Final Technical Report

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Title: Use of baculovirus control agents within an integrated pest management strategy against teak defoliator, *Hyblaea puera*, in India

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Production System: Hillside Production System

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1. Executive Summary

The current project has been concerned with development of an Integrated Pest Management (IPM) strategy against teak defoliator moth, *Hyblaea puera*, in India. A key element of the work has been the use of a baculovirus, originally isolated from moth populations in India, as a microbial control agent within the IPM strategy. Reductions in moth populations are necessary to reduce damage to teak trees and, thence, to increase the ultimate yield of teak in plantation forests. The programme was divided into several elements, namely development of improved moth monitoring and prediction, gathering of basic knowledge on virus-host interactions and virus production, quantification of elements within a *Control Window* conceptual model and, finally, field testing of the parameters predicted by the model to calculate optimal field dosage rates. The main results and conclusions are described below.

1. Moth monitoring.

Both solar powered light trapping and, particularly, visual observations through a network of field observers were used to monitor and predict field populations. Results from Kariem Muriem indicated that in the three years from 1995 to 1997, there were up to three area-wide infestations per year in the study area, but that most infestations were small and localised.

2. Dosage-mortality relationships

Analysis of the relationships between virus dosage and host mortality revealed that responses of the five larval instars varied with larval age and weight. Young larvae were more susceptible than older larvae such that the LD_{50}s were 17, 70.2, 72.7, 3932 and 20125 Polyhedral Inclusion Bodies (PIBs) for instars I to V respectively. This indicated that dosages for later larval instars were likely to be too high for practical use in the field and, therefore, it was advisable to concentrate on the third instar stage for targeting in the field. An anomalous response was observed for fourth instar larvae; responses up to 72 hours post infection had a similar slope to other instars but, thereafter, the slope flattened progressively, indicating that even relatively low dosages were inducing significant mortality. This unusual finding was not due to experimental error and indicated that a greater than expected response could be observed in field application even against this larval stage. Larval weight explained virtually all the variation in response between instars, thus enabling biomass to be used as a predictor of larval response to virus.

3. Virus productivity

Tests with individual larvae indicated that up to $7x10^8$ PIBs could be produced within a single fifth instar larva. This is within the expected range of similar sized larvae of other species infected with a baculovirus. Initial tests of virus productivity methods indicated that bacterial contamination was a problem and that virus yield was generally $<1x10^8$ PIBs per larva, indicating considerable loss of virus potential. Various methods of mass production were tested and ultimately a combination of individual feeding on virus contaminated leaf, followed by feeding on semi-synthetic diet gave a much improved yield with minimal bacterial contamination. The mean yield achieved was approximately $2x10^8$ PIBs per larva for semi-purified virus. There is, however, room for further improvement in both productivity per larva and in reduction of labour input.

4. Virus persistence

Once virus is applied in the field it is subject to environmental degradation from factors such as ultra-violet light (uv), rainfall and chemical composition of the leaf substrate. The

potential effects of these factors were investigated in laboratory experiments.

Ultra-violet light

Comparison of virus persistence and activity in direct sunlight and in darkness was made both in 1996 and 1997. The results indicated that when exposed to full sunlight, mortality of third instar larvae fed known quantities of virus dropped from 97% to 70% over a six-hour period. This rate of decay continued until approximately 24 hours, after which it stabilised at 13% mortality for up to 12 days. These results indicate that loss of virus can be very high during the first few hours of exposure to uv light but that, surprisingly, there is relative stability from 24 hours onwards. It is likely that the rate of decay in the first 24 hours would be less severe in field conditions because of the shade afforded by leaves and also by the fact that much of the virus applied in the field would be deposited on the shaded underside of leaves.

Rainfall

When subjected to various levels of artificial rainfall (up to 200 mm per hour) there was a rapid loss of virus activity leading to a decline from over 90% to around 15% mortality in test larvae. However, these data represent the extreme situation where virus is fully exposed on the upper leaf surface. It is likely that lower attrition rates would be observed in the field where the leaves would provide an umbrella effect against direct rainfall, particularly on the lower leaf surfaces.

Leaf surface effects

Tests of both young, tender leaves and more mature leaves indicated that there was no direct or indirect effect of leaf structure or chemistry on viability of virus.

5. Spray technology

Following initial evaluation, it was decided to employ ultra low volume (ulv) controlled droplet application (cda) for field testing of virus. Three sprayers were employed for experimental and field use:

Micron Sprayers Ulva+ rotating disk atomiser: this relies on natural wind and turbulence to project droplets into the canopy.
Micron Sprayers UlvaFan rotating disk atomiser; this employs both natural wind and fan assisted airflow to direct the droplets to the target.
Stihl SR400 motorised mistblower with Micronaire AU8000 rotating cage atomiser; this uses petrol driven forced air to project droplets up to 14 m from the spray head. This can be used to spray tall trees and in still air conditions.

Tests were carried out on potential carrier fluids to provide a formulated virus preparation that did not evaporate quickly under the high temperatures experienced in India. The final formulation was a stable emulsion of 94% coconut oil, 5% water and 1% emulsifier (Laboleine or Tween80).

Incorporation of a fluorescent dye into the spray fluid allowed direct observation of droplets on leaves under uv lighting. Data were gathered on foliage coverage under incremental spraying for the Ulva+ and UlvaFan sprayers and for direct spray application for the SR400/AU8000 sprayer. It was shown that good coverage of foliage could be achieved using both spray methods, such that sufficient droplets were present on the foliage to ensure that a third instar larva would encounter at least one droplet during a six hour feeding period. These data were used to predict field dosages within the *Control Window* model.

6. Field testing

Three trials were carried out during the project, although because of low field populations in years 2 and 3, the final trial was carried out after the official end of the project.

Small-scale trial at Nedungayam, 1996.

The target stage in this trial was the first instar layer. Results indicated that, employing dosages as low as $8.5x10^8$ PIBs per ha (only four larval equivalents of virus), up to 97% infection was achieved in larvae feeding on the trees. Difficulties in obtaining sufficient larvae meant that it was not possible to carry out a full statistical analysis of the data. However, it is likely, from knowledge of the very rapid rate of kill of virus, that the percentage infection figures actually under-represented the true accumulated infection rate.

Preliminary trial at Valluvassery, Nilambur, 1997

This trial employed a fully randomised layout and targeted the third instar larval stage on the trees. Results indicated an increasing response with increasing dosage, giving close to 90% mortality at the highest two dosages ($4x10^{11}$ and $8x10^{11}$ PIBs per ha (2000 and 4000 larval equivalents, respectively). The observed mortalities for these dosages were close to those predicted from the *Control Window* equations developed in this study. However, the responses for the lowest dosages were higher than predicted, indicating that virus was still effective (>70% mortality) even at a dosage 10 times lower than the top dose tested. This may have been due to the anomalous response of the fourth instar and/or to the rapid release of secondary inoculum on death of infected larvae arising from the initial dose applied. The result indicates that it may be possible to use considerably lower dosages in the field without a significant loss in efficacy.

Final trial at Valluvassery, Nilambur, 1998

This trial, carried out after the official end of the project, has not been analysed completely but is reported here to indicate that the trends observed in 1997 were essentially repeated in 1998, showing that the *Control Window* predictions were robust. This trial employed a randomised complete block design with four virus dosages up to $4x10^{11}$ PIBs per ha. Results were very similar to those obtained during 1997. Around 90% infection was achieved at the highest dose, compared with around 70% infection at the lowest dose. Assessment of damage was carried out by visual observation of percentage loss and by fresh weights of leaves taken at random from the sprayed trees. Analysis revealed that significant differences between treated plots and the untreated control plots were achieved, particularly for the highest dose. The trend of foliage weight indicated a direct response between dosage and weight in relation to time post spray, the greatest weight gain being achieved at the highest virus dose. The untreated control leaves lost weight, reflecting the continuation of feeding by *H. puera* larvae over the duration of the experiment.

Conclusions

Overall, the project has demonstrated that *HpNPV* can be a key component of an IPM strategy against teak defoliator moth, *H. puera*. The majority of parameters to underpin the planned *Control Window* concept were evaluated successfully and it was possible to use the equations developed during the study to predict field dosages with a high degree of accuracy. Further work is required to develop the IPM system for routine use and negotiations are underway with the Department of Biotechnology, India and with the DFID Western Ghats Project to fund the next phase.

2. Background

Teak defoliator moth, *Hyblaea puera*, (Lepidoptera: Hyblaeidae) is the most significant insect pest of teak (*Tectona grandis*) through most of its range in the tropics and sub-tropics. Studies carried out by scientists at Kerala Forest Research Institute (KFRI) have indicated losses averaging 44% of potential growth increment over a five year study on trees from four to eight years old (Nair *et al.*, 1985; Nair *et al.*, 1996). By using insecticides to protect trees from attack, it was shown that mean annual volume increment was reduced from 6.7 m^3 to 3.7 m^3 for unprotected trees. Projection of these data to more mature trees suggested that prevention of regular attacks by *H. puera* could potentially reduce the rotation for commercially grown teak from 60 to 26 years (Nair *et al.*, 1985).

A range of techniques for management of *H. puera* has been investigated over the past 100 years or so, but it is only during the past 11 years that a systematic research programme has been put in place. This approach towards Integrated Pest Management (IPM) has been led by KFRI and has considered the potential of natural enemies (parasitoids, predators and diseases) (Mohamed-Ali *et al.*, 1992; Nair *et al.*, 1995), silvicultural management through exploitation of relative susceptibility at the tree level (Nair *et al.*, 1997) and studies of the dynamics of *H. puera* outbreaks (Nair, 1988; Mohanadas, 1997). Among these factors, the most promising appeared to be a baculovirus isolated from *H. puera* larvae in Kerala (Sudheendrakumar *et al.*, 1988). Baculoviruses are double stranded DNA viruses that have been isolated only from arthropods. They have been used extensively in pest management programmes worldwide and have been shown to be highly specific, with no recorded effects on non-target organisms outside the arthropods. The *H. puera* baculovirus was identified to be a nuclear polyhedrosis virus (NPV) (*HpNPV*) that affects the nuclei of infected cells and in which the virions (virus particles) are occluded within a crystalline protein coat called a polyhedral inclusion body (PIB). NPVs of Lepidoptera typically affect the majority of organs in the insect body, resulting in large production of PIBs, providing a mechanism for survival of the virus outside the host, within and between larval generations. For a full treatise on their ecology and use in pest management see Hunter-Fujita *et al* (1998).

Initial work at KFRI indicated that *HpNPV* could be propagated in *H. puera* larvae in the laboratory and that a low volume application of a crude, unformulated preparation applied to populations of the moth in the field gave damage reduction of up to 76% (Nair *et al.*, 1998). Some progress was made in methods for production of virus, although this was relatively ineffective and gave low yields per larva. It was recognised that the methods used to evaluate *HpNPV* in both laboratory and field were hampered by lack of experience in handling baculoviruses. Consequently, contact was established between KFRI and the Forestry Commission Research Agency (FCRA). This led to the submission of a proposal to the Forestry Research Programme of DFID for development of IPM against *H. puera*, building on the experience of KFRI scientists and adding the expertise on baculoviruses offered by Dr H F Evans, FCRA. The approach was based on the *Control Window* concept (Evans, 1994) successfully pioneered for baculovirus control of pine beauty moth, *Panolis flammea* (Lepidoptera: Noctuidae) and pine sawfly, *Neodiprion sertifer* (Hymenoptera: Diprionidae) in Scotland (Entwistle *et al.*, 1990). Evans (1998) has developed this concept further to provide a framework for determining optimal dosages of microbial insecticides to reduce or eliminate the need for *ad hoc* experimentation in the field. The essential components are provision of quantitative measures of host susceptibility, host age/size distribution, host feeding rates, host feeding sites on the tree, rates of attrition of virus in the field and detailed knowledge of droplet emission and field capture rates for given spray applicators. There was little or no prior information on any of these components and, therefore, the current programme commenced with an essentially clean sheet in relation to *HpNPV* itself, but benefited from the extensive information already gathered by KFRI on the dynamics and distribution of *H. puera*

in teak forests.

3. Project Purpose

The purpose of the project was to develop an IPM strategy for management of *H. puera* populations, based primarily on improved monitoring and prediction of pest outbreaks and on the use of the baculovirus, *HpNPV*, as the main mortality factor acting on the larvae of the moth. As identified in the original submission, the intention was to quantify the following principal features:

- Insect biology: Monitoring systems for adult moths (moth behaviour, aggregation pheromones, spatial dynamics, choice of host tree and oviposition site), larval biology (feeding sites, feeding rates, within and between plant distribution, development rate), pupal biology (choice of pupation site, duration).
- Virus biology Dosage-mortality and virus productivity data for all larval stages, linked to larval weight, rate of decay of virus in the field.
- Plant factors Tree age, leaf phenology, relationships to other host plants, physical and chemical characteristics of leaves of different ages.
- Environment: Ultra-violet light, temperature, humidity, rainfall, pre- and post-monsoon effects on adult dispersal.
- Application: Equipment (LV and ULV), formulation for stability and flowability, optimisation of droplet production and deposition in the field, matching droplet deposition to insect feeding sites, investigation of "seeding" methods of introducing virus to moth populations.
- Output: Quantitative development of the "Control Window", development of decision-support systems, production of training manuals, training of beneficiaries.

Potential beneficiaries would be teak producers at both the commercial (Government and, increasingly, privately owned) and at the family scales. The principal socio-economic impacts would be the ability to grow teak on a shorter rotation and, as a result of the increased productivity generated, to make the crop more attractive economically. The results would have applicability not only in India but also in most other teak growing areas including Bangladesh, China, Indonesia, Papua New Guinea, Philippines, Malaysia, Solomon Islands, Sri Lanka and Thailand. Teak is one of the few high quality hardwoods to have been successfully domesticated and this project adds to its potential as a productive crop.

As described in the remainder of this report, the majority of the above fundamental factors have been successfully quantified, although one of the important assumptions in the Logical Framework, namely presence of suitable moth densities in the field, has, unfortunately, not materialised on all required occasions. Moth populations have been unusually low and relatively sporadic during years 2 and 3 of the project. This has limited the number of field trials that could be carried out during the duration of the project.

