this aim in mind most of the of the work was concentrated on the isolation of a gene(s) from the parasitic wasp *Bracon hebetor*. However, changing opinions during the course of the project on the patentability of HaNPV caused us to look towards the genes of other toxins. These genes are from a predatory mite, a scorpion and a cone-snail and were made available to us from other sources. HaNPV recombinants were successfully generated that contain the first two of these genes. Both recombinants were found to improve the speed of action of the virus. However, it has subsequently become apparent that the partners in the program do not have a proprietary position on these toxins and so work is still continuing to isolate the gene(s) encoding the *B. hebetor* toxin.

2.1 Isolation of Suitable Insert Genes

All studies to date indicate that for both biological and proprietary reasons the toxin from *Bracon hebetor* is still the most promising candidate for insertion into HaNPV. However, isolation of the gene(s) encoding these toxin have been continually hampered by:

1) Its very high specific activity and low abundance in the wasp and,
2) The fact that the toxin is composed of four subunits.

Despite these difficulties, considerable progress was made in the last year of this project. Most notable is the isolation of the genes encoding three of the four subunits and evidence to suggest that one of the subunits may have toxic activity in its own right - thereby obviating the need to insert all subunit genes into the eventual recombinant. The provisional patent on this toxin completes on the 27 November 1995, by which time it is hoped that the final subunit will have been cloned and the identity of the (putative) toxic subunit will have been established. This patent, if granted, will give us a strong intellectual property position on the insert gene and the recombinant product developed from it.

Table 2. Biological activity of Clonal Virus Isolates against *Helicoverpa armigera* and *H. punctigera*.

	H. armigera			*H. punctigera*		
	LC_{50}[1]	Lower Limit[2] Upper Limit[3]	b[4] (+/-)	LC_{50}[1]	Lower Limit[2] Upper Limit[3]	b[4] (+/-)
A44EB1[5]	0.217	0.117	1.527	0.238	0.157	1.366
		0.381	0.198		0.345	0.102
A44EB1[6]	0.664	0.414	1.278	0.334	0.263	1.713
		1.095	0.128		0.417	0.173
E7F10	0.099	0.071	1.601	0.487	0.308	1.383
		0.139	0.155		0.754	0.121
E7C12	0.761	0.441	1.051	1.613	0.544	1.335
		1.643	0.111		4.877	0.189
E7B9	0.164	0.131	1.629	0.516	0.359	1.615
		0.205	0.128		0.726	0.166
H10B3	0.155	0.119	1.854	0.550	0.330	1.392
		0.201	0.184		918	0.144
H10B10	0.650	0.492	1.820	1.265	0.846	1.635
		0.876	0.165		1.832	0.155
H10B12	0.285	0.193	1.615	0.591	0.353	1.358
		0.413	0.140		0.988	0.156
D12B4	0.408	0.335	1.971	2.410	1.580	1.626
		0.501	0.175		3.442	0.209
D12D8	0.462	0.341	1.543	0.800	0.487	3.555
		0.648	0.143		1.132	0.710
D12F12	0.086	0.061	1.205	0.173	0.130	2.001
		0.119	0.113		0.224	0.150
C9B3	>10.0[7]	N/A	N/A	>10.0	N/A	N/A
		N/A	N/A		N/A	N/A
C9A12	>10.0[7]	N/A	N/A	>10.0	N/A	N/A
		N/A	N/A		N/A	N/A
C9H3	0.127	0.072	1.200	0.277	0.168	1.535
		0.213	0.120		0.403	0.126

(1) LC_{50}'s are expressed PIBs/mm^2. Estimates of LC_{50} were obtained by probit analysis using the POLO program and are derived from the pooled data of at least three separate replicate experiments in which four virus concentrations between 10.0-0.001 PIBs/mm^2 were used.
(2) Lower 95% fiducial limit of LC_{50} estimate
(3) Upper 95% fiducial limit of LC_{50} estimate
(4) Slope of probit mortality line
(5) Parental virus (A44EB1) recovered from insects
(6) Parental virus - 3rd passage in cells
(7) Lower than 50% mortality recorded for highest concentration of virus used in the assay i.e. 10.0 PIBs/mm^2

Overall, the delays in isolation of the *Bracon* toxin gene have pushed the timeframes of activities 2.2 and 2.3 back by approximately 18 months.