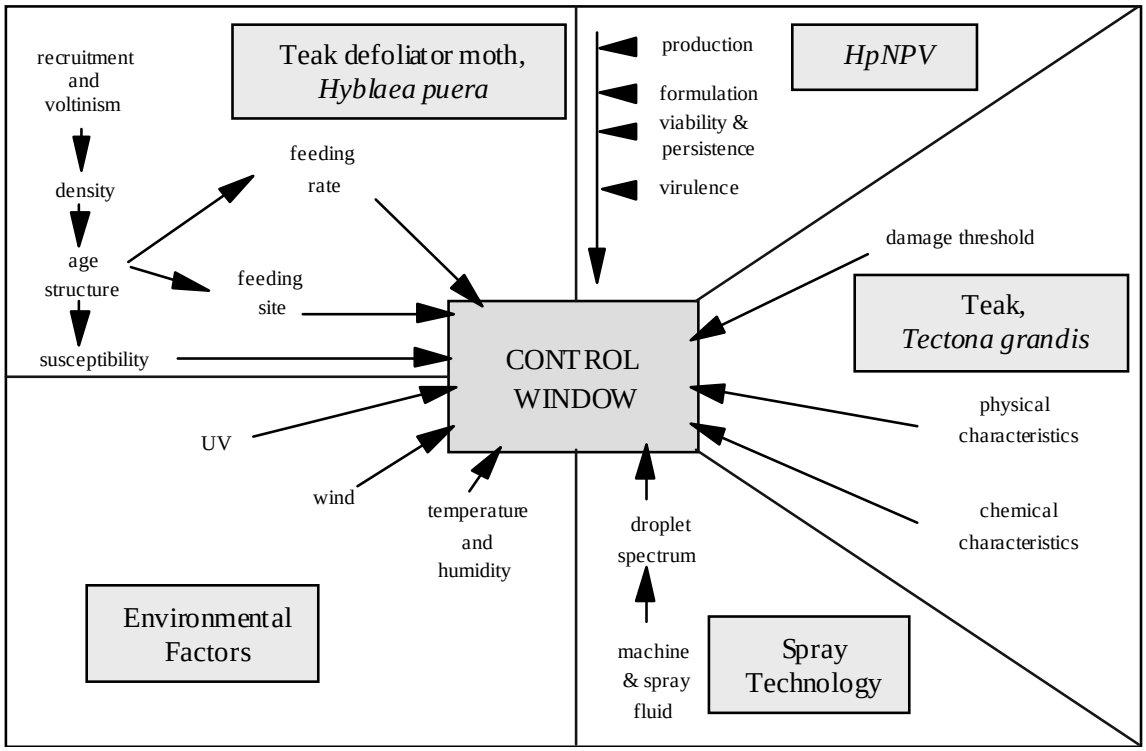
4. Research Activities

The Control Window concept provides a useful visual representation of the factors that have been studied during the current research programme (Figure 1). It concentrates on the five primary variables of *Host (Hyblaea puera)*, *Virus (HpNPV)*, *Environmental Factors*, *Tree (Teak)* and *Spray technology*. The *Control Window* is a conceptual model that provides a means of bringing these primary variables together quantitatively.

Research into each of the key components in Figure 1 was carried out and most of the work

outlined in the original research proposal was initiated and successfully completed.

Figure 1: The interactions of host, pathogen, crop, environment and spray technology in defining a Control Window for use of HpNPV in IPM of H. puera (from Evans (1994)).



Experiment plans were produced for each component of the work and, depending on results obtained, were modified at least annually during the duration of the project. Titles, aims and objectives of the experiment plans are shown in Appendix I. For brevity, Appendix I provides greater detail than the information included below.

4.1 *Moth population monitoring.*

The original aims for this part of the programme were:

- Assessment of existing KFRI data and determination of the most appropriate techniques for further development within the moth-monitoring programme.
- Robust monitoring techniques to pinpoint epicentres of moth population growth developed, linking to physical and meteorological features, using GIS as an analytical tool (UK and India).
- Beneficiaries trained in basic monitoring methods.
- Critical population density thresholds for use of baculoviruses within the *Control Window* approach determined and clear guidance given to beneficiaries.

The research programme itself was split into four inter-related components as outlined below.

4.1.1 Light trap assessments

Four solar powered light traps to attract adult moths were purchased for use in the project. These were located initially at Nilambur, Kunnamkulam, Kottayam and Konni but have since been concentrated only at Nilambur to aid the location of infestations for use in field trials. Although a valuable monitoring tool for local use, the cost of installation and manning make them less useful for extensive monitoring over wide areas.

4.1.2 Establishment of a survey network

The defoliator infestations which occurred in about 1000 ha teak plantations at Kariem Muriem (Nilambur North Forest Division) were monitored and mapped during the period 1995-1997. Two trained field observers were deployed to visually detect defoliation in the plantation area, which was divided into twenty Observation Units and one round of observation was completed in a fortnight.

4.2 *Baculovirus production and field application.*

The original aims for this core section of the programme were, broadly:

- Appropriate methods of baculovirus production, quality control and formulation for large-scale use developed.

- Methods for application of baculoviruses, by wide-area spray application or by seeding, developed and appropriate technology identified. Training of beneficiaries carried out using relevant methods for technical expertise and equipment availability.

- Storage and distribution systems for baculovirus preparations developed and beneficiaries trained in appropriation methods.

More specifically, the work has been divided into components that provide quantitative data on most of the elements in Figure 1.

4.2.1 The Host

This component of the programme was concerned with aspects of host biology, distribution, feeding rate, etc. that provide data to determine dosage rates, requirements for field droplet distribution, etc. Much of this information was already available from the previous work carried out at KFRI. Additional data, specific to the current project, were determined during the first year of the project.

As a general rule it is the larval stages of Lepidoptera that are susceptible to baculoviruses, reflecting the fact that the virus has to be ingested to become infective in the host individual. PIBs are ingested during feeding and, in the highly alkaline conditions of the mid-gut, are dissolved (dissoluted) to free the virions embedded within the crystalline protein coat. The free virions migrate to the outer walls of the mid-gut cells and, if the host is susceptible, enter the cells and migrate to the basement membrane. The virions then “bud” through the basement membrane and move to cells of the internal organs where they enter the nuclei and begin replication and eventually form new PIBs. On death of the host, these PIBs are released to the environment and the cycle can recommence. It is important to be able to determine how much food the host ingests to help determine dosage per unit of leaf. It is also necessary to quantify larval weight, which is directly linked to both susceptibility and to virus productivity.

4.2.1.1 Growth rates, including weight relationships

Insects grow through a series of moults, each stage being termed an instar. *H. puera* has five larval instars, each of which feeds on the leaves of teak. Instars I and II are able to eat only newly expanded, tender leaves whereas the remaining instars are capable of feeding on leaves of all ages. Although weight varies considerably within each instar, the width of the chitinous head capsule remains fixed until the next moult and is, therefore, a useful measure of which instar is being handled. Weight, head capsule width and duration of each instar was measured for each instar.

4.2.1.2 Larval distribution on the host tree

Studies on the population dynamics of *H. puera* have been carried out for many years at KFRI (Mohanadas, 1997). This information was used to aid the understanding of larval distributions on host trees that, in turn, provided the necessary information for directing the spray cloud during testing of *HpNPV*.

4.2.1.3 Larval feeding rates over time

Experiments were carried out to measure the surface area of teak leaf eaten by each larval instar within a six hour period selected as the upper limit of time for the larvae to encounter and consume a lethal dose of virus. This reflected the possible short duration of virus persistence in the field and also the rapid growth rates of the larvae that would potentially lead to equally rapid decreases in susceptibility to virus.

4.2.2 The Virus

There was very little prior information on the ecology and epizootiology of *HpNPV* and, thus, a comprehensive programme of research was initiated to provide the necessary data to determine field dosages. During this work, attention was also paid to improving the recognition of symptoms of virus infection over time, thus distinguishing virus infection from other mortality factors such as bacterial and fungal attack.

4.2.2.1 Dosage-mortality relationships for larval stages

A knowledge of how larval stages respond to challenge by virus is essential in determining dosage rates for both laboratory and field use. Research concentrated on three main steps (96.5, 97.2 – Appendix I):

i. Development of methods for carrying out quantitative bioassays.

The data from the experiments in section 4.2.1.3 were used to determine the amount of teak leaf that each instar could consume in six hours and, hence, the leaf surface area that could be employed to feed a precise dose of *HpNPV* PIBs to each instar during that time. Experiments concentrated on leaf disks and, using precision micro-pipettes, on accurate placement of known virus dosages on these disks.

ii. Ranging assays to determine approximate limits of dosage-mortality relationships

Within each instar, 10 larvae within a narrow weight range were each fed with a precise dose of *HpNPV* and left for 6 hours, after which they were transferred to individual tubes containing semi-synthetic diet. Larvae were allowed to feed and were observed twice daily until death or pupation. In these ranging assays the dosages were spaced at logarithmic intervals in order to ensure that the full range of responses from 0% to 100% would be

accommodated. The approximate LD_{50} was then calculated for each instar (using probit analysis) and the value used as the central dose in the detailed assays described below.

iii. Detailed bioassays to quantify the precise relationships between dosage and mortality.

Detailed bioassays used the same methods to present the virus as for the ranging assays. In these cases the dose range employed (at least five dilutions) was much narrower (approximating 0.2 logarithm steps) and at least 30 larvae were employed per dose. At least 30 larvae taken at random from the same set of larvae were used to provide untreated controls to determine natural mortality rates. All data were subject to probit analysis, using a maximum likelihood routine to calculate the slope and associated statistics of the relationships.

4.2.2.2 Virus productivity relationships for larval stages

There was no prior information on the expected productivity of *HpNPV* PIBs per larval instar. Data for other Lepidoptera, particularly those within the family Noctuidae, indicated that yields between approximately 5×10^8 and 5×10^9 PIBs per larva could be expected when the largest instar larvae were killed by virus (Evans, 1986). Current investigations concentrated on quantifying and optimising virus yield per larva (96.1, 97.4 – Appendix I).

Third and fourth instar larvae were fed with a known lethal dose of *HpNPV* and observed until the point of death. They were then macerated individually with a known volume of distilled water. Larvae at this late stage of infection are virtually bags of virus and contain almost no intact body tissues. Maceration, therefore, yields mainly virus and it is simple to discard any insect debris, particularly the outer skin, which does not become infected. The numbers of PIBs in the remaining suspension can then be counted using either a haemocytometer or, more accurately in this situation, a dry counting procedure that allows the PIBs to be stained *in situ* with Buffalo Black (Wigley, 1980; Hunter-Fujita *et al.*, 1998). The data were used to determine optimal virus yields per mature larva for use in mass production methods.

4.2.2.3 Methods for mass production of *HpNPV*

Experiments were set up to consider how to rear *H. puera* larvae, to feed them, *en masse* or individually with virus, to determine the ideal time for harvesting and to develop efficient methods for virus extraction (96.4 – Appendix I). The latter attribute, in particular, was an important step because it had been shown that harvesting at the optimal time (on the point of death or very soon after) provided a virus preparation of high purity with very little bacterial or other contamination. The aim, therefore, was repeat this during routine harvesting so that virus purification could then be concentrated only on low speed centrifugation to remove insect debris, followed by high speed centrifugation to pellet the virus for later re-suspension in distilled water. The use of non-ionic surfactants such as sodium dodecyl sulphate (SDS) was also investigated to try and reduce or prevent clumping of the final virus suspension. Finally, storage of the virus suspensions was evaluated, which led to the purchase of a deep freeze specifically for this purpose.

4.2.3 Environmental effects

There is a considerable body of literature to indicate that baculoviruses and other microbial agents, such as *Bacillus thuringiensis* (*Bt*), are inactivated by environmental factors such as ultra violet light (uv), chemical composition of leaf exudates and, through physical losses, rainfall (Ignoffo & Garcia, 1992; Sundaram *et al.*, 1994). In particular, uv light is regarded as the most damaging environmental factor, often leading to half lives of baculoviruses or *Bt*

exposed to natural or artificial uv light, measured in hours or, at most, days. It is, therefore, important to ascertain the rate of degradation of microbial insecticides so that the need for formulation to reduce this effect can be evaluated fully (96.6, 97.3 – Appendix I).

4.2.3.1 Effects of sunlight (ultra-violet (uv) light) on viability of *HpNPV* on foliage

In the absence of laboratory uv simulators, all experiments concentrated on comparing exposure of *HpNPV* to sunlight with virus kept in shaded conditions indoors. Known quantities of virus were applied to precisely defined areas on the upper leaf surfaces of potted plants and either left in direct sunlight or in shaded conditions. Leaves were removed at intervals and the entire known dosages fed to individual *H. puera* larvae that were then fed until death or pupation. All larvae were diagnosed for cause of death. The initial experiment in 1996 was carried out over 12 days with 3-hour intervals during the first 12 hours (i.e., daylight hours), followed by 24-hour intervals. The repeat experiment during 1997 was restricted to 4 days but used 3-hour intervals during daylight hours for the first 2 days.

4.2.3.2 Effects of simulated rainfall on persistence of *HpNPV*

H. puera infestations are associated with flushing of teak leaves that are, in turn, associated with the arrival and duration of monsoon rains. Application of *HpNPV* for management of *H. puera* is, therefore, likely to take place during a period when heavy rain can be expected soon after spraying. Experiments were carried out to assess the rainfastness of known quantities ($\equiv LD_{95}$ dosage for third instar larvae) of *HpNPV* applied to the surfaces of leaves and exposed to artificial rain equivalent at intervals up to a maximum of the highest average rainfall recorded in Kerala State (210 mm in 1 hour). The virus was then assayed using third instar larvae and the results analysed using probit analysis.

4.2.3.3 Assessment of the potential effects of plant structure and chemistry on viability and persistence of *HpNPV*

There are great differences in structure between tender and mature teak leaves. This difference has a clear effect on the ability of larvae to utilise the leaves so that first and second instars are only able to feed on tender leaves. On other plants, such as cotton, leaves have been shown to exude alkaline dew that has a direct inactivating effect on baculoviruses (Elleman & Entwistle, 1985). Experiments were, therefore, carried out to test the potential of leaf chemistry on virus viability.

In the study of the effect of leaf volatiles on virus deposits on teak leaves the following experiment was set up just before dusk, in order to remove any possible uv effects.

1. PIBs of *HpNPV* were applied directly to the surface of tender leaves.
2. PIBs were applied directly to the surface of mature leaves.

PIBs were applied on the inner surface of a polypropylene cover and then,

3. Tender leaves were covered with the virus treated cover but avoiding contact between the leaf and the virus
4. Mature leaves covered with the virus treated cover, again avoiding direct contact between leaf and virus.
5. Virus was applied to an inert polypropylene cover and exposed to the air

This experimental set up was left overnight and, after 12 hours, virus PIBs were removed by washing with a known volume of 0.1% SDS, and then counted using a haemocytometer. A

total of 1000 PIBs from each sample was dispensed onto individual leaf disks and bioassays conducted using third instar larvae of teak defoliator.

4.2.4 Spray technology

During the past 20 years or so there have been considerable advances in the technology of spray application for both chemical and microbial insecticides (Matthews, 1992; Picot & Kristmanson, 1997). Foremost in this technology is ultra low volume (ulv) controlled droplet application (cda). The principles are based on producing large numbers of small droplets (usually in the range 50 μm to 150 μm diameter) while, most importantly, keeping the range of droplet sizes around the average as small as possible (known as the span of the droplet distribution). In particular, large diameter droplets, that carry disproportionately large volumes and, hence, active ingredient, are avoided. This minimises waste and reduces the probability of contamination of the environment. The very small droplets employed in ulv cda applications have a high surface to volume ratio and, if they are based on aqueous formulations, are prone to rapid evaporation and loss of droplets, especially at high temperatures. In such situations, it is advisable to employ anti-evaporant carrier fluids such as mineral or vegetable oils.

All carrier fluids must also be tested under the local conditions of temperature and humidity, concentrating on flow rates through the sprayers and on actual field performance in terms of droplet deposition on targets or directly on leaves (96.2 – Appendix I). Droplet generation and the size spectrum of the spray cloud is a specialised area that cannot be easily measured in the field and, therefore, the opportunity was taken to train Dr K Mohanadas and Mr T V Sajeev in spray technology at the International Pesticide Application Centre (IPARC), Silwood Park, UK. This internationally renowned spray technology centre has considerable expertise in application of pesticides in the tropics. Droplet spectra for the sprayers employed during this project were analysed on laser droplet equipment while at IPARC.

4.2.4.1 Development of anti-evaporant carriers for spray application

A range of vegetable oils was tested for flowability through the gravity fed sprayers to be employed in the project (Micron Sprayers Ulva+ and UlvaFan spinning disk atomisers). Tests were also carried out in the Stihl SR400 mist blower purchased for the project, although in this case a pressurised pump delivered the fluid, rather than gravity feed. The virus extracted from infected larvae was re-suspended in distilled water and, therefore, it was necessary to produce an emulsion to enable the virus to be dispersed evenly through the oil-based carrier fluid. Tests were carried out to determine the precise mixtures and surfactants necessary to produce a stable emulsion that did not result in rapid settling and/or aggregation of the viral PIBs.