2.2 Generation of Recombinants

As discussed above (Activity 2.1) genes from a predatory mite (Tox-34), a cone-snail (KK-0) and a scorpion (AaHIT) were made available to the project to ascertain:

1) That "arming" of an HaNPV recombinant could be achieved by insertion of a toxin-encoding gene and,
2) If any potential intellectual property position could be generated with these insert genes.

As it arose, the latter did not eventuate. However, recombinants were generated using AaHIT and Tox-34, both of which showed improved speed of action (of between 25 and 40% over the wild type virus). As both of these toxins have relatively high LC_{50}'s for lepidopteran species (at least 100 fold higher than *B. hebetor* toxin) they gave strong indications that even greater improvement in kill-time may be obtained by the insertion of the *B. hebetor* toxin.

2.3 Testing and Optimisation of Recombinants

The delays experienced in the isolation of suitable genes meant that this activity did not commence during the term of the project.

3. TOXICOLOGY

Zeneca have carried out some preliminary toxicological studies on the wild-type virus prior to the first field trials carried out in 1993/94. Obviously full-scale toxicology studies will not commence until a final toxin-virus has been decided upon.

4. DEVELOPMENT OF PRODUCTION SYSTEMS

The work carried out in this activity was funded (see Appendix), in part, by the Grains Research and Development Corporation through the project "Genetic Engineering of Helicoverpa Nuclear Polyhedrosis Virus: Development of Large-Scale Production Systems" - Project Number CSE 24F.

4.1 Development of Small-scale Systems

Up until the late 1980's most studies carried out on the production of insect viruses in cell culture systems had been based around cell lines that require the addition of foetal calf serum (FCS) to the cell growth medium. However, with the development in the late 1980's of expression systems based around insect viruses, cell culture media became available that do not require the addition of FCS. As these media are much cheaper to produce than FCS-containing media, they are an important part of the development of cost-effective production systems for viral insecticides. Work in this activity was therefore focussed on testing our *Helicoverpa* cell lines for growth in the available serum-free media.

By the end of 1992 the parental *H. zea* cell line had been adapted to growth in static culture in two commercially available serum-free media Sf900-II and Excell-401. During 1993 the cells were further adapted to growth in suspension culture along with a cell line that has been adapted to the serum-free media Insect Xpress - which became commercially available in Australia around the middle of 1993.

4.2 Optimisation of Small-scale Production Systems

While work in Activity 1.1 was underway, parallel work was carried out in this activity to gain insights into the factors that may control virus production in small-scale systems. Work in this activity was therefore focussed primarily on the following components:

a) Assess cell lines adapted to serum-free media for productivity of polyhedral inclusion bodies (PIBs) and non-occluded virus (NOV)
b) Assess the biological and genetic stability of virus constantly passaged through the parental *H. zea* cell line.

a) PIB production in serum-free media

Cell lines that had been adapted to static culture in Sf900-II, ExCell 401 and Insect Xpress were assessed for their relative levels of PIB and NOV production. Results indicated some differences between the commercially available serum-free media against these criteria, but none of these differences were consistent between experiments (Table 2). In a further series of experiments a number of factors that may be responsible for this variation were eliminated e.g. batch to batch serum/media variation, variation in the amount of virus used to initiate infection, and the only variable strongly implicated was the growth phase of the cells (see also Activity 1).

Table 3 Productivity of PIBs in commercially available serum-free media compared with the serum-containing TC199MK.

	Polyhedral Productivity(1)	
	Experiment 1	Experiment 2
Media		
TC199MK	144.66	267.15
Excell 401	61.83	18.16
Insect Express	160.15	ND
Sf900 II	ND	206.09

(1) Polyhedral productivity is expressed in terms of PIB produced/cell. Each figure is an average of six replicates with each replicate being a $25cm^2$ tissue culture flask seeded at a density of $1x10^6$ cells and infected with passage 2 of the parental A44EB1 at an multiplicity of infection m.o.i.of 0.1 $TCID_{50}$'s/cell. Note, 1 $TCID_{50}$ is the amount of virus necessary to establish an infection in 50% of the wells of a microtitre plate exposed to it.

ND = not determined

b) Biological and genetic stability of virus

Studies were completed in early 1993 to assess the biological and genetic stability of virus constantly passaged through the parental *H. zea* cell line. Over the course of several passages it was found that the biological activity of recovered PIBs did not change significantly, although the activity of the recovered PIBs was consistently lower than the virus produced in insects. Despite the encouraging results with the PIB production we did however find the yield of NOVs fell throughout the course of the experiment. In two separate experiments these yields had fallen so low by passage 7 that it was not possible to continue with the experiment, until a suitable suspension culture system was available.

When the serum-free suspension lines became available in early 1994, initial experiments were carried out to assess the lines for PIB and NOV production. As previously suspected (see above), results were more reproducible than in static systems and so we proceeded with further experiments.

As with the static cultures above, we found that PIB production could be maintained in these systems but NOV productivity fell very rapidly. Within a few passages, there were insufficient NOVs produced to continue the experiments. At this point we decided to investigate further the kinetics of NOV production. Contrary to expectation we found that much higher NOV yields could be produced by:

1) Lowering the initial seeding density of cells and,
2) Lowering the m.o.i. used the establish an infection.

These data became available at a relatively late stage of the project and so subsequent experiments to assess the productivity of the suspension systems are awaiting full analysis. Preliminary analysis has shown that under equivalent conditions, productivity of PIBS is approximately the same in the three different lines. With this discovery we then proceeded to test some of the clonal virus isolates (C9A12, E7F10 and H10B12 -

selected on the basis of the range of biological activities they exhibit; see Table 2) and observed basically the same results. Initial analysis has also indicated that the biological activity of the clonal isolates is retained after passaging in the suspension systems.

In addition, further studies have also found that biological activity of clonal viruses with an intrinsically low level of biological activity (e.g. C9A12 - see Table 2), can be "rescued" by passaging through insects. This has offered the exciting prospect, that even under conditions where the biological activity of a virus falls through passaging, the activity can be maintained/recovered by alternate passaging through insects. Experiments are currently underway as part of the third phase of the program to ascertain if this alternate passaging process has value, while analysis of the remaining material from this project is being completed. It is anticipated that the full analysis of the material from this project will be completed within the next six months.