4.2.4.2 Calibration of spray equipment, including flow rates, rpm, etc.

Flow rates of spray fluid (water, oil or emulsion), rpm rates (free rotation or under load) using a range of flow restrictors were tested for all spray equipment.

While calibrating the Stihl SR400 using various spray formulations, the time taken for spray out was noted and the volume of liquid sprayed was determined by subtracting the dead volume of liquid remaining in the tank from the initial volume added. The flow rates were assessed using various restrictors that determined the maximum potential flow. Flow rates were assessed for gravity feed and during use of a circulation pump that produced a constant pressure, forcing the spray liquid through the spray head. The volume of liquid delivered was measured for 60 seconds and converted to generate a flow rate per minute.

Assessment of droplet distribution during field application of spray fluid, using the Stihl 400 mistblower with Micronaire AU8000 spinning cage atomiser.

A Stihl SR400 sprayer with AU8000 was used in the field application. Walking speed (0.5 m/sec.) with sprayer on the back was determined before the actual spray was carried out. The average tree height was 5.40 meters. Keeping the spray head at a 45^{o} angle, spray was applied to the trees perpendicular to the direction walked. Leaf samples were taken from 8 trees located in the middle of the walked distance (20m) in two rows, from two levels, i.e. top and middle. One leaf from the front and back of each tree was collected, i.e. 4 leaves per tree, and observed for droplet distribution. The spray fluid was water mixed with lumogen (a water mixable fluorescent dye). The droplet distribution on the leaves was observed in a dark room in the presence of fluorescent light. To record the presence of droplets, the following scale was used for the number of droplets per cm^2.

No droplets = 0
< 5 droplets = 1
< 10 droplets = 2
> 10 droplets = 3

4.2.4.3 Assessment of droplet spectra for selected spray equipment

Droplet spectra were assessed using laser droplet analysis equipment at IPARC, Silwood Park, UK while Dr K Mohanadas (1997) and Mr T V Sajeev (1998) were on training placements in the UK. All spectra were produced on a Malvern laser analyser which produced computer printouts of size distributions in μm and also calculated volume median diameter (D[v,0.5]), span and various other parameters.

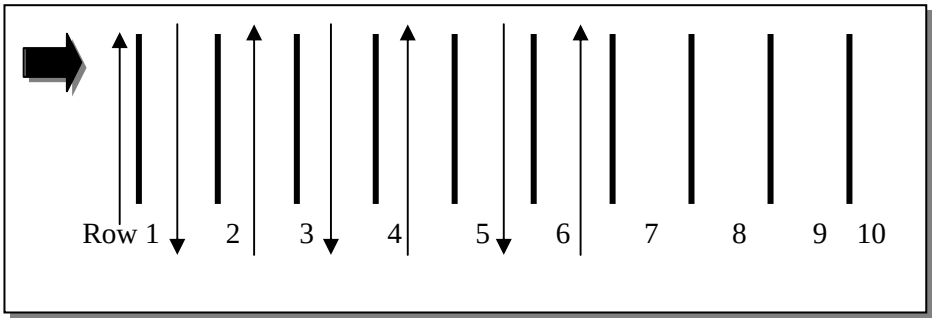
4.2.4.4 Assessment of droplet distribution during field application of spray fluid

Droplets generated by the spray equipment are too small to be visualised directly on leaf surfaces. Fluorescent particulate dye (Lumogen suspension fine ground into oil) was added to the oil emulsion at a concentration of 2.5% (vol: vol). Spraying was carried out on individual trees and also in a simulation of full incremental spraying at 2 m intervals across the prevailing wind. Leaves were removed from sprayed trees at three heights and from front to back of the tree relative to the spray head. Numbers of droplets were counted under uv light to visualise the fluorescent particles within the droplets (bright yellow under uv). In the tests of incremental sprays, samples were taken from trees at intervals downwind from the spray lanes. Examination of leaves *in situ* at several heights up the trees and throughout the plots was made at night using portable uv lamps.

Estimation of spray coverage during incremental spraying.

The layout of the plot where the estimation was made is shown in Figure 2.

Figure 2: Layout of incremental spray test plots for Ulva+ sprayer.



Spray direction

Spray path

Row of trees

Assessment of spray height achieved using the Stihl SR400 with Micronaire AU8000 spray head.

In order to assess the spray height capacity of the SR400/AU8000, a trial was carried out at Nilambur, using water with lumogen (water-soluble fluorescent dye). Restrictor No.5. was used during this spraying trial (VMD=120 μm).

Two trees of 12 m and 14.45 m height were sprayed using Restrictor No.5 for 30 seconds and 45 sec respectively, standing 2 m away from the tree base. This spray was done in the morning in still air conditions. The height to which the spray droplets reached was noted visually and, after completion of spraying, leaves were collected from various tree heights. Three pairs of leaves were taken from the bottom, middle and top sub divisions, taking account of the front and back of the tree and dorsal and ventral surfaces of the leaves. These leaves were observed under fluorescent light in a dark room. The number of droplets per cm^2 on both the ventral and dorsal sides were counted and recorded. The scoring was done using the following system.

Scale:- No spray droplets = 0
< 5 droplets/cm^2 = 1
< 10 droplets/cm^2 area = 2
> 10 droplets /m^2 area = 3

4.2.5 Calculation of field dosage rates

Data from the separate experiments were generated to provide the individual quantitative elements for the *Control Window* so that a theoretical dosage per ha could be calculated. The sequence of calculations is shown below.

4.2.5.1 Determining the theoretical dosage rate per ha to achieve >95% mortality of target larvae

Calculations of tank mix can be made using simple relationships between virus, host and droplet parameters (Evans, 1998). Although there are many other physical and biological parameters that could be included, experience has demonstrated that the predictions from these equations provide a reliable first approximation to the necessary field dosage:

N = Numbers of droplets emitted by atomiser per litre: for convenience use Volume Median Diameter (VMD) (if span is small)

CE = Capture Efficiency defined by the number of droplets required to ensure at least one droplet per host feeding area, expressed in terms of droplets per unit ground area, based on Leaf Area Index (LAI), loss to ground, etc.

Determine feeding rate (*fr)*, LD_{95} (*d*) and virus activity loss (*a*) to give initial dose D_i expressed as PIB/mm^2

Dose per ha and final concentration of the tank mix is determined by:

$$CE = (1\times10^{10})LAI\,\frac{1}{s\times fr} \text{ droplets per ha} \qquad \text{(ii)}$$

where $1x10^{10}$ = area of 1 ha in mm^2

LAI = Leaf Area Index, a multiplier to express surface area of leaves in units of ground area

s = Loss of spray fluid to non-target area

Theoretical minimum volume, $V = \frac{CE}{N}$ litres per ha (iii)

Dose per ha, $D_{ha} = CE \times D_i$ expressed in PIB/ha (iv)

Dose per litre $D_l = N \times D_i$ expressed in PIB/litre (v)

The principle of tank mix calculation can be illustrated by use of hypothetical data for the above equations. In this case the initial dosage, D_i is 1000 PIBs.

Using equation (ii), *CE* = 4 x 10^{10} droplets per ha (assume *fr* = 4 mm^2, LAI = 8, loss to non-target area = 50%)

Let *N* = 1.53 x 10^{10} droplets per litre (assume 50 μm VMD)

Let D_i = 1000 (assume d = 500, a = 0.5 (50% loss))

Using equation (iii), *V* = (4 x 10^{10}/1.53 x 10^{10}) = 2.6 litres

Using equation (iv), D_{ha} = (4 x 10^{10} x 1000) = 4 x 10^{13} PIBs per ha

Using equation (v), D_l = (1.53 x 10^{10} x 1000) = 1.53 x 10^{13} PIBs per litre

Equation (v) determines the actual tank concentration of PIBs that relates to total volume of spray fluid in the tank, including any volume attributed to formulation products.

4.2.5.2 Assessing the practical dosage per ha in terms of cost vs. projected mortality

The equations in 4.2.5.1 provide guidance on the theoretical dosage to achieve a given mortality (usually aiming at >95%) that can be achieved, taking account of the calculated parameters. However, it is often the case that the dosage calculated is much higher than is

economically viable and, thus, there may have to be compromises in setting the dose parameters. Aspects, such as mitigating factors that reduce uv losses in the field, knowledge of potential impacts of secondary inoculum arising from early mortality from the original applied dose, etc. can all reduce the required dose. This process can only be carried out after all the data are available for calculating the theoretical tank mix.

4.2.6 Trials of *HpNPV* against field populations of *H. puera*

Early data on some of the parameters, such as ranging bioassays, preliminary assessment of uv degradation and droplet capture estimates enabled a small trial to be carried out during 1996. The remaining two, more comprehensive, trials were carried out in 1997 and 1998, respectively. Low larval populations in the field during 1996 and, particularly, during 1997 had a severe effect on the planned field programme so that it was not possible to carry out the definitive trials planned originally. However, the commitment to the work at KFRI is very high and, therefore, although the project funding finished officially at the end of May 1998, a further trial was carried out during June when a suitable field population was detected.

4.2.6.1 Small scale trial at Nedungayam

A small trial was conducted at Nedungayam teak plantation in October 1996. Virus was applied using the Ulva+ sprayer on an incremental spray pattern in plots of 7 x 5 trees, each occupying an area of 140 m^2. The spray head was held at head height and spraying commenced on the downwind side of the plot. Details of the spray trial are given in Table 1. Average tree height was 2.25 m.

Table 1: Parameters for small-scale trial at Nedungayam, October 1996

Dose per plot (PIBs)	Equivalent dose per ha (PIBs)	Number of replicates
Untreated control	Untreated control	1
1.19×10^7	8.5×10^8	3
9.04×10^7	6.46×10^8	3
6.15×10^8	4.39×10^{10}	2

Larvae were collected from leaves taken at random from the centre 5 x 5 trees within the plot, classified for instar, alive or dead and smeared for the presence of virus. The larval samples represented total larval counts per tree. However, high levels of wasp predation reduced the numbers of larvae obtained in some of the plots.

4.2.6.2 Preliminary trial at Valluvassery, Nilambur

A larger scale trial, using parameters from the *Control Window*, was set up at Valluvassery, Nilambur during June 1997. Although originally planned as a complete randomised block design, the terrain and distribution of larval infestations meant that the final design was a complete randomised layout. Although this reduced the capacity to allow for blocking of the design across the study area, the distribution of plots was such that all doses were represented in all sections of the plantation, but the number of replicates in each section differed. Statistical analysis was, therefore, based on one way analysis of variance without blocking.

Control Window parameters were based on targeting the third instar stage, using dosages that included a theoretical 90% kill rate. The plots were based on a 7 x 7 tree layout and virus was applied using an Ulva+ sprayer for incremental spraying across the wind, commencing on the downwind edge of the sprayed area. Dosage and replication parameters are shown in Table 2. Sampling was carried out at 0, 48, 72 and 96 hours post spray and employed a sequential

sampling regime to remove 30 live larvae per tree removed at random from within each row of each plot, taken from the central 5 x 5 tree area. Live larvae were classified to instar and then smeared for diagnosis of virus. All dead larvae found on the leaves during sampling were collected and also assessed for presence of virus.

Table 2: Parameters for small-scale trial at Valluvassery, June 1997

Dose per ha (PIBs)	**Number of replicates**
Control (untreated)	5
5×10^{10}	5
1×10^{11}	5
2×10^{11}	5
4×10^{11}	5
8×10^{11}	5

4.2.6.3 Final trial at Valluvassery, Nilambur

Problems in identifying suitable populations of the moth meant that a second trial planned for 1997 was not possible. The final trial was, therefore, carried out after the official end of the project (which ended on 31 May 1998). The site employed was the same as that used in 1997 at Valluvassery, the trial being carried out during mid-June 1998. A randomised complete block design was used, employing the same dosages as in Table 2, but excluding the highest dose. PIBs were suspended in coconut oil emulsion prepared with 94% oil, 5% aqueous virus stock and 1% Tween80 (emulsifier). Spraying was carried out using the Ulva+ attached to a bamboo pole to take the spray head to the top height of the canopy. Spray was carried out across wind on an incremental pattern. A full destructive sampling regime was used whereby all larvae, classified as live or dead, were collected from a single tree taken at random per row per plot. Samples were carried out at 0, 48, 72 and 96 hours post spray. Larvae were stored at -20^{o}C until they were diagnosed, by smearing, for the presence of virus. Shortage of time and resources meant that only 25% of all live larvae collected were smeared. A visual assessment of damage, expressed as % loss of leaf surface and measurement of leaf loss by leaf weight over time post spray were taken to assess the potential damage reduction from application of the virus.

5. Outputs

5.1 *Moth population monitoring.*

5.1.1 Light trap assessments

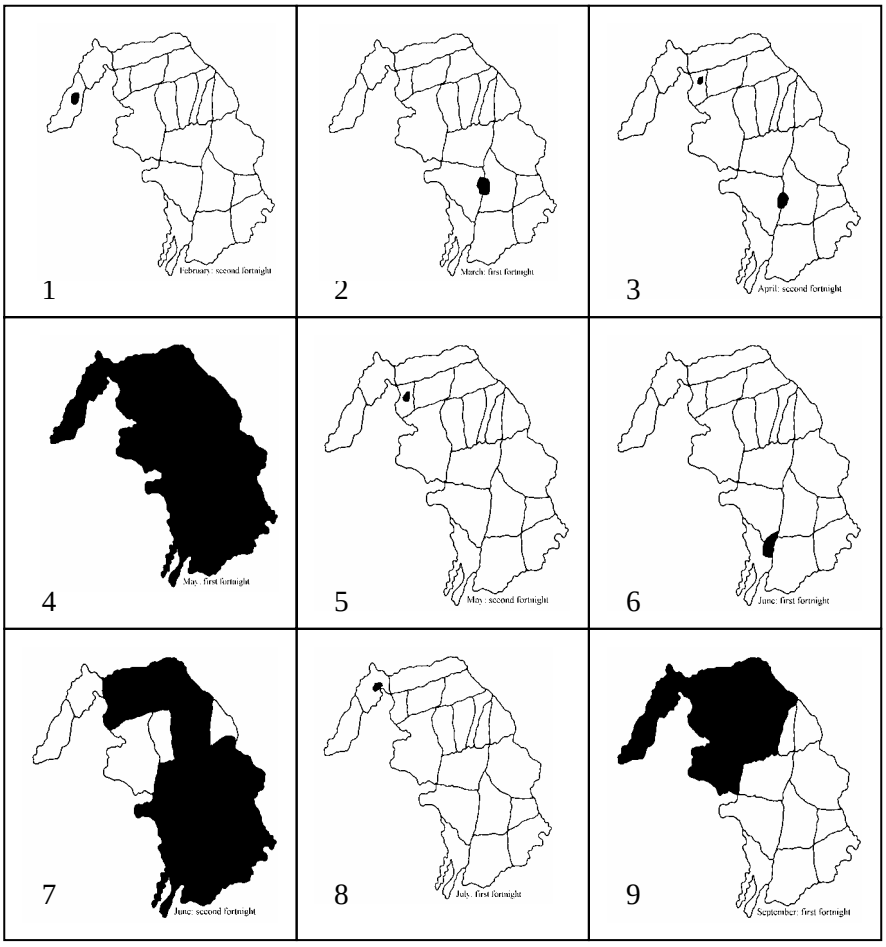
Solar powered light traps have been constructed and installed at key locations in the main infestation areas of Nilambur, Kunnamkulam, Kottayam and Konni. These have been used to monitor adult flight activity to provide early warning of potential infestations. This is being supplemented by larval monitoring carried out by local personnel trained for the purpose. This is carried out at fortnightly intervals to provide a reliable picture of local moth population build-up and to aid tracking of population movements between locations.