4.3 Development of Scale-up Procedures

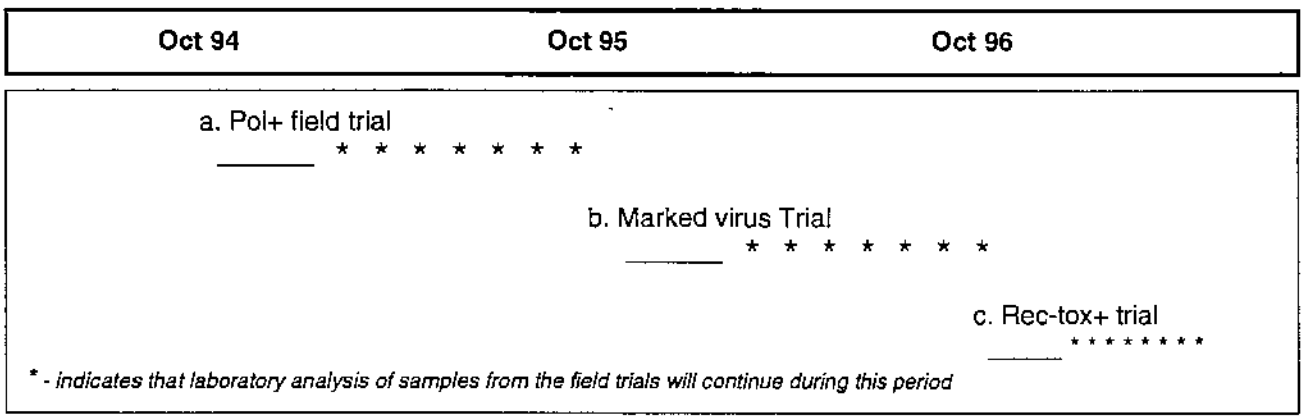
We adapted three cell lines to growth in suspension in the three commercially available serum free media (Sf900-II, Excell 401 and Insect Xpress). All three lines were found to grow at approximately the same rate and to reach densities of between 2.0-4.0 x 10^6 cells/ml. However, after several passages the line growing in Sf900-II demonstrated a tendency to attach to the culture flask more readily than the other two lines, and has subsequently proven quite difficult to work with.

Notwithstanding these problems, the work in this activity demonstrated that *Helicoverpa* cell lines could be readily adapted to growth in suspension culture. This therefore gave us the ability to progress onto experiments to assess and optimise the productivity of virus in such systems.

5. ENVIRONMENTAL IMPACT STUDIES

Work in this activity was planned to commence in 1993/94 with the aim of carrying out a planned release of a toxin containing recombinant in the 1994/95 season. However, due to problems with the isolation of a proprietary insert gene (see Activity 2) the timescales for release trials have been amended to those shown in Figure 2.

Figure 2: Revised Time-schedule for field trials



5.1 Development of Monitoring Protocols

The aim of this work has been to develop protocols by which the environmental fate of viruses used in field studies can be monitored, thereby enabling data required for experimental release/registration of recombinant viruses to be generated.

The work was originally focussed around the development of a physical detection technique namely, the polymerase chain reaction (PCR). Good progress was made on the development of this technique, although by early 1995 it had become obvious that there would also be a necessity for data in which the amount of biologically active material could be assessed. At this point the emphasis on the detection methodology changed from a PCR based method to a bioassay based technique.

Since this change in direction, good progress has been made in this activity, aided in no small part by the strong collaboration with the CRC funded project "Environmental Impact Assessment for Genetically Engineered Viruses". We have developed a relatively simple bioassay procedure that relies upon the incorporation of either foliar or soil samples directly into *Helicoverpa* diet; with mortality giving a direct measure (by extrapolation from standard curves) of the amount of viable virus present in the sample.

5.2 Contained Glasshouse Studies

Because of the delays experienced in the isolation of suitable insert genes, this activity will not commence until the recombinants are available.

5.3 Field Studies

Field trial with wild-type virus commenced in 1993/94, with the primary aim to test the suitability of some simple formulations (see Activity 6), to provide baseline data for carrying out future trials. These trials generated some preliminary data on the persistence of virus on the cotton leaf surface and the studies were expanded in 1994/95 to gather more data on the persistence/rate of decay of virus on leaf surfaces and in soil. To summarise, we have found that the rate of decay on leaf surfaces is relatively rapid with, over 90% of the original activity having been lost within 48 hours on the plant surface, while in soil the virus has a half life in excess of several months.

Field studies in 1994/95 also assessed the:

1) Distribution of virus (HaNPVs) in cotton cropping systems
2) Dispersal of virus through and across the soil profile.

We found that HaNPVs are widely distributed in cotton cropping systems and can reach relatively high loadings in the soil. Importantly, we also found that once the virus comes into contact with the soil it has little ability to disperse vertically through the soil profile but that there is some horizontal dispersal of the virus associated with irrigation. Laboratory studies have confirmed these results and the indications are that the virus binds very rapidly (and almost irreversibly) with clay particles in the soil.

Virus isolated from material collected during the field studies has also been the subject of genetic analysis to ascertain the intrinsic levels of variability that are present in the Australian environment. Much of this analysis is still on-going as we have linked it with the PCR detection technology developed as part of activity 5.1 (fragments of DNA generated from the PCR detection can also be used for restriction enzyme analysis, which when compared against a set of standards can give a measure of genetic variability). Initial results suggest that most of the HaNPVs isolated are very closely related at the nucleotide level.

5.4 Laboratory Host Range Studies

The primary aim of this activity to date has been to generate baseline data on the host-range of wild-type virus and hence, the potential host range of recombinants. We have now built up a good baseline of data on the host range of HaNPV. The results are summarised in Table 4.

In addition to the data presented in Table 4 we have also tested the susceptibility of *Heliothis subflexa*. As similarly reported for the ELCAR™, we found that *H. subflexa* was relatively insensitive to A44EB1 with concentrations of 100 PIBs/mm2 producing only 84% mortality.

5.5 Planned Release of Recombinant HaNPVs

Figure 2 shows that the time frame for the first field trial with fully active (rec-tox+) virus has been pushed back to 1996/97 due to problems encountered in other activities.

6. FORMULATION RESEARCH (Zeneca)

After initial problems during 1992 (which precluded field trials going ahead in 1992/93) Zeneca produced enough formulated virus for field studies during the 1993/94 season. These trials were carried out (in collaboration with CSIRO staff) in the Toowoomba area and tested two basic formulations - a suspension

concentrate (SC) and oil-based ULV formulation. Results were encouraging but did demonstrate that there was relatively rapid inactivation/loss of virus after application to the cotton plant.

Table 4 Biological activity of HaNPV (A44EB1) against the heliothine species *Helicoverpa armigera, H. punctigera, H. zea, Heliothis virescens* and *H. (Neocleptria) puctifera.*

	LC_{50}(1)	Lower Limit(2)	Upper Limit(3)	b(+/-)(4)
Species				
H.armigera	0.217	0.117	0.381	1.527(0.198)
H.punctigera	0.238	0.157	0.345	1.366(0.102)
H.zea	0.018	0.011	0.026	1.586(0.175)
H.virescens	0.072	0.039	0.132	1.687(0.185)
H.punctifera	0.207	0.112	0.352	1.358(0.177)

(1) LC_{50}s are expressed PIBs/mm^2. Estimates of LC_{50} were obtained by probit analysis using the POLO program and are derived from the pooled data from three at least separate replicates in which four virus concentration between 10.0-0.001 PIBs/mm^2 were used.
(2) Lower 95% fiducial limit of LC^{50} estimate
(3) Upper 95% fiducial limit of LC^{50} estimate
(4) Slope of probit mortality line

6. DISCUSSION

As part of a larger program to bring an engineered HaNPV insecticide to the marketplace the aims of this project were to optimise the design of recombinant *Helicoverpa* viruses with increased insecticidal activity; to develop suitable large-scale production systems, and; to assess the potential impact recombinant viruses may have upon the environment. With the timescale of the overall program being to have a commercial product around the turn of the century, it made good progress throughout the last three years. However, the specific milestones and aims at particular points in the CRDC project have been amended to meet the longer-term goals of the program.

In broad terms there are four major components to the overall program that need to come to fruition at the same time to bring the product to the marketplace:

1) Selection and generation of the most suitable virus-toxin recombinant
2) Development of suitable large-scale production systems
3) Development of an appropriate registration/regulatory package
4) Development of suitable formulation and delivery systems.

The project performed well in all of the of the above areas throughout it's duration, keeping the project on course with the projected release of a commercial product. A brief discussion against each of these major program components are presented below.

1) Selection and Generation of Suitable Virus-toxin Recombinant

This component has proven to be the most problematic area of research to date. Although good progress has been made on the development of polyhedrin-based expression systems in HaNPV, work planned to exploit other promoters has been delayed. In part this has been due to the uncertainty that has evolved around the use of such promoters, but has also been due to the fact that resources have had to be moved into isolation of the *Bracon* toxin genes.