5.1.2 Establishment of a survey network

Data from the detailed survey network have been entered on a database and used to generate maps of the infestations over time. These are described below.

Infestations in 1995

Figure 3: Sequence of teak defoliator outbreaks at Kariem Muriem in 1995



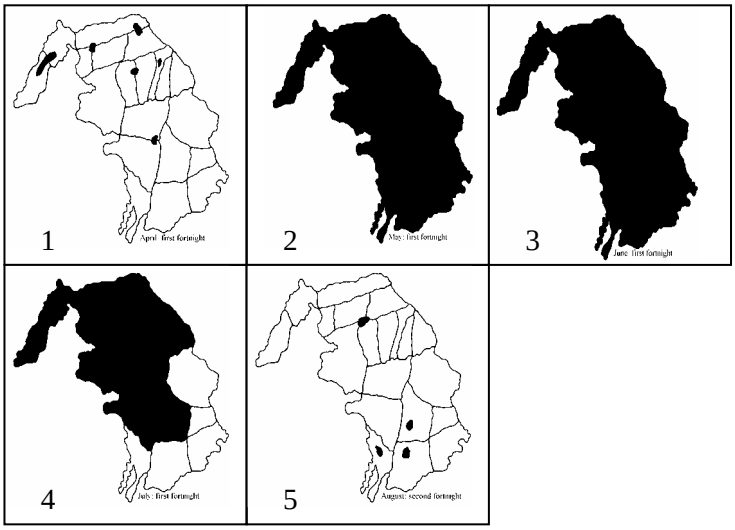
The sequence of defoliator infestations during 1995 is shown in Figure 3. The first visible defoliation occurred at a small patch in the second fortnight of February. Subsequent small patch infestations occurred during March (first fortnight) and April (second fortnight). During the first fortnight of May, there was a major outbreak covering the entire area. After this, only two small patches were defoliated, one during the second fortnight of May and the other during the first fortnight of June. The outbreak, which occurred during the second half of June, was widespread. A small area was infested in July and in September the third widespread outbreak occurred in almost all the northern Observation Units in the study area.

Thus, there were nine distinct infestations during the year, of which three were widespread.

Infestations in 1996.

The first infestations during the year were found in six small patches during the first fortnight of April (Figure 4). Following this, the entire area was infested during May and June. In July, the infestation was widespread. The last infestations of the year were the four small patches, which occurred during August. Overall there were five distinct infestations, of which three were widespread.

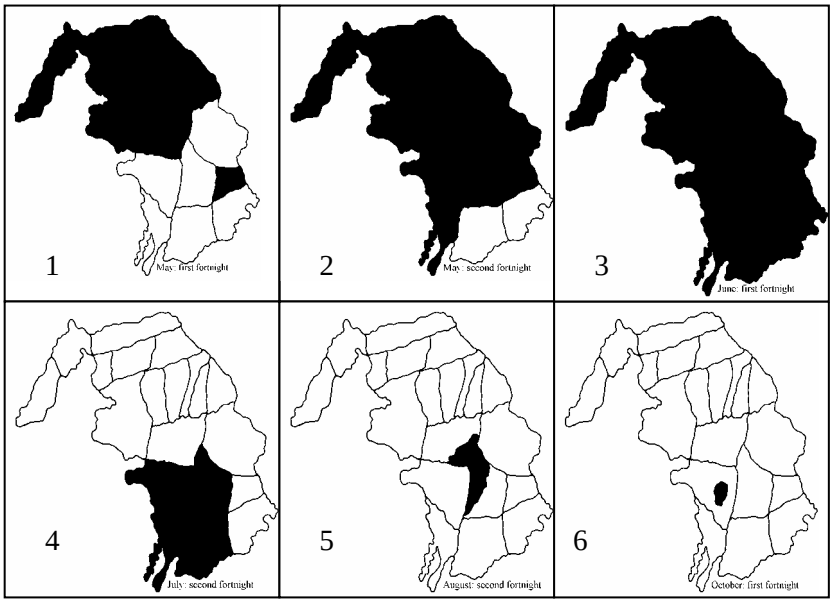
Figure 4: Sequence of teak defoliator outbreaks at Kariem Muriem in 1996



Infestations in 1997

In contrast to the early two years in which the initial infestations were confined to small patches, in 1997, the very first outbreak was widespread (Figure 5). There were two distinct patches, which occurred during the first fortnight of May. The second and third infestations, which occurred during May and June, were more widespread, covering almost the entire area. A major outbreak, confined to the southern Observation Units, occurred during July. During August and October, the infestations were confined to smaller areas. In summary, there were six infestations, of which four were widespread.

Figure 5: Sequence of teak defoliator outbreaks at Kariem Muriem in 1997



The pattern of infestations described above shows that, although the majority of them were distributed over minor areas, there were several widespread infestations during the period May – September. There were thus three widespread infestations during each year surveyed. However, although widely distributed, the populations were numerically too low for use in repeat field trials, with the exception of the trial described in section 4.2.6.1.

5.2 *Baculovirus production and field application.*

5.2.1 The Host

5.2.1.1 Growth rates, including weight relationships

The rates of development of larvae are remarkably constant and take an average of 2 days for each stage, including the final instar prior to pupation. Average weights and head capsule widths (an accurate indicator of larval stage) are given in Table 3. Mean weights were used for assessment of the relationships of lethal dosage to larval development.

Table 3: Mean weights and head capsule widths for *Hyblaea puera* larvae

Instar	**Mean weight**	**Head capsule width +/- SD (mm)**
I	0.1 mg	0.334 +/- 0.018
II	2.0 mg	0.590 +/- 0.032
III	9.0 - 12.0 mg	1.007 +/- 0.006
IV	27.0 - 36.0 mg	1.673 +/- 0.106
V	80.0 - 110.0 mg	2.622 +/- 0.170

5.2.1.2 Larval distribution on the host tree

The dispersion of immature stages of *H. puera* within the teak tree crown was examined by Dr K Mohanadas (PhD study). The aspects looked into were (i) the within-shoot dispersion of eggs and larvae, (ii) the age structure of populations of immature stages and (iii) the distribution of different instars within the tree crown.

The following is a summary of findings:

1. In general, eggs are laid only on tender leaves, and the larvae feed preferentially on the 1st to 3rd pairs of leaves. Neonate (newly hatched) larvae do not survive on older leaves.

2. High-density infestations are characterised by the dominance of one developmental stage at any given time and there is no overlap of generations. However, low-density populations have a mixed age structure.

3. Although it was suspected that early instars might concentrate in the upper tree crown and late instars in the lower crown, no preferences were noticed in this study, all instars being present at all levels of the crown.

5.2.1.3 Larval feeding rates over time

Results of larval feeding tests are shown in Table 4.

Table 4: Leaf area consumed in 6 hours by each *Hyblaea puera* larval instar

Instar	**Leaf area consumed (mm^2)**
I	2.0
II	12.0
III	18.0
IV	200
V	300

The laboratory tests provided accurate descriptions of the average leaf area consumption for each instar during a six-hour period. The results were used to assess the required coverage per unit area of droplet applications for use in the *Control Window*.

5.2.2 The Virus

5.2.2.1 Dosage-mortality relationships for larval stages

Following a series of ranging bioassays that were used to develop methodology and to determine the approximate LD_{50} and slopes of the relationships, detailed assays were carried out for all five larval instars. The results were analysed using Probit analysis and are presented in Table 5 and Figure 6.

Table 5: Calculated LD_{50} values and associated statistics for instars I to V of *H. puera* in relation to dosage of nucleopolyhedrosis virus.

Instar	LD_{50} (PIBs)	95% fiducial limits (PIBs)	Slope	Intercept
I	17	*	1.09	-.83
II	70.2	*	1.07	-1.48
III	72.7	25-271	1.10	-1.55
IV	3932	2810-6157	1.17	-3.71
V	20125	9206-34488	1.26	-4.94

There are difficulties in methodology in applying doses to first and second instar larvae, arising from their small size and high sensitivity to virus. In these cases the variability was too high to calculate 95% fiducial limits. However, because the slopes were not significantly different (chi-square 1.31, df = 4) it was possible to calculate LD_{50} and associated statistics for the common slope, using all data combined. The results are shown in Table 6. Here the effect has been to increase the limits for instars III to V, but has also enabled fiducial limits to be calculated for instars I and II, in all cases without substantially altering the calculated LD_{50}s.

Table 6: LD_{50} and associated statistics for instars I to V of *H. puera* exposed to nucleopolyhedrosis virus based on a common slope for all instars.

Instar	LD_{50} (PIBs)	95% fiducial limits (PIBs)	Common slope value
I	17.7	8.5-43.0	1.14
II	74.3	39.3-130.7	
III	73.3	39.0-135.6	
IV	3936	2168-7165	
V	18673	8323-39398	

Figure 6 illustrates how the responses of larvae to dosage of virus decrease with larval age and emphasises that the differences in response are a function of age rather than any major change in the type of response. In other words, the parallel slopes indicate that the population as a whole responds in a similar way to virus dosage but the absolute dosage is determined by some factor related to larval age. In fact, the principal factor is larval weight, as has been found for many other lepidopteran virus-host interactions (Evans, 1986). This is illustrated in Figure 7 in which both LD_{50} and LD_{50}/mg body weight are plotted against body weight. Although the surprising similarity of dosage between the second and third instar larvae introduces variability, it is clear that weight accounts for the majority of the variation in observed LD_{50} with larval age.

Figure 6: The relationships between Log dosage of nucleopolyhedrosis PIBs and mortality (in probits) of the five larval instars of *H. puera*.

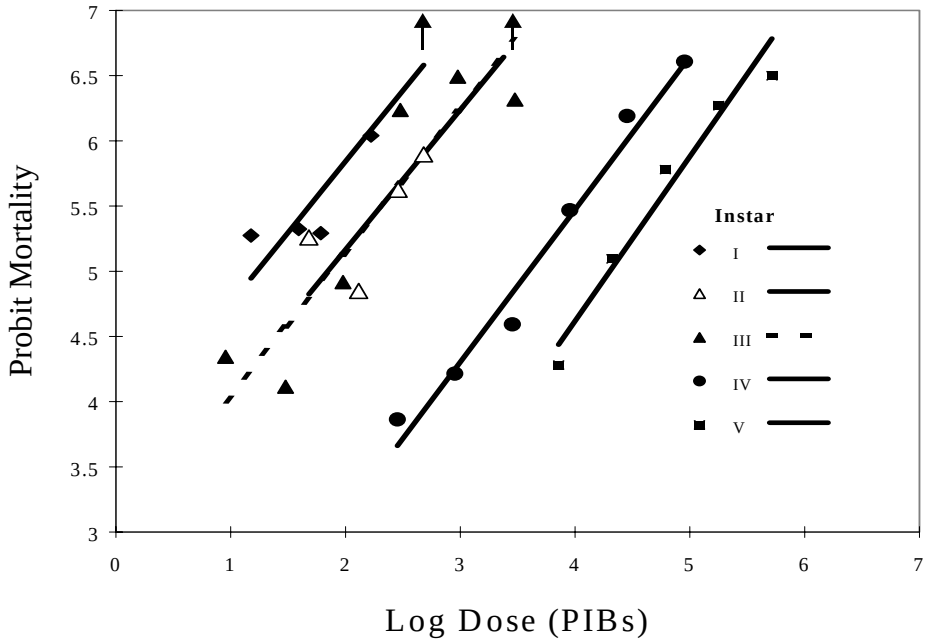
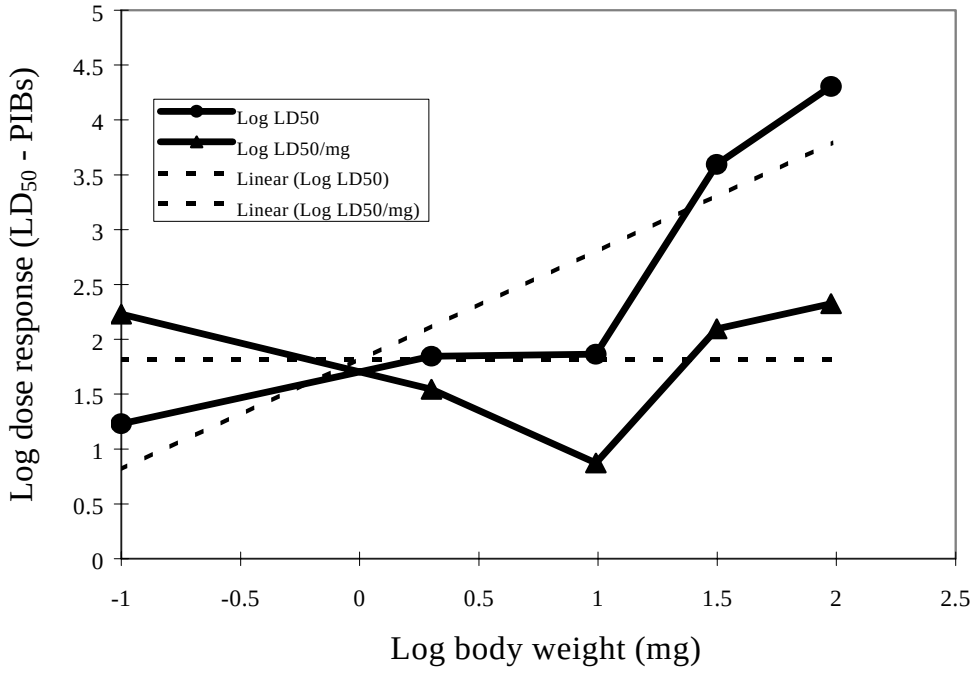


Figure 7 The relationships between Log LD_{50} and Log LD_{50}/mg and body weight of *H. puera* larvae in relation to infection by nucleopolyhedrosis.

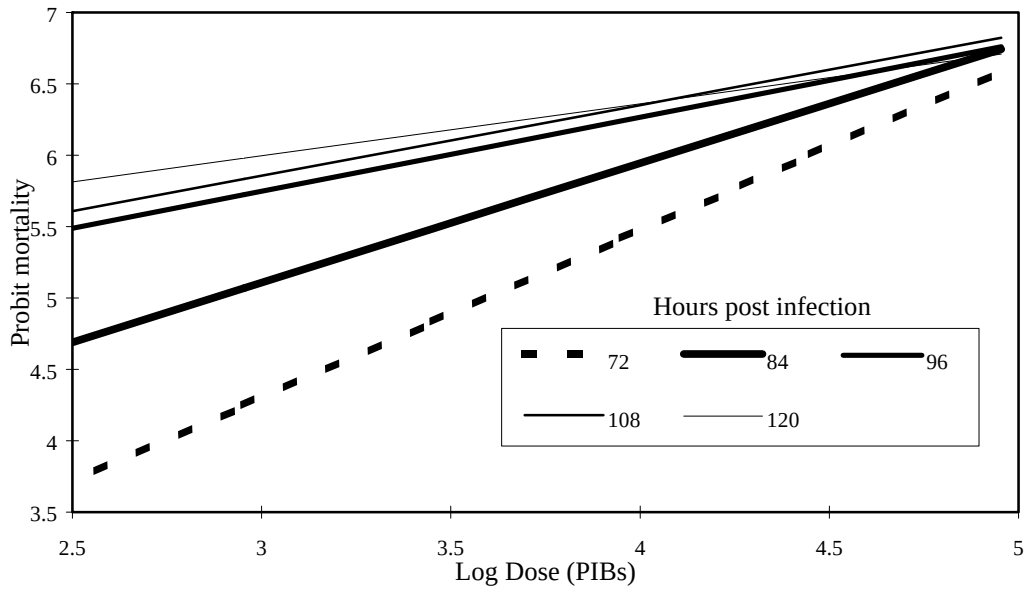


In relation to targeting of larvae in the field, the virtually identical response of second and third instar larvae was a favourable result in determining field dosage. The third instar is a much easier target than the first or second instars for a number of reasons; it eats more foliage

and, thus, has a greater likelihood of taking in a virus dose, it is more open in its feeding habits and is capable of feeding on older foliage. Field dosage rates were, therefore, based on Control Window parameters for the third instar, although some preliminary trials were also carried out on populations that were predominantly in the first, most susceptible, instar.