To date we are only aware of three toxins that have been successfully inserted into baculoviruses to produce an increased speed-of-action. None of these toxins are in the public domain i.e. all are tied up with existing

patents owned by other parties. A much stronger intellectual property position will eventually be achieved if the ownership of the insert gene resides within the program partners. We have therefore continued to work with the *Bracon* toxin, despite the difficulties that it has presented. Most notable of these difficulties are the fact that it is extremely toxic and therefore produced in very small amounts by the insect, and the fact that it is also composed of four proteins. To isolate the genes for these proteins it has been necessary to generate sequence data from the protein itself; all of the subunits have therefore had to be isolated independently and protein sequence generated from them.

However, we now have three of the four subunit genes cloned and good protein sequence data from the fourth. Work is already underway to begin insertion of the first three genes into baculoviruses to test for their ability to improve speed-of-kill. We have also generated data to indicate that one of the subunits has toxicity in its own right and we are therefore hopeful that only one of the subunit genes will need to be inserted into the HaNPV genome to improve its activity.

The toxin/insert for a recombinant HaNPV insecticide is just one component of the system and the virus selection and construct design are other important factors that need to be taken into consideration. Our virus selection work to date has given us good access to a range of isolates that show a range of responses in *in vitro* and *in vivo* systems. Contrary to previous studies carried out with other baculoviruses we were able to isolate clones that retained biological activity after an *in vitro* selection process and which, just as importantly, seem stable in *in vitro* systems. The results of this research will now be incorporated into the production systems component of the next phase of the program.

During the course of this project we have also produced a first generation of pol+ viruses i.e. in which polyhedrin is still expressed and the virus is still produced in it's occluded form. These transfer vectors are still at a relatively early stage of their development but give us a strong indication that our eventual recombinant can be produced in a format compatible with the preferred formulation and application options.

2) Development of Suitable Large-scale Production Systems

Over the three years of this projects' duration, the general field of *in vitro* of production of baculoviruses has grown dramatically. This has been fuelled in no small part by the adoption baculovirus expression systems by the pharmaceuticals industry and the research community in general. This has had a direct flow-on effect to baculovirus production *per se* such as has been seen in the area of serum-free media development. There are now a large number of media products available for testing, and several companies around the world that specialise in the development and formulation of serum-free media.

For this reason, the objectives set for the project in 1992 to develop our own serum-free media were obviated by rapid developments in the field. Work was therefore focussed more onto the biology of growing *Helicoverpa* cells and HaNPV in these types of production systems, and the consequences that the biology of the systems may have upon the development of a suitable production system.

We have been successful in demonstrating that:

1) *Helicoverpa* cells adapt readily to culture in serum-free media and retain their ability to produce HaNPV
2) Viruses with good intrinsic levels of biological activity can be selected by *in vitro* cloning procedures
3) The kinetics of virus production in suspension systems are very different to static systems and need to be taken into account when optimising virus production
4) Recovery of biological activity can be relatively stable over several passages and the potential for the recovery of any biological activity lies in alternating between *in vitro* and *in vivo* production systems

The above lay down a good baseline of information with which to take forward the development of large-scale production systems. With the selection of the best combination of cell line and virus we can expect to generate robust production systems.

Within the overall program there has been additional effort invested in the generation of a wider base of cell lines from which to select. Zeneca, through an EC funded project, have worked in collaboration with teams in Germany and The Netherlands to generate new *Helicoverpa* cell lines, while we ourselves have worked in collaboration with Dr Art McIntosh of the USDA to develop new cell lines. Assessment of these new cell lines

will be integrated into the next phase of the project, along with our clonal viruses, to select the best virus-cell line upon which to base a production system.