Responses for the fourth instar were extremely unusual. The data presented above incorporated responses up to 72 hours post infection, a period sufficient for the great majority of larvae to have become infected and to die. Up to this stage the population response is virtually identical to other instars (parallel slopes for the dosage-mortality regression lines). However, if the fourth instar larvae were left for longer periods, mortality at lower dosages became significant, finally resulting in high mortality at even the lowest dose tested. Contamination of the larvae was ruled out by the demonstration of no virus mortality in the untreated control larvae taken from the same stock of insects. Dosages and dilutions were also checked carefully to eliminate the possibility of incorrect administration of PIBs. The results over time are illustrated in Figure 8.

Figure 8: The change in response of fourth instar *H. puera* larvae with time post infection. Note the progressive flattening of the slope as infection increases even with low dosages.



This response was noted in a number of repeat bioassays and was recorded only in the fourth instar. Although this result might be predicted from the theory that only a single virus particle is required for infection, it is, nevertheless, extremely unusual to have more mature larvae dying at such low dosages. However, assuming that the same response applies in the field, it does imply that very low dosages of virus will be sufficient to kill a significant proportion of fourth instar larvae remaining on the trees and, hence, will enhance the impact of any virus applied for management of the moth.

5.2.2.2 Virus productivity relationships for larval stages

Although initial work carried out during the first year of the project provided base-line data on virus productivity, it was not possible to complete this aspect of the work as planned. This was due to difficulties in obtaining sufficient numbers of larvae in both laboratory and field and the need to place priority on mass virus production for field trial use. However, the initial

data indicated that yields could be improved through more efficient harvesting and that virus productivity in mature larvae was sufficiently high to justify further efforts to optimise production systems.

Data on basic virus productivity per larva are shown in Table 7.

Table 7: Summary of virus yields achieved during virus production in preliminary trials of virus production systems.

	Trial 1	Trial 2	Trial 3
No. of dead larvae retrieved	19206	42151	35600
Approx. larval equivalent of NPV (PIBs)	6.2×10^5	5.7×10^4	1.1×10^8
Approx. available quantity of NPV (PIBs)	1.2×10^{10}	2.4×10^9	3.91×10^{12}

The increase in yield per larva (larval equivalent – LE) was encouraging and was confirmed in experiments in which individual larvae were assessed to determine maximum yield as a target for final productivity. This demonstrated that up to 7×10^8 PIBs could be produced. It was therefore, decided that a mass production target yield of 3×10^8 PIBs per larva would be a realistic target.

5.2.2.3 Methods for mass production of *HpNPV*

Arising from the results above, a method for mass production of *HpNPV* was developed and standardised within the study period, although there is still room for further development and scale-up.

Materials required:

NPV stock
Teak defoliator larvae in the early fifth instar stage
Tender leaves of teak
Semi-synthetic larval diet
Plastic tubes (10cm x 2.5cm) with perforated cap
Micropipettes (0.5 -10µl),
Camel brushes
Scissors,
Glass plate (60cm x 60cm).

Larval sources: Teak defoliator larvae reared on artificial diet or healthy larvae collected from the field. Fifth instar larvae are preferred for virus production because they maximise virus yield in relation to inoculum used to induce infection.

Inoculum feed

1. 2 ml of semi-synthetic larval diet dispensed in plastic tubes at least 3-4 hours before inoculation.

2. Leaf discs (2 cm x 2 cm) prepared from tender teak leaves placed on a sterilized glass plate with sufficient space in between to prevent touching of the leaves.

3. Using a micropipette, 2µl of NPV stock at a concentration of 5 x 10^8 PIBs/ml is spotted over each leaf disc, to obtain a deposit of 1 x 10^6 PIBs/leaf disc. The leaf disc is left for some time until the virus deposit is dry.

4. Individual leaf discs are then transferred to the rearing tube and placed over the diet.

5. Individual early fifth instar larvae are then introduced into each plastic tube. The Larva first feeds on the leaf disc containing the virus and, after completely consuming the leaf, switches to feeding on the diet.

Incubation and retrieval of the dead larvae

1. Larvae that have died within 48 hours of inoculation are discarded, as the death could be due to bacterial infection.

2. Larvae that die between 48 and 96 hours post inoculation are collected and transferred to a deepfreeze where they are maintained at –20^{o}C.

3. After 108 hours all remaining larvae, both dead and live are retrieved and transferred to the deep freeze.

Harvest of virus

1. The frozen larvae are macerated in a pestle and mortar with chilled 0.1% SDS solution.

2. After maceration, the virus suspension is filtered using a muslin cloth to remove coarse insect debris. The filtrate is again filtered, this time using muslin cloth folded into three layers.

3. The virus suspension is centrifuged at 8000 rpm for 20 minutes to produce a pellet, composed mainly of virus PIBs. The supernatant is removed and discarded.

4. The pellet is again suspended in chilled distilled water, and centrifuged at 1000 rpm for 5 minutes. The pellet containing any remaining impurities is removed and the supernatant, containing virus PIBs, retrieved and stored.

Purification of virus

1. The semi-purified virus stock is further purified by ultra centrifugation in a sucrose gradient comprising 48%(w/w), 51%(w/w), 54%(w/w), 57%(w/w) and 60%(w/w) of sucrose solutions layered (6ml from each stock) into a Sorvall centrifuge tube using a disposable syringe (5ml). The prepared sucrose gradient is kept at 4^{o}C overnight to stabilise the sucrose across the range of concentrations.

2. 3 ml of the polyhedral preparation are layered on top of the gradient centrifuged in AH627 swinging bucket rotors in a Sorvall ultra centrifuge at 25000 rpm for 40 minutes.

3. The *HpNPV* forms a band in the region of 52 to 54% of sucrose concentration. The band is collected with a syringe and needle.

4. The PIBs suspended in sucrose solution are diluted in distilled water and pelleted at 8000 rpm to remove the sucrose. The pellet of PIBs can be re-suspended in distilled water. This procedure is repeated once more to remove any remaining residues of sucrose.

Merits of the method:

Freezing of the dead larvae and the virus stock minimises bacterial contamination of the virus suspension. Similarly elimination of dead larvae prior to 48 hours post inoculation also avoids bacterial contamination because the premature death of such larvae could be due to bacteria.

Drawbacks of the method:

Since the larvae are not allowed to putrefy, the PIBs are not always completely released from the nuclear groups within the infected cells, resulting in reduced yield of free virus. Clumping of the inclusion bodies may also be high.

Summary of virus yield data

The virus yield, based on semi-purified stock, from 5 trials carried out as per the above methods is given in Table 8 below.

Table 8: Results of experiments to determine virus yield using the standard virus production protocols.

Trial No.	Dosage	No. of larvae used	Instar	No. of virus infected larvae retrieved +	Total yield	Larval equivalent
1	$5x10^5$	330	V	311	1.28×10^{11}	$4.115x10^8$
2	$1x10^6$	1558	V	1340	2.128×10^{11}	$1.559x10^8$
3	$1x10^6$	822	IV	659	1.344×10^{11}	$2.0394x10^8$
4	$1x10^6$	769	IV, V	628	8.096×10^{10}	$1.289x10^8$
5	$5x10^5$	228	V	198	1.6×10^{10}	$8.08x10^7$
					Mean larval equivalent	$1.9621x10^8$

+ larvae dead before the required incubation period discarded.

Comparison with early methods of mass production of virus

Method 1 (1995-96 trials): Group rearing of field-collected fourth instar larvae fed on whole leaves treated with virus in a yard. This method was abandoned due to high risk of contamination and virus wastage at the time of inoculation and difficulties in retrieving the dead larvae. Required high labour.

Method 2 (1996 -97 trials): Individual rearing of larvae on virus treated leaf disc and then rearing on artificial diet until death. This method was abandoned and switched to the present method (1997 trials) which is more advantageous – it saves labour and minimises the risk of contamination.

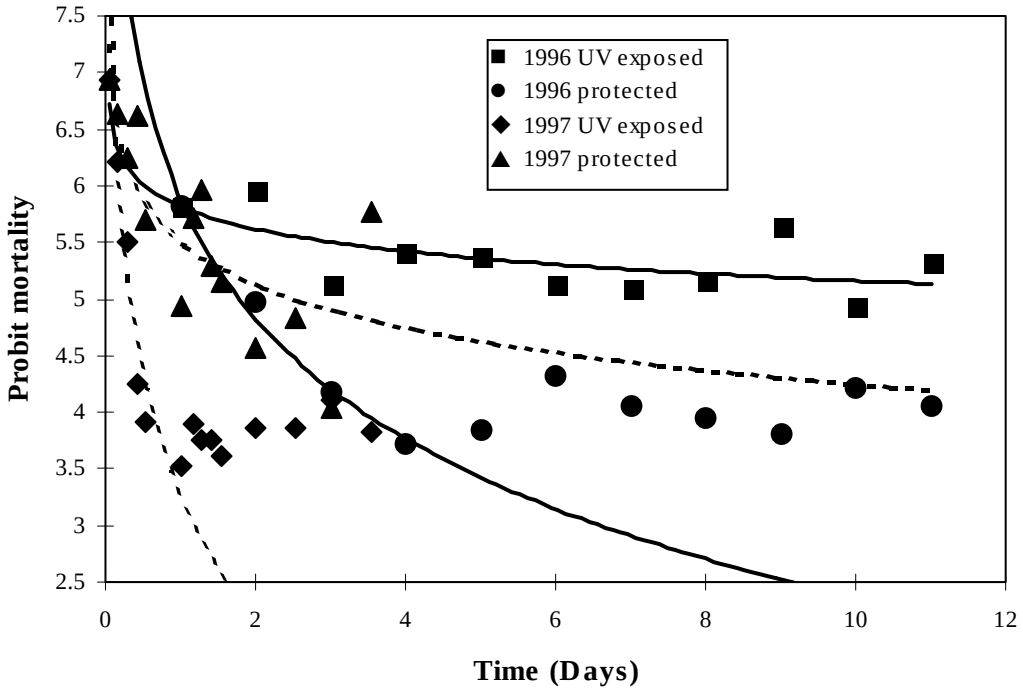
5.2.3 Environmental effects

The key elements that are known to lead to degradation of virus in the field are uv light, rainfall and possible effects from the host plant (Evans & Harrap, 1982). Results from this part of the study are important in determining loss rates during the vital first six hours after spray application in the field.

5.2.3.1 Effects of sunlight (ultra-violet (uv) light) on viability of *HpNPV* on foliage

The results of the two series of experiments, carried out in 1996 and repeated in 1997, are combined in Figure 9 which shows the rates of decay of virus when exposed to uv and when protected in virtual darkness. The patterns of decay are similar for both series of experiments, showing that sunlight has a dramatic early effect on virus survival so that mortality dropped from an initial 97% to around 70% (probit 5.5) in six hours. This is not unexpected and represents the maximum that would be experienced in the field, considering that the experiments were carried out in full sunlight with no protection for other leaves or from being on the underside of leaves.

Figure 9: The effects of exposure to ultra violet light on activity of *HpNPV* assessed by bioassay against third instar larvae.



The rate of decay continued on a predicted line as indicated in Figure 9 but, unexpectedly, stabilised at approximately 13% mortality (probit 3.9) for the remaining period of the experiment. This was repeated in both years. There are several possible explanations for this result, although it was not possible to carry out experiments to verify the hypotheses:

- Presence of a small amount of insect debris may have afforded some protection against uv. The fact that the virus was virtually free of debris tends to rule out this explanation.
- The period of uv exposure is restricted to around 12 hours each day and, therefore, the virus is not continually exposed to harmful wavelengths of light.
- It is known that baculovirus DNA can repair itself after exposure to uv light and it is possible that the 12 hours of darkness in each 24-hour period may enable the virus to retain a base-line activity level.
- There may be some characteristic of teak leaves that provides protection against loss of virus activity, although this is not suggested by the results in section 5.2.3.3.

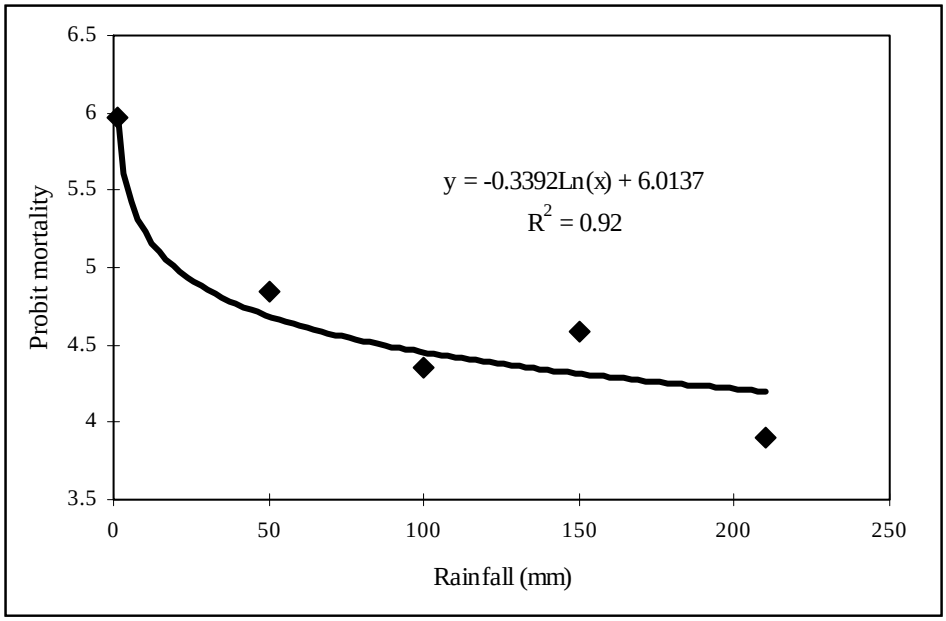
Overall, therefore, retention of activity over an 11-day period under direct exposure to tropical sunlight is an extremely surprising result that warrants further study. However, the demonstration of such an effect indicates that *HpNPV* has a remarkable capacity to remain

viable despite apparently adverse conditions, increasing its potential as a pest management tool.

5.2.3.2 Effects of simulated rainfall on persistence of *HpNPV*

The linking of *H. puera* population dynamics to the presence of monsoon rains poses the obvious question of whether *HpNPV* can remain on the leaves during periods of high rainfall. Results from experiments to test this question are presented in Figure 10, where proportionate mortality has been transformed to probits.

Figure 10: The effects of simulated rainfall on persistence of *HpNPV* on teak leaves, measured by bioassay against third instar larvae



The highest rainfall results in a decline from over 90% mortality to approximately 15% mortality, indicating that under extremes of rainfall, losses of virus can be dramatic. However, these data represent the situation when virus is fully exposed on the upper surfaces of leaves. In nature, virus infected larvae tend to be found on the undersides of leaves and are, at least to some extent, protected from the direct effects of monsoon rains. Nevertheless, there is likely to be some loss of applied virus if heavy rainfall is experienced within six hours of spray application, a factor that must be considered in timing spray applications.

5.2.3.3 Assessment of the potential effects of plant structure and chemistry on viability and persistence of *HpNPV*

The results of the experiment are presented in Table 9, which indicates that there is no effect of either leaf surfaces or of leaf volatiles on *HpNPV*. This possibility can, therefore, be discounted in determining field persistence of virus applied for control of teak defoliator moth.

Table 9: The effects of leaf surfaces and leaf volatiles on persistence of *H. puera* NPV

Type of leaf	Virus applied on leaf surface			Virus exposed to leaf volatiles on inert surface			Control		
	larvae	dead	% mortality	larvae	dead	% mortality	larvae	dead	% mortality
Mature leaves	47	46	97.9%	53	52	98.1%	50	3	6.0%
Tender leaves	50	48	96.0%	50	46	92.0%	65	5	7.7%

Virus applied to inert surface of polypropylene cover					
Virus treated			Control		
larvae	dead	% mortality	larvae	dead	% mortality
39	32	82.1%	45	2	4.4%

5.2.4 Spray technology

5.2.4.1 Development of anti-evaporant carriers for spray application

Tests of vegetable oils locally obtainable in Kerala were carried out in relation to flow rates and ability to form stable emulsions with water and an emulsifier. It was found, by trial and error, that stable emulsions could be formed with proportions of 94% oil, 5% water and 1% emulsifier. Both Labolein, a commercial detergent preparation, and Tween80, a non-ionic detergent, proved to be suitable emulsifiers. Flow rates of oil alone and oil emulsions were greater using coconut oil compared with sunflower oil. It was, therefore, decided to use coconut oil for all field spray applications.

5.2.4.2 Calibration of spray equipment, including flow rates, rpm, etc.

Many tests were carried out to assess flow rates for both Ulva+ and Stihl SR400 sprayers, the former under gravity feed and the latter under both gravity and pump feed. Average RPM rates varied with the power applied to the respective sprayers. For the Ulva+ the revolution rates varied with voltage as per the manufacturer's specifications. However, the trials were carried out with the maximum 12 v setting, delivering 11000 rpm (see section 5.2.4.3). The Stihl SR400 airflow through the Micronaire AU8000 spray head delivered up to 13000 rpm at maximum throttle settings.

Experiments to assess flow rates under both gravity feed and pump-assisted conditions were carried out for the SR400 sprayer and are presented in Table 12. As expected, the flow rates are considerably greater when assisted by the pressure pump within the SR400. Flow for oil alone or the oil emulsion was slower than for water alone, a result that is compatible with other data on gravity feed for the Ulva+ sprayer.

5.2.4.3 Assessment of droplet spectra for selected spray equipment

The majority of field trials were carried out with the Ulva+ sprayer and, therefore, data on droplet spectra obtained at IPARC, Silwood Park, using the Malvern laser droplet analysis equipment are reproduced in Table 10. Results indicate that droplet diameter drops with increases in rpm. The maximum rpm rate for the Ulva+ produces droplets with volume median diameter of 55 μm, providing large numbers of droplets per litre of spray fluid and improvements in droplet coverage on the foliage.

Table 10: The relationships between rpm and volume median diameter for Ulva+ sprayer with oil emulsion carrier. Data obtained on Malvern laser droplet analyser at IPARC, UK

Revolution rate (rpm)	Droplet volume median diameter (μm)
3000	116.49
4000	104.42
5000	92.74
6000	81.96
7000	74.35
8000	71.82
9000	62.01
10000	60.01
11000	55.00

5.2.4.4 Assessment of droplet distribution during field application of spray fluid

Table 11 shows the spray coverage in a young teak plantation (average tree height 5.40 m) using a Stihl SR400 sprayer with Micronaire AU8000 rotating cage atomiser.

Table 11: Droplet distribution (score) for Stihl SR400 with Micronaire AU8000 atomiser.

Row No. 1.				
Tree No.	Tree level			
	Top		Middle	
	Front	Back	Front	Back
1	1	1	3	3
2	1	0	3	0
3	0	0	0	0
4	0	0	0	0
Row No. 2				
1	1	1	1	1
2	0	1	3	3
3	1	0	1	1
4	0	1	1	0

High foliar coverage (score 3; > 10 droplets/cm^2) was achieved in the middle layers of trees in the first and second rows from the spray head, but few droplets penetrated horizontally to rows 3 and 4. Therefore, despite the velocity applied to the droplets, it would still be necessary to spray at least every other row in a full-scale application.

Table 12: Calibration of Stihl SR400 sprayer under a range of conditions and carrier fluids.

	Water alone				Coconut oil alone				Emulsion			
	Engine				Engine				Engine			
	Off		On		Off		On		Off		On	
Restrictor No.	Ml/ min	Time for 1 litre flow out Min.Sec	Time for 1 litre flow out Min.Sec	Ml/min	Ml/min	Time for 1 litre flow out Min.Sec	Time for 1 litre flow out Min.Sec	Ml/min	Ml/min	Time for 1 litre flow out Min.Sec	Time for 1 litre flow out Min.Sec	Ml/min
1	32.2	36.25	6.20	157.9	4.0	252.7	9.4	110.29	6.5	167.8	9.43	102.0
2	67.0	17.45	2.2	491.8	12.5	84.5	5.7	195.44	14.0	76.1	4.34	219.0
3	145.0	7.48	1.21	740.7	40.0	25.5	4.1	249.94	46.0	21.5	3.1	331.49
4	244.0	5.10	1.12	833.3	49.8	21.7	3.2	329.67	40.0	25.8	2.7	472.4
5	337.0	4.47	1.5	923.1	54.0	19.3	1.3	645.16	64.0	17.9	1.15	798.0

Incremental spraying with the Ulva+ sprayer.

The results of the incremental spray trials are shown in Table 13 where the numbers of droplets/cm^2 on leaves in various positions on the tree are recorded. The results indicate that average coverage is good, the incremental spraying building up from rows 1 to 3, after which is remains remarkably constant until the final two rows, neither of which was sprayed (as is standard practice in incremental spraying).

The results, therefore, indicate that good coverage of foliage can be obtained using the Ulva+ sprayer and coconut oil emulsion as the carrier. In fact, the coverage obtained was greater than required to ensure that third instar larvae obtained a suitable number of droplets during their six hour feeding periods, as defined in the *Control Window*.

Table 13: Numbers of droplets captured during incremental spraying with the Ulva+ sprayer.

Row No.	Level	Droplets/cm^2	Droplets/cm^2
1	Top	21.65	7.95
	Middle	2.22	
	Bottom	0.00	
2	Top	0.27	16.95
	Middle	34.20	
	Bottom	16.39	
3	Top	0.00	20.11
	Middle	37.90	
	Bottom	22.45	
4	Top	3.01	10.29
	Middle	4.61	
	Bottom	23.25	
5	Top	3.81	19.63
	Middle	17.19	
	Bottom	37.90	
6	Top	17.64	31.14
	Middle	37.90	
	Bottom	37.90	
7	Top	23.25	22.98
	Middle	23.25	
	Bottom	22.45	
8	Top	6.21	18.79
	Middle	23.25	
	Bottom	26.91	
9	Top	2.46	7.40
	Middle	13.53	
	Bottom	6.21	
10	Top	2.22	8.54
	Middle	19.59	
	Bottom	3.81	

Assessment of droplet distribution during field application of spray fluid at Nilambur to assess the height reached by the spray droplets.

These tests were carried out using the Stihl SR400 mistblower that has a manufacturer's recommended maximum spray height of approximately 12 m. The trials were carried out using the booster pump to ensure even flow of spray fluid through the AU8000 spray head. The first trial was carried out on trees 12 m tall, as indicated below and reported in Table 14. The second trial, on trees 14.45 m tall is reported in Table 15.

Table 14: Numbers of droplets (score) captured at different tree heights when using a SR400/AU8000 spray system to spray trees 12 m tall (Restrictor 5, 30 secs).

Height level of tree	Front		Back	
	Sides of the leaves observed			
	Ventral	Dorsal	Ventral	Dorsal
11.5 m	0	0	1	0
10.0 m	0	0	1	0
9.0 m	3	3	1	1
8.0 m	1	0	2	0

Table 15: Numbers of droplets (score) captured at different tree heights when using a SR400/AU8000 spray system when used to spray trees 14.45 m tall (Restrictor 5, 45 secs).

Height level of tree	Front		Back	
	Sides of the leaves observed			
	Ventral	Dorsal	Ventral	Dorsal
14:15 m	1	0	0	1
13.0 m	0	0	0	0
12.0	1	0	2	2
9.0 m	3	2	1	1

Note: Scores used to record the droplet numbers per cm^2 area.

No. Droplet = 0
< 5 droplets = 1
< 10 droplets = 2
> 10 droplets = 3

Spray coverage obtained with the Ulva+ sprayer

Estimation of spray coverage on individual trees.

Date of spray	11-04-1996
Place of spraying	Kariem Muriem
Duration of spray	5 seconds
Wind speed	9.6 km/hour
Height of spray head from ground	9 metres
Sprayer	Ulva+ (with 8 cells)
RPM	11000
Nozzle	Pink
Spray fluid	Coconut oil emulsion with suspended fluorescent dye

The spray coverage obtained on three trees, which were at three different distances from the spray head, was estimated. The details are given in Table 16, Table 17 and Table 18.

Table 16: Droplet capture on trees 9 m from the spray head, using the Ulva+ spray system.

Height of the sampled shoot	Position of the sampled shoot	Leaf pair	Surface	Mean droplets/cm^2
8.5 metres	Front	1	upper	2.0
			lower	15.4
		2	upper	5.85
			lower	4.35
		3	upper	10.05
			lower	2.45
	Back	1	upper	0.0
			lower	0.0
		2	upper	0.15
			lower	1.15
		3	upper	2.0
			lower	0.3
5.5 metres	Front	1	upper	0.75
			lower	10.2
		2	upper	4.95
			lower	0.3
	Back	1	upper	0.8
			lower	4.2
		2	upper	5.95
			lower	1.9
		3	upper	2.25
			lower	4.5
2.5 metres	Front	1	upper	0.1
			lower	0.0
		2	upper	0.0
			lower	0.05
	Back	1	upper	0.0
			lower	0.0
		2	upper	0.02
			lower	0.0
		3	upper	1.8
			lower	0.0

Results indicated that adequate coverage was obtained only on the trees closest to the sprayer, confirming that incremental spraying is necessary to achieve full coverage during operational spraying.

Table 17: Droplet capture on trees 15 m from the spray head, using the Ulva+ spray system.

Height of the sampled shoot	Position of the sampled shoot	Leaf pair	Surface	Mean droplets/cm^2
7.5 metres	Front	1	upper	0.0
			lower	0.0
		2	upper	0.45
			lower	0.05
		3	upper	0.0
			lower	0.0
	Back	1	upper	0.1
			lower	0.0
		2	upper	0.0
			lower	0.0
		3	upper	0.1
			lower	0.0
		4	upper	0.0
			lower	0.0
4 metres	Front	1	upper	0.45
			lower	0.05
		2	upper	0.4
			lower	0.0
		3	upper	0.0
			lower	0.0
	Back	1	upper	0.2
			lower	0.0
		2	upper	0.6
			lower	0.0
		3	upper	0.0
			lower	0.0

Table 18: Droplet capture on trees 21 m from the spray head, using the Ulva+ spray system.

Height of the sampled shoot	Position of the sampled shoot	Leaf pair	Mean droplets/cm^2
8.5 metres	Front	1	0.0
		2	0.0
		3	0.0
	Back	1	0.075
		2	0.025
		3	0.0
5.5 metres	Front	1	0.0
		2	0.075
	Back	1	0.0
		2	0.0
		3	0.0
2.5 metres	Front	1	0.0
		2	0.5
	Back	1	0.0
		2	0.0
		3	0.0

5.2.5 Calculation of field dosage rates

5.2.5.1 Determining the theoretical dosage rate per ha to achieve >95% mortality of target larvae

Using the equations described in section 4.2.5.1, incorporated into an Excel spreadsheet, it was possible to calculate a number of potential dosage rates per ha, depending on assumptions of larval target stage, rates of attrition, feeding rates, etc. A typical outcome is shown in Table 19 which includes calculated values for third instar larvae, assuming spraying in mid to late afternoon (to reduce uv attrition) and young teak trees (2-4 m tall) with relatively low leaf area indices. The result indicates that, in this case, a theoretical 90% kill of target larvae should be achievable using a dose equivalent to 5.49 x 10^{11} PIBs per ha.

Table 19: Spreadsheet printout of equations to calculate projected dosage per ha for *HpNPV* applied against third instar *H. puera* larvae in teak plantations.

Equations to calculate theoretical dosage per ha using pre-determined parameters			
(From Evans 1998)			
	Target stage	**Third instar**	
Capture efficiency = (area)*(LAI*(1/(s*fr))	CE	2.47E+09	
Number of droplets per litre:	N	1.53E+10	
Initial dose (PIB/mm^2) = LD_{90}*a	D_i	222	
Theoretical Volume = CE/N litres per ha	V	0.16	
Dose per litre	D_l	3.40E+12	
Dose per ha	D_{ha}	**5.49E+11**	
Area of ha in mm^2	area	1.00E+10	
Calculation of CE	Calculate CE	2.47E+09	required droplets per ha
Feeding rate of larvae (mm^2)	fr	18	
Leaf Area Index (ratio to ground area)	LAI	4	
Loss of spray fluid to ground area (1/propn.)	s	0.9	
Virus attrition rate (propn of original)	a	0.9	
LD_{90} for target larval stage	LD_{90}	200	

5.2.5.2 Assessing the practical dosage per ha in terms of cost vs. projected mortality

Although the projected dosage for 90% was within the range tested in verification trials described below, there are a number of potential options for reducing the quantity of virus to be used in practical management of *H. puera*. Bearing in mind that the maximum larval equivalent achieved during mass production of *HpNPV* has, so far, been around 2 x 10^8 PIBs (Table 8 on page 27), the projected dosage calculated in Table 19 represents approximately 2700 larval equivalents per ha. Clearly, any measures that can reduce the number of larvae required for virus production will reduce costs and logistics. Thus, it may be possible to accept a lower initial level of mortality, allowing for the potential release of secondary inoculum arising from initial deaths.

Further gains can be obtained by:

- formulation to reduce losses from uv light degradation,
- making allowance for the anomalous dosage response of the fourth instar stage,
- accounting for the massive production of secondary inoculum arising from early deaths of larvae attributable to the applied field dose.

As indicated in the results of the field trials, it would appear that both fourth instar responses and production of secondary inoculum may have had an influence on the high responses obtained using relatively low dosages in the field.

5.2.6 Trials of *HpNPV* against field populations of *H. puera*

5.2.6.1 Small scale trial at Nedungayam

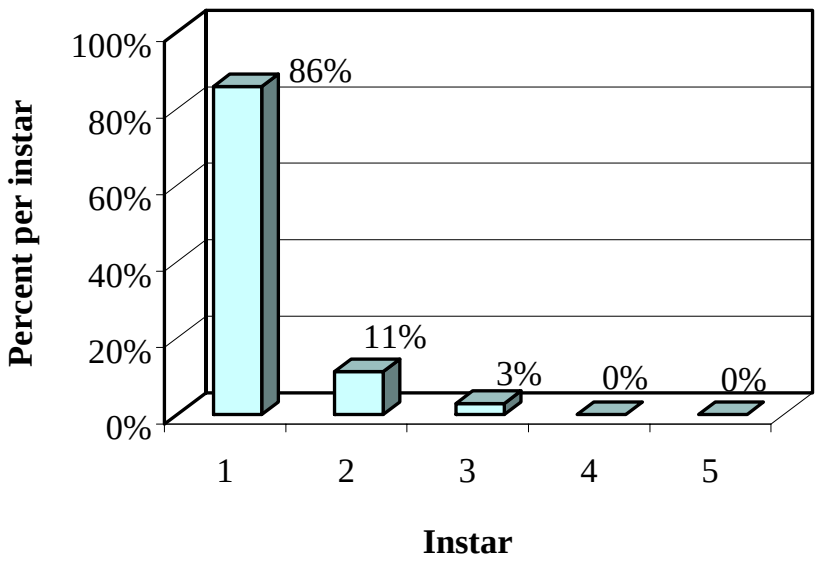
Data from larval assessments for the presence of virus infection in field collected larvae are presented in Table 20.