3) Development of an Appropriate Registration/Regulatory Package

The registration and regulatory aspects of the program are some of the most complex that will need to be resolved to bring a product to the marketplace. Obviously, most of the toxicological component of the registration package will not be carried out until a a final virus/toxin combination is decided upon. However, to get the eventual virus/toxin combination to this stage it will be necessary to go through field trails, and to reach this point there are a number of studies and regulatory requirements that will need to be met. The studies in this component have therefore concentrated, thus far on generating components of a regulatory package that will be necessary to carry out field trials as soon as possible after a commercially viable virus/toxin combination has been generated.

In the first instance we have been concentrating on defining the following components of this package:

1) Ability of the virus to persist and disperse in the environment
2) Relationships to other virus present in the environment and the distribution of these viruses

Results so far have shown that HaNPVs (as previously suggested for ELCAR™) are inactivated once they have been applied to the cotton plant. However, the virus is capable of persisting in the soil for quite long periods. Notwithstanding the latter observation, data indicate that once the virus is in the soil it has very little capability for dispersal.

We have found that viruses are widely distributed in the environment and that overall there is a very low level of variation within the virus populations. In addition, we had previously carried out studies that showed that Australian HaNPVs are quite closely related to the virus which formed the basis of the product ELCAR™. As ELCAR™ was registered for use in both the USA and Australia we should be able to use much of the literature on ELCAR™ to support our regulatory and (later) registration submissions.

4) Development of Suitable Formulation and Delivery Systems.

Like *in vitro* production research, formulation research for biological insecticides is a rapidly expanding area. There are now a number of biological-based products coming into the marketplace with the realisation that the "old" technologies - based around those developed for chemical insecticides aren't necessarily appropriate. The results from our first field trials with an oil based ULV formulation were encouraging and more effort will be placed in this activity during the third phase of the program.

The factors that need to be addressed in future formulation studies include:

1) u/v stability
2) "Stickability" of the formulation i.e. getting the applied virus to stay on the surface to which it is applied
3) Application technology i.e. can simple modifications be made to the way the virus is applied and/or the virus is formulated to ensure that it reaches a site where it is readily accessible to the insect.

Studies on U/V stability will commence in this forthcoming season when Dr Marty Shapiro will be taking up a McMasters Fellowship in the Division to look at the use of fluorescent brighteners in u/v protection and activity enhancement.

7. CONCLUSIONS AND RECOMMENDATIONS

The biopesticides industry has begun to gather momentum over the last three years and there are now a number of options becoming available to the agricultural sector. The benefits that will accrue from this project will come from several quarters:

1) The use of biologicals *per se* will more readily allow for the move toward more integrated systems of pest management that obviate the need for widespread chemical usage
2) Through providing new options they will also relieve the pressure on some of the chemical groups e.g. pyrethroids that suffer from major resistance problems.

Because of the nature of the program the project has a relatively long time-frame. To date the majority of the research costs for the program have been borne by CSIRO, although as the program progresses towards a commercial product the input of our commercial collaborator will obviously increase dramatically. As a results of these long time-frames and large downstream costs eg. registration and construction of production facilities , the only pest control companies capable of undertaking such venture are large multinationals. For this reason it is important that the available Australian resources are focussed in a way that ensure maximum Australian ownership of the intellectual property and that products are tailored to suit Australian conditions.

To date, we have managed to achieve this balance, with most of the intellectual property being Australian owned and with the overall research program focussed around an Australian HaNPV which will significantly speed the route through the regulatory framework in this country.

8. COMMUNICATION OF RESULTS

Due to its commercially sensitive nature much of the research undertaken in this project has not been published. When the intellectual property has been suitably protected the results of the research will be made available to the both the scientific and wider community.

Notwithstanding these problems, we have tried to advertise as widely as possible the work that we are undertaking and the involvement that the CRDC have had in the project. The project has been featured in two issues of the video magazine "Cotton Report" and a number of articles have appeared in popular journals (Australian Cotton Grower)

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