Table 20: Infection data for the small-scale trial at Nedungayam.

Dose (PIBs/ha)	Hours post spray	Number of larvae infected	Total larvae sampled	% larvae infected
0	0	0	19	0.00%
	24	0	23	0.00%
	48	0	7	0.00%
	60	0	18	0.00%
	72	0	2	0.00%
8.5×10^8	0	0	39	0.00%
	48	40	52	76.92%
	60	66	68	97.06%
	72	27	32	84.38%
6.46×10^9	48	16	18	88.89%
	60	45	50	90.00%
	72	4	5	80.00%
4.39×10^{10}	48	12	21	57.14%
	60	28	33	84.85%
	72	2	4	50.00%

The time period between samples was shorter than for subsequent trials and, therefore, it is difficult to make allowance for the very rapid mortality of infected larvae. Data are presented as instantaneous infections, but with the proviso that samples at 60 and 72 hours post spray are underestimates of total population mortality. This preliminary trial targeted predominantly first instar larvae (Figure 11) and this is reflected in the calculated dosage per ha which is considerably lower than for later trials in which third instar larvae are targeted. The data provide encouraging results that high mortality can be achieved using ulv technology. Unfortunately, larval numbers were affected by high rates of predation by wasps at the field site, resulting in unreliable percent infection data for the 72-hour sample for the highest dose.

Figure 11: instar distribution in small-scale field trial at Nedungayam



Infection rates were remarkably high, even in the lowest dosage (equivalent to 8.5 x 10^8 PIBs per ha) and there was no significant dosage response with increasing dose per ha. This shows the potential value of targeting the first instar stage. However, the difficulty of obtaining sufficiently early warning of impending infestations makes it impractical to consider targeting the first instar for routine application. If the opportunity does present itself, it would appear that very large savings in virus requirements could be achieved relative to targeting the third instar stage, as described below.

5.2.6.2 Preliminary trial at Valluvassery, Nilambur

Assessment of the effects of virus application was confined to a standardised sample of 30 larvae per tree and collection of all dead larvae seen during the sampling process. Problems with layout meant that the planned randomised block arrangement was not employed and was replaced by a fully randomised arrangement. Thus, it was not possible to use blocking to allow for potential differences in population sizes, tree sizes, etc. across the experimental plots. Nevertheless, the data were collected in a consistent manner and provide a measure of infection that can be compared across treatments.

A factor that also had to be taken into account was the very rapid rate of mortality once larvae were infected, as measured by larval smearing. In effect, larvae that were diagnosed with definite presence of baculovirus PIBs in one sample would have died by the following sample, 24 hours later. This reflects the observations made in laboratory assays, at temperatures similar to those observed in the field, that larvae died within 24 hours of showing overt symptoms of infection, revealed by light microscope diagnosis. In view of this information, two measures of infection were recognised in assessing field results; (1) presence of PIBs was recorded in each larva as an “instantaneous” measure of infection in the existing population of insects at the time of sampling, (2) on the assumption that larvae in the previous sample would have died by the time of the next “instantaneous” sample, percent infection was expressed as a proportion of the population that had survived from previous samples. This measure was then added to the previous percentage to provide an accumulated total percent infection. At the same time the numbers of larvae found dead and diagnosed as positive for

virus infection were also assessed, thus providing an independent measure of accumulated mortality in the sampled populations.

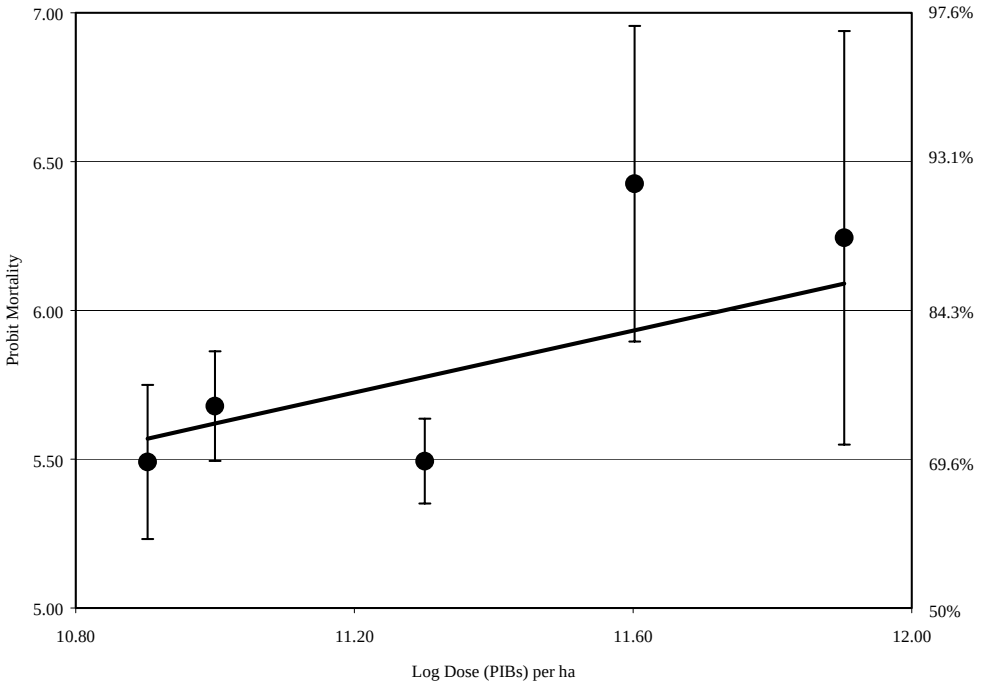
A summary of the data gathered in the trial is shown in Table 21. The percentage infection data have not been transformed in the table itself, but statistical analysis of the data employed probit transformation to determine standard deviations.

Table 21: Summary of results from small-scale field trial at Nilambur, 1997.

Dose	Replicate	Hours	No. larvae	No. infected	% infected	Accumulated percentage	NPV dead	Accumulated dead larvae
0	1	0	6	0	0.00%		0	
0	1	48	29	2	6.90%		0	
0	1	72	30	0	0.00%		0	
0	1	96	20	1	5.00%		11	11
1	All	48	149	52	34.90%		0	
1	All	72	116	50	43.10%		16	
1	All	96	112	31	27.68%	73.21%	89	105
2	All	48	149	51	34.23%		3	
2	All	72	149	68	45.64%		76	
2	All	96	93	24	25.81%	73.47%	64	143
3	All	48	136	37	27.21%		0	
3	All	72	149	60	40.27%		60	
3	All	96	138	44	31.88%	70.38%	83	143
4	All	48	147	54	36.73%		0	
4	All	72	144	85	59.03%		73	
4	All	96	111	61	54.95%	88.32%	113	186
5	All	48	145	46	31.72%		0	
5	All	72	149	95	63.76%		106	
5	All	96	123	53	43.09%	85.92%	127	233

Although there was a small incidence of natural infection in the untreated control plots, this was insignificant relative to the effects of virus application. The key features of these data are the confirmation of the flat dose response curves observed in laboratory experiments. This has resulted in high infection and mortality rates across all treatments but, as predicted by the laboratory results, did not reach 100% infection in any of the plots. The data are summarised in Figure 12 in which both probit and percentage scales are indicated. Variability was high for the highest dosages tested, resulting in no significant difference in the mean percentage infections achieved. However, application of probit analysis to the data did result in a significant regression line at the 95% probability level. The slope of this line (0.521) was considerably flatter than that observed for the third instar in laboratory bioassays (slope = 1.10). However, account must also be taken of the potential effects of secondary inoculum, released on death of the larvae infected from the initial spray application, and also of the anomalous dose response for the fourth instar observed in the laboratory.

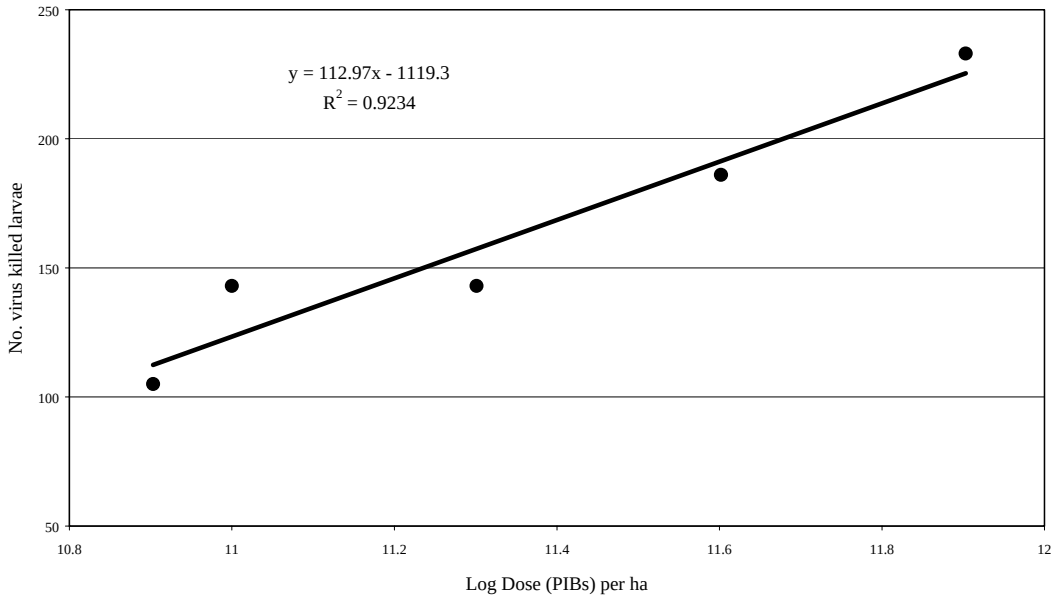
Figure 12: Percent infection (probit transformed) in relation to dosage applied during the preliminary field trial at Nilambur.



A further trend emerges when the data on accumulated numbers of virus-killed larvae in the plots are analysed. In this case the data represent all larvae in this category found on leaves while collecting live larvae and, thus, is a quantitative measure of accumulating mortality over time post infection. The data are presented in Figure 13, showing a clear linear relationship between dosage and accumulated host mortality. However, because the methods of collection are not directly comparable, it is not possible to make a direct correlation between the two measures of the impact of virus on *H. puera* populations. Nevertheless the trends are similar and confirm that the infection data observed in live larvae do result in rapid, accumulated mortality on foliage. The latter measure is undoubtedly an underestimate of actual mortality because of factors such as monsoon rainfall, wind abrasion, disintegration of the cadavers, etc. that will reduce the numbers of bodies remaining on foliage between sample occasions.

The relationship in Figure 12 can be used to predict the dosage required to kill a particular proportion of the host population. For example, the dosage required to kill 95% of larvae is predicted to be 1.05×10^{13} PIBs per ha. Such a quantity of virus represents the productivity from approximately 50000 infected larvae (larval equivalents) and is clearly unrealistic. However, the flat slope of the regression also indicates that significant mortality can be induced by doses very considerably less; for example 80% mortality requires a predicted dose of 2.53×10^{11} PIBs per ha, which is over 40 times lower than the 95% dosage.

Figure 13: Total accumulated number of virus-killed larvae in relation to dosage of baculovirus applied in the Nilambur 1997 trial.



5.2.6.3 Final trial at Valluvassery, Nilambur

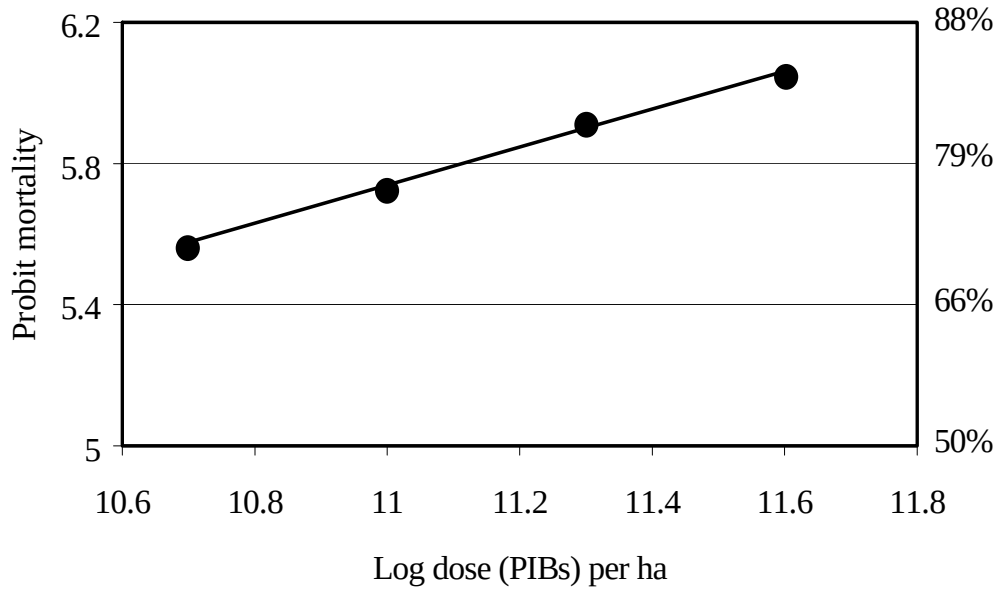
The final trial at Valluvassery was carried out after the official end of the project and funds set aside for production of a colour manual were diverted to cover some of the costs of work. However, it was not possible to complete all smearing and data analysis and the results presented here must, therefore, be regarded as preliminary. It is hoped that further funding from the DFID Western Ghats project will enable full analysis to be carried out in the near future.

Using the same procedures as the preliminary trial, described in section 5.2.6.2, accumulated percentage infection was calculated for the four dosages tested (note that the highest dose in this case was $4x10^{11}$, which is 50% lower than the highest dose used in the 1997 trial). The data were subjected to probit analysis to provide the dosage mortality relationship in Figure 14.

The slope (0.538) is very similar to that obtained in the preliminary trial in 1997 (slope = 0.521) indicating very similar responses to the dosages applied. However, there is less variability in the 1998 trial. It appears, therefore, that over 80% mortality can be achieved with dosages over $2x10^{11}$ PIBs per ha. This represents approximately 1000 larval equivalents, based on the data in Table 8 on page 27.

A further measure of the success of virus application is the extent of damage reduction that can be achieved following larval mortality from the virus. This was measured in two ways, namely a visual assessment of percentage defoliation and a direct measure of fresh leaf weight. The latter measure clearly has a high degree of variability because of the different sizes of leaves, but was still felt to be a useful measure on the basis that sufficient leaves were weighed to provide a comparable mean weight for all leaves taken.

Figure 14: Percent infection (probit transformed) in relation to dosage applied during the final trial at Nilambur.



In view of the fact that the trial targeted the third instar stage, there was already some defoliation evident in the plots by the time the virus was applied. This was, therefore, measured as a base line against which to assess further defoliation as the trial progressed.

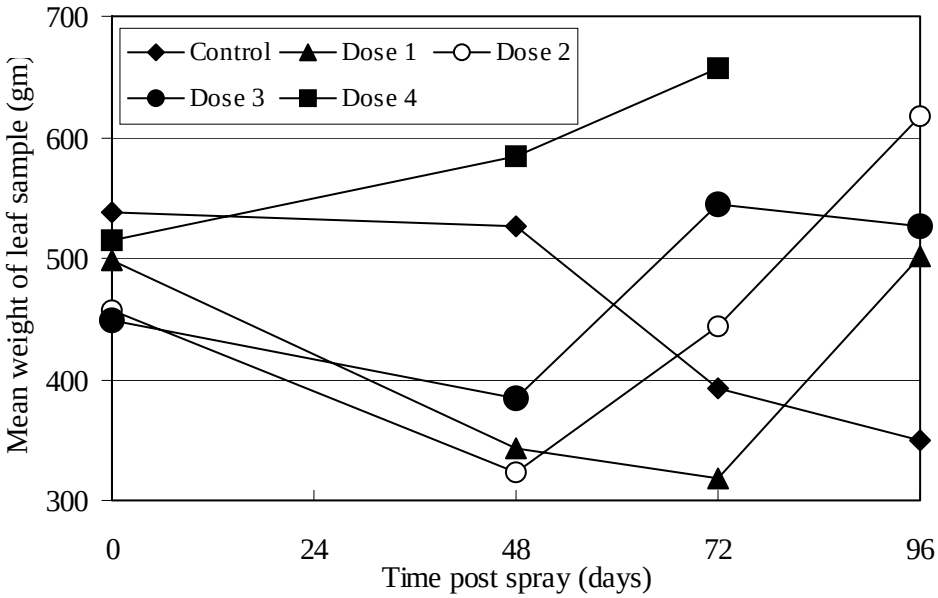
Table 22: Percentage damage recorded in the final trial at Valluvassery, 1998.

	Percentage damage (loss of foliage)		
Dose per ha	Initial	Final	Difference
Control	37.74%	81.68%	43.94%
5×10^{10}	42.28%	73.44%	31.16%
1×10^{11}	33.56%	61.12%	27.56%
2×10^{11}	35.52%	67.08%	31.56%
4×10^{11}	24.24%	50.36%	26.12%

Table 22 summarises the data from the trial and demonstrates that, when compared separately against the untreated controls, all treatments provided a significant reduction in damage (t-tests, $p<.05$), but there was no significant difference between virus treatments. However, the variability in the percentage values was high and, using Duncan's multiple range test for the final percentage damage recorded, the only significant difference was between the highest dose and the untreated control ($p=0.05$). This trend is confirmed when the mean weights of the leaves are compared. Figure 15 shows the trend of mean weights against time for each dose tested (no measurements were taken for the highest dose at 96 hours). The characteristics of the weight changes reflect the trends in Table 22. During the first 48 hours the mean weights of leaves (with the exception of the highest dose) dropped, reflecting the feeding of larvae on leaves prior to any noticeable effects of the virus on insect mortality. The only exception was the highest dose in which larvae were killed more rapidly than lower doses. After 48 hours, mean weights for virus treated leaves increased again, indicating recruitment of new leaves (with little or no damage) to the sampled cohort and also the

increasing effects of virus on the larvae. By contrast, the mean weights of leaves in the untreated controls continued to decline, despite recruitment of fresh leaves to the sampled cohort.

Figure 15: Mean weights of leaf samples collected from the final trial at Valluvassery, 1998.



These data indicate that there was a significant decrease in the quantity of foliage consumed, reflected both in visual assessments and in the fresh weights of leaves sampled. Shortage of resources meant that it was not possible to take measurements of other parameters such as tree diameter in order to assess effects on growth increment. However, it is likely that the greater photosynthetic area on the treated trees would have resulted in greater growth, at least at the highest dose tested.

6. Contribution of Outputs

6.1 *Contribution towards DFID's developmental goals*

The research funded under this topic was within the DFID Hillside System, which has a primary goal of increasing the productivity of plantation forestry on hillsides. Within this goal the purpose is to develop strategies for the efficient management of plantation forestry on sloping lands. The approach adopted within the present study has been to develop environmentally benign methods of reducing populations of teak defoliator moth, minimising damage to the trees and, hence, increasing growth and productivity.

Despite some setbacks, which reduced the number of field trials and demonstrations of efficacy, the project has been successful in its main aims of;

- improved monitoring of moth populations,
- development of improved knowledge on virus dosage-mortality relationships,
- development of virus mass production systems,
- assessment of individual parameters within a *Control Window* concept,
- prediction of field dosages,
- testing of predicted dosages in the field.

It was shown that use of virus is a realistic IPM strategy that provides relatively cheap and safe pest management, using sophisticated spray technology that is, nevertheless, cheap and easy to use. Reductions in moth populations and in damage to trees were demonstrated and, although it was not possible to equate these directly to changes in volume increment, there is every prospect that significant increases in yield could be achieved by using the virus IPM strategy routinely. The work has attracted considerable interest from both the private teak plantation sector in India and also from the Department of Biotechnology, India. Indeed, the latter organisation is likely to fund a pilot scale virus production plant to supply virus to both commercial and government teak plantations in Kerala and to neighbouring states where teak is grown actively.

The full IPM strategy requires labour inputs at a number of levels, including field monitoring, virus production (partially by field collection of larvae) and in final use in the field. This presents opportunities for job creation in remote areas and, therefore, contributes to the DFID aim of alleviating poverty and creating employment opportunities. The use of coconut oil as the base for the formulated virus product also supports indigenous industry in Kerala and elsewhere.

6.2 *Further action to establish a working Integrated Pest Management regime against teak defoliator moth, Hyblaea puera*

As indicated in this report and in earlier quarterly reports, a number of facets of the work have not been achieved as planned. In part, this has been a result of building the data on virus-host interactions from scratch, thus requiring a number of changes to the original plans. There has also been difficulty in locating suitable populations of the moth in the field at appropriate times for field trials. This has meant that it has not been possible to carry out field demonstrations, along with appropriate training courses and illustrative materials for likely beneficiaries. Although the accumulating information through the duration of the project indicated that success in use of virus was likely, the time-scale was too short to complete the full task. However, there are prospects for taking the work to fruition through the following routes:

1. Department of Biotechnology, India is likely to fund a pilot scale virus production plant at Nilambur. This would enable scale-up, quality control and formulation to be carried out in purpose-built laboratories, thus providing the potential for production of virus for a wide range of beneficiaries.

2. Assuming the pilot plant goes ahead, it is projected that at least 50 ha of teak plantations will be treated per annum over the next three years. These spray operations will be used to train beneficiaries and to allow further assessment of impacts on growth of teak. Illustrated manuals will be produced to accompany these training courses. The main target areas will be in Kerala and Karnataka States.

3. Negotiations are proceeding with DFID, New Delhi for consultancy funding for Dr H F Evans to continue his association with the next phase of the work. Mr J Gayfer, First Secretary (Forestry) at New Delhi has indicated that there are good prospects for funding of Dr Evans' involvement under the Western Ghats Project.

4. Regardless of further funding, the results already achieved under the project are being written up for publication in scientific and lay journals. Two papers are already in late drafting stage and it is proposed that at least three more will be produced. Most will be produced by the end of the first quarter of 1999.

Overall, this programme has been a fruitful collaboration between Forest Research in the UK and Kerala Forest Research Institute in India. Considering that the information on *HpNPV* was scant, progress towards practical use of the virus in the field has been remarkable. It is to be hoped that the final phase of the work will be funded to complete a most productive collaboration.

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Appendix I

Experiment plan titles, aims and objectives.

No.	Title	Aims and objectives
96.1 97.4	Laboratory assessment of virus	An important basis for working with NPV is a precise knowledge of the quantities of virus that are being worked with, both within the larvae and in the preparations of virus used for bioassay and other purposes. This should be quantified at all stages during virus production and in quality control of batches of virus prepared for further use. 1. To assess the degree of clumping in virus preparations. 2. To carry out counts, preferably using the dry count method, of the concentration of NPV PIBs in given preparations of virus. 3. To determine the potential for contamination from other occluded viruses, especially cytoplasmic polyhedrosis virus (CPV). 4. To determine precisely the symptoms that arise from larval infection from NPV and to count the quantity of virus produced at each stage of infection up to and including larval death. 5. **97.4** Quantification of the production of virus at different times post infection (Virus Growth Curve (VGC)) is an important component for optimisation of virus production methods and to aid the understanding of the ecology and epizootiology of *Hyblaea puera* NPV in the field, especially in relation to secondary inoculum. VGC should be developed for instars III to V initially and, if time permits, for instars I and II.
96.2	Calibration of sprayers for use in small scale field trials	Using data on LD_{50} and other variables such as virus persistence, the required dosages for field application of virus to populations of *H. puera* are being developed. An essential part of this process is accurate information on the performance of spray machinery and the degree of Foliar coverage achieved in the field. Calibration of the sprayers is, therefore, necessary before further field work can be commenced: 1. To assess basic parameters of the Ulva+, UlvaFan and AU8000 sprayers (rpm, flow rate) and to relate to published droplet sizes for given combinations of rpm and flow rate. 2. To assess foliar coverage using fluorescent marker dyes. 3. To investigate the development of oil-based formulations using emulsifiable oil preparations.
96.3 97.1	Field trials for the control of *H. puera* using NPV	LD_{50} and other parameters are known for instars I to V of *H. puera*. This information requires to be integrated with other variables, such as UV degradation, rainfastness and droplet coverage to produce precise dosage requirements for small-scale field applications of virus: 1. To use the known feeding rates of instars I to V as basic parameters for predicted area of leaf eaten by each instar in 6 hours in the field.

		2. To predict from UV and rainfastness experiments the rate of loss of virus over a 6-hour period following application. 3. To determine the leaf area index of teak for different sizes of tree, taking account of the ratio of tender leaf to old leaf in each case. 4. To use the data from above to integrate with known droplet distribution parameters for Ulva+, UlvaFan and AU8000 sprayers (see experiment plan 96.1) and to predict required dosage per ha. (including PIB load per VMD-sized droplet) for each of instars I to V. 5. To design small scale trials for both large and small trees, making use of the different chronologies of attack during the rainy season. 6. **97.1** Experiment Plan 96.3 provides the basis for design of field dosage requirements and for plot layouts for both small and large scale field trials. The small-scale field trial protocols were used to set up a trial in May 1997. Results from this study indicated a number of areas that require more detailed procedures to ensure that data gathered during the trials are quantitative and statistically valid. The current experiment plan outlines the procedures that should be adopted in further small or large-scale field trials. In particular the following components should be addressed: 7. Design of a sub-sampling system to allow quantitative larval samples to be taken at regular intervals. It is important that the sample can be extrapolated to give a measure of the whole larval population on a per tree and per plot basis. Virus control is concerned both with analysing infection rates in larvae and in assessing decline of the target insect population. 8. For small tree trials, plot layouts should be based on randomised block design with adequate buffer zones between treatments. Statistical texts and/or discussion with statisticians should be incorporated in the design so that the layout and methods of analysis are understood fully. 9. Design of a damage assessment protocol to be used as a further measure of efficacy of the virus treatments.
96.4	Mass Production of *H. puera* NPV for 1997 spray trials	1. To produce partially purified virus for stock inoculum. 2. To produce highly purified virus inoculum at NRI 3. To scale-up virus production using the improved working stock. 4. To produce a working stock of NPV for use in the 1997 field trials, totalling an estimated minimum of 5.0×10^{12} PIBs but preferably exceeding this figure. 5. To produce reference collections of NPV, covering different production batches and different geographical origins of insects used to grow the virus.
96.5 97.2	To quantify the dosage-mortality relationships between *H. puera* larvae and	To carry out further tests on the dosage-mortality relationships for each instar and to relate the results to larval weight. Work will concentrate on instars I and V but it is desirable to repeat the results from the 1995 work in view of the uncertainty

	its nuclear polyhedrosis virus (NPV)	about diagnosis of NPV. To investigate the relationships of LD_{50} and LT_{50} against larval weight. 1. Experiments carried out during 1995 provided information on the feeding rates and weight relationships for each instar. These data were used to set up leaf disk assays using the correct amount of leaf for consumption by each instar within 6 hours. The subsequent bioassays provided reasonably precise dosage-mortality relationships but there were doubts concerning the diagnosis of cause of death in both the treated and control larvae. A more precise diagnostic routine is, therefore, necessary for the assays to be carried out during 1996. 2. Bioassays of instars I to V. Based on the results from the studies in 1995, five dosages should be selected centered on the LD_{50} and equally spaced to the LD_{95} and LD_5 values calculated in the probit analysis. Purified virus should be used for the assays so that potential effects from contaminating bacteria can be removed. The dosages should be tested on a minimum of 30 larvae per dose and per control. The individual treated larvae should be examined twice daily and any deaths recorded and assessed for NPV on stained whole body smears under a high power microscope (an essential step in view of recent findings of indeterminate symptoms). Results should be analysed using probit analysis and both the LD_{50} and LT_{50} calculated. It is important that weight is assessed so that detailed analysis of larval response against weight can be calculated and relationships produced. Assays of instars IV and V and a comparative assay of the infectivity of aqueous and emulsion-oil based virus (for III instar only) should be carried out to complete this phase of the work. 3. **97.2** Results for all five instars have been obtained. Instars I to III provided statistically valid dosage-mortality relationships but, because there was an error in calculation of the probit analysis, the dosages used in the trials were too high and resulted in all mortality exceeding 50%. Although this still gave a valid regression line, it had a very flat slope and, thus, there is some uncertainty about the validity of the results for extrapolation to field dosages. Instars IV and V were also affected and, particularly for the former, did not give significant results. Consequently, it is desirable to repeat the assays for instars III to V and, if results of these new assays are satisfactory to consider whether it is also necessary to repeat instars I and II.
96.6 97.3	To carry out detailed studies on field persistence of NPV	Occluded baculoviruses produce polyhedral inclusion bodies (PIBs) that provide a means of virus persistence outside the host, within and between generations. This inoculum is also used in virus control programmes and it is important to quantify the dynamics of this persistence in designing microbial control programmes. 1. To determine the precise form of the UV degradation curve using shorter time intervals than the 1995 tests. 2. To determine the relative rainfastness of oil-based and

		water-based spray deposits using comparative bioassay. 3. To assess the effects of spraying virus at different times of the day on the overall persistence of the virus (if time and materials permit). 4. To determine the role of soil and soil movement by termites on persistence of NPV (if time and materials permit).
96.7	To improve methods for rearing of *Hyblaea puera* in laboratory culture	Existing methods of rearing are prone to large, unexplained mortalities in larvae. There is also a relatively high incidence of viral infection, pointing to the need for more stringent hygiene regimes. These experiments will investigate systematically ways of improving rearing success and quality control. 1. To assess the potential effects of microclimate in rearing tubes on the survival of *H. puera* larvae, concentrating on factors that induce NPV-like symptoms at time of death. 2. To analyse published information on insect rearing as an aid to designing improved methods of rearing. 3. To monitor symptoms and the precise causes of mortality so that more precise targeting of those mortality factors can be achieved.
96.9	To study the pathology of *Hyblaea puera* NPV in infected larvae.	There is little information on the pathology of *H. puera* NPV. The virus is unusual in that it kills its host very rapidly (2-3 days) and, thus, there may be unusual pathology associated with this. 1. To study the gross pathology of *H. puera* NPV in infected larvae. 2. To assess the tissue specificity of the virus in infected larvae. 3. To study the detailed cytopathology of *H. puera* NPV